Mitochondrial DNA Fragmentation as a Molecular Tool to Monitor Thermal Processing of Plant-Derived, Low-Acid Foods, and Biomaterials

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Abstract: Cycle threshold (Ct) increase, quantifying plant-derived DNA fragmentation, was evaluated for its utility as a time-temperature integrator. This novel approach to monitoring thermal processing of fresh, plant-based foods represents a paradigm shift. Instead of using quantitative polymerase chain reaction (qPCR) to detect pathogens, identify adulterants, or authenticate ingredients, this rapid technique was used to quantify the fragmentation of an intrinsic plant mitochondrial DNA (mtDNA) gene over time-temperature treatments. Universal primers were developed which amplified a mitochondrial gene common to plants (*atp*1). These consensus primers produced a robust qPCR signal in 10 vegetables, 6 fruits, 3 types of nuts, and a biofuel precursor. Using sweet potato (*Ipomoea batatas*) puree as a model low-acid product and simple linear regression, Ct value was highly correlated to time-temperature treatment ($R^2 = 0.87$); the logarithmic reduction (log CFU/mL) of the spore-forming *Clostridium botulinum* surrogate, *Geobacillus stearothermophilus* ($R^2 = 0.87$); and cumulative *F*-value (min) in a canned retort process ($R^2 = 0.88$), all comparisons conducted at 121 °C. D_{121} and *z*-values were determined for *G. stearothermophilus* ATCC 7953 and were 2.71 min and 11.0 °C, respectively. D_{121} and *z*-values for a 174-bp universal plant amplicon were 11.3 min and 9.17 °C, respectively, for mtDNA from sweet potato puree. We present these data as proof-of-concept for a molecular tool that can be used as a rapid, presumptive method for monitoring thermal processing in low-acid plant products.

Keywords: mitochondrial DNA, quantitative PCR, thermal processing

Practical Application: This method could be used as another tool for thermal process validation and monitoring. It is especially useful for thermal processes over 100 °C, since temperatures above boiling rapidly damage DNA. Its advantages over enzymatic assays are that mtDNA is highly stable and can be stored at freezing temperatures for long periods. Mitochondrial DNA can be used for all plant products tested. Processors will be able to validate processes and track process deviations using rapid molecular methods. Processors can use this presumptive test prior to shipping out a product.

Introduction

Mitochondria are organelles that supply power in the form of ATP to eukaryotic cells. Foodstuffs, such as vegetables, nuts, and fruits contain multiple intracellular copies of nonnuclear DNA, mitochondrial DNA (mtDNA), which can be amplified via quantitative polymerase chain reaction (qPCR). Each mitochondrion possesses its own genome in multiple copies (Anderson and others 1981; Foury and others 1998). These properties make mitochondrial DNA sequences (mtDNA) excellent targets for qPCR amplification in terms of specificity, sensitivity, and robustness due to multiple copies per cell (approximately 1000) (Bogenhagen and Clayton 1974; Gerber and others 2001; Andreasson and others 2002). Rapidly dividing, continuous cell lines, such as HeLa cells, were found to have four times the amount of mtDNA as mouse L cells (Bogenhagen and Clayton 1974). Therefore, the advantages of targeting mtDNA in living and respiring plant-derived foods with qPCR are substantial.

mtDNA genes are used as identifiers in many scientific disciplines. They have been adopted for bar-coding almost all groups of higher animals (http://www.barcoding.si.edu/). mtDNA is also used in human typing for forensic analysis (Hopwood and others 1996; Andreasson and others 2002; Budowle and others 2003) using tissues such as bones, teeth, and hair shafts for DNA extraction. mtDNA primers/probes have been developed for source tracking fecal contaminates in wastewater influents and effluents using multiplex qPCR (Caldwell and others 2007; Caldwell and Levine 2009; Caldwell and others 2011). In the food industry, PCR-based mtDNA analyses were used in the authentication of meats and to trace contamination of other animals in the food products (Meyer and Candrian 1996; Lahiff and others 2001; Zhang and others 2007; Fujimura and others 2008). The development of these molecular tools has improved the monitoring of food quality, preventing fraudulent description of food content, and identifying adulterants. The commercial success of using mtDNA as identifiers in heterogeneous food matrices led to the idea of using intrinsic foodstuff mtDNA as indicators of thermal processing efficacy.

The drastic effect of high temperature on DNA degradation is well established. Above 100 °C, denaturation, depurination,

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deamination, and loss of secondary structure occurs (Gryson 2010). However, autoclaving a foodstuff at 121 °C for 15 min does not destroy all DNA available for PCR (Lipp and others 1999). Other researchers have reported reduced recovery of DNA via qPCR from cornmeal boiled for 60 min at 100 °C (Murray and others 2007). Increased Ct (threshold cycles) values occurred in DNA from heat-treated corn grits and corn flour when compared to untreated corn and resulted in distortions of qPCR assays for detection of genetically modified organisms (Moreano and others 2005). Therefore, DNA is degraded but still detectable by qPCR, when using thermal processing techniques suitable for preserving vegetables and fruits.

The effect of heat treatments on the quantification and detection of meat DNA by qPCR was found to be dependent on the temperature and duration of the treatment, as well as the size of the amplicon (Sakalar and others 2012). Smaller amplicons were less likely to be affected by heat treatment and the degree of DNA fragmentation was directly correlated to time and temperature (Sakalar and others 2012). A relationship between amplicon size and detection after heat treatment or mechanical processing was cited in other meat assays (Hird and others 2006) and plant products, such as soybeans, maize, peas, and white potatoes (Bauer and others 2003; Kharazmi and others 2003; Hrncirova and others 2008).

Thermal validation and monitoring can employ microbial culture methods for verification of sterility (Pflug and others 1980; Smith and Kopelman 1982; Marcy 1997; Guan and others 2003). For low-acid products (pH 4.6 to 6.0) the target organism is the *Clostridium botulinum* spore (Pflug and others 1985) due to its heat resistance and the catastrophic effects of its toxin when ingested. The minimum botulinum cook has been determined based on a *D*-value of 0.21 min at 121 °C (Esty and Meyer 1922; Townsend and others 1938; Stumbo 1965). For the canning industry to achieve a 12-log reduction (12D) for a low-acid product, the convention is to round up to an F_0 of 3 min (Tucker and others 2008), *F* being the cumulative time-temperature treatment at 121 °C. Destruction of spore-forming *C. botulinum* surrogates such as *Geobacillus stearothermophilus* have been used to monitor heat processes in low-acid foods such as sweet potato puree (Smith and Kopelman 1982; Brinley and others 2007; Steed 2010). Problems with using a culture approach include tracking and recovering surrogate spores, and time required to culture (48 h). Molecular methods, such as qPCR, are able to detect spores and vegetative bacteria but are unable to differentiate between live and dead cells.

Other biological techniques such as enzymes have been used or proposed as time-temperature indicators: Beta-glucosidase from *Pyrococcus furiosus* (Yen 2009), alpha-amylase from *B. licheniformis* (De Cordt and others 1994; Guiavarch and others 2004) or *B. subtilis* (Guiavarch and others 2005), algal R-phycoerythrin (Smith and others 2002; Orta-Ramirez and others 2001), glucose oxidase (Reyes-De-Corcuera and others 2005), and endogenous muscle proteins such as lactate dehydrogenase in meats (Veeramuthu and others 1998) and alkaline phosphatase and lactoperoxidase in milk (Claeys and others 2004). Advantages of using endogenous DNA, intrinsic to the plant food, over enzymes are many. DNA is more stable than protein enzymes and can be stored for long time periods at −20 °C. Therefore, one can return and assay the process at a later date, thus having a record of past events. Most enzyme techniques are extrinsic to the process and invasive. Enzymes must be added or containerized then recovered from the system. A great deal of energy is exerted to find the proper carrier for the exogenous enzyme (De Cordt and others 1994; Guiavarch and others 2004; Reyes-De-Corcuera and others 2005; Wang and others 2010). Endogenous enzymes are specific to the product and not universal like DNA.

Table 1–Initial quantitative PCR primers tested.

Primer name	Sequence
174F (forward)	5′ –TTTCCGCGATAATGGAATGCACGC-3′
174R (reverse)	5′ –TCCGATCGTTTAGCCGCTCTTTCT-3′
108F (forward)	5′ –CGCCTTTGCTCAATTTGGCTCAGA-3′
108R (reverse)	5′ –GGCAGTGGTGCATATTGTGGTTGT-3′
81F (forward)	5′ –CGCCTTTGCTCAATTTGGCTCAGA-3′
81R (reverse)	5′ –AGTACTTCTGTCAGCCTTGCACCT-3′
141F (forward)	5′ –GAATTTGCCAGCGGTGTGAAAGGA-3′
141R (reverse)	5′ –TCCCGCAGGAACATCCACAATAGA-3′

Quantitative PCR has several advantages over culture techniques and conventional PCR: it is rapid (4 to 6 h), does not require gels or plates and is directly measurable in real time. For this study, all mtDNA qPCR assays developed met minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines (Bustin and others 2009) which features a quality control checklist. Conformity to MIQE guidelines makes qPCR operator- and laboratory-independent and allows comparisons between results from different production runs and different locations.

Because of the many advantages of using intrinsic mtDNA from foodstuffs directly, we propose monitoring and process validation of thermally processed low-acid plant foods using mitochondrial DNA fragmentation via qPCR by graphing its thermal destruction over time. This approach to monitoring food safety represents a paradigm shift by using qPCR to quantify the fragmentation of foodstuff mtDNA over time due to thermal processing, and compare the kinetics of this protocol to the *D*- and *z*-values of spore-forming bacteria.

Materials and Methods

Primer design and validation

Primers were designed using consensus sequences to target a wide variety of plant foods. Four sets of qPCR primers were designed with Primer Quest software (http://scitools.idtdna.com/Primerquest/) targeting the *Ipomoea batatas* F1-ATPase alpha subunit (*atp*1) mitochondrial gene (GenBank AY596672.1). Amplicons for primer sets ranged from 81 to 174 base pairs (Table 1). Four primer sets were purchased from IDT (http://www.idtdna.com). Oligonucleotide primers were reconstituted in TE buffer (pH 7.5) and stored at -20 °C prior to use. All primer sets matched the *atp*1 gene with 100% identity, not only in *I. batatas,* but in a wide range of common fruits and vegetables when subjected to NCBI BLAST searches

Standard curve

Standard curves were generated using gBlocks™ Gene Fragments (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments) which are double-stranded, sequence-verified oligonucleotides of the *atp*1 gene. Tenfold serial dilutions of *atp*1 copies (10^8 to 10^1) were performed and PCR amplification efficiency (*E*) was determined using the slope of the standard curve:

$$E = (10^{-1/\text{slope}}) - 1.$$

Data analysis of the qPCR standard curve was performed using goodness-of-fit linear regression correlation coefficient (R^2) (Figure 1).

Assessing universality of primers

The *atp*1 gene was found to be highly conserved among plant species. Primers were created which could be used universally to test plant-based foods, both singly and in mixtures, such as soups (Table 1). Fresh, uncooked fruits, vegetables, and nuts were purchased from a retail grocery store and processed immediately by grinding in a Hamilton Beach coffee mill. The mill was thorough cleaned with distilled water and 70% ethanol between samples and reps to prevent DNA cross-contamination. Three separate individuals were used for each variety of plant tested. Six reps were tested in all, 3 uncooked controls and 3 autoclave treatments (20 min at 121 °C). The tissue culture protocol of the MasterPure DNA purification kit (Epicentre, Madison, Wis., U.S.A.) was used. DNA was quantified and qualified using a spectrophotometer (Nanodrop, Wilmington, Del., U.S.A.). DNA was normalized to 5 to 10 ng/well using the qPCR assay as below. Each sample was run in duplicate wells. Mean Ct values for uncooked and autoclaved plant materials were recorded as well as the increase of Ct due to autoclave treatment and the slope of the line formed by the graph of the 2 values (Table 2).

Sweet potato puree

Sweet potato puree was prepared according to the method of Truong and Walter 1994. Briefly, sweet potatoes were cured and stored at 13 to 16 °C and 80% to 90% relative humidity prior to use. Roots were washed and peeled by immersion in a boiling solution of 5.5% NaOH for 4 min. Peeled roots were hand trimmed and cut into slices, then steam-cooked for 20 min in a thermo-screw cooker (Rietz Manufacturing co., Santa Rosa, Calif., U.S.A.) and reduced in size in a hammer mill (Model D, Fitzpatrick Co., Chicago, Ill., U.S.A.) with a 0.15 cm screen. The puree was stored in polyethylene bags at −20 °C until used in thermal trials.

Sporulation

Lyophilized *G. stearothermophilus* (NRRL No. B-1102, equivalent to ATCC 7953) was obtained from the ARS Culture Collection (USDA-ARS; Peoria, Ill., U.S.A.), reconstituted, verified by microscopy and Gram-staining, and placed in freezer stocks containing 20% glycerol. To sporulate, 10 mL BHI broth (Becton Dickinson, Sparks, Md., U.S.A.) was inoculated directly from freezer stocks or 1% volume from liquid culture and incubated overnight at 55 °C, static. Bacillus heat resistant agar containing 13 g nutrient broth, 0.51 g $MgSO_4(7H_2O)$, 0.97 g KCl, 0.2 g $CaCl_2(2H_2O)$, 0.003 $MnSO_4(H_2O)$, 0.00055 g $FeSO_4(7H_2O)$, and 15 g agar brought up to 1 L with distilled water and autoclaved (Stam 2008), was spread with 100 μL overnight culture. Over 20 plates were spread in this fashion. Plates were incubated at 55 °C for 5 d. Spores were harvested by applying 10 mL cold, sterile, distilled H_2O containing 0.1% Tween 80 (Sigma-Aldrich; St. Louis, Mo., U.S.A.) directly to each plate and scraping with a sterile cell scraper (van Melis and others 2011). The resulting liquid, containing both vegetative cells and spores, was aspirated from plates via modified pipette tip and placed in 50 mL centrifuge tubes and spun at 6000× *g* for 10 min at 4 °C. Enzymatic cleaning to eliminate vegetative cells (modified from Foegeding and Busta 1983) was performed by adding lysozyme (200 mg/mL) to each 200 mL spore pellet and incubating at 45 °C for 30 min with occasional vortexing, then adding trypsin (final volume; 100 mg/mL) and incubating at 45 °C for an additional 2 h with occasional vortexing. Spore solutions were rinsed 10× with 25 mL cold water/0.1% Tween 80 (6000× *g* for 10 min at 4 °C). After a final spin, spores were re-suspended in 10 mL water/Tween 80 solution. Spores were evaluated by dark phase microscopy with a target ratio of spores to vegetative cells of 9:1 or greater. Spores were stored at 4 °C long term, but centrifuged and re-suspended weekly to maintain and prevent germination.

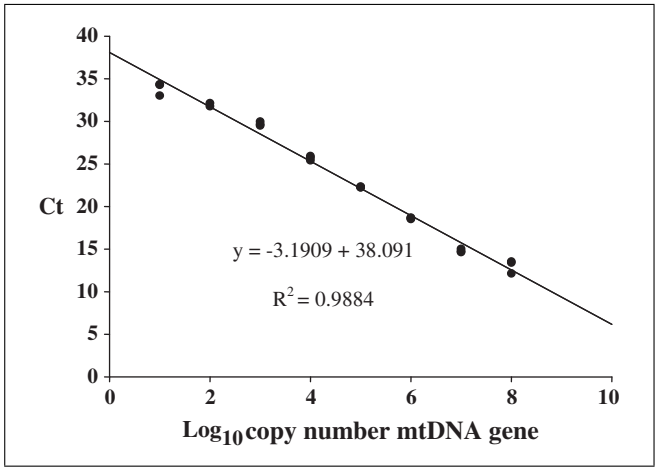

Figure 1–Mitochondrial DNA qPCR standard curve for 174 bp amplicon.

Hot oil bath trials

In an effort to mimic and quantify values in a 12D thermal process, the reduction curve of *G. stearothermophilus* (*C. botulinum* surrogate) spores with resulting *D*- and *z*-values were compared to Ct values of sweet potato puree in a hot oil bath at the following temperatures: 116, 121, 123, and 126 °C. A hot oil bath (EW-111, Neslab Instruments, Newington, N.H., U.S.A.) filled with 8 L white mineral oil (Therminol XP, Solutia, Inc, St. Louis, Mo., U.S.A.) was used to maintain each target temperature for substances placed in a thermal death tube (TDT). This laboratory system was used to replicate conditions in an industrial retort, heat exchanger, or microwave thermal process. The TDT was composed of a ¾ inch aluminum screw post (Screwpost.com, Muskegon, Mich., U.S.A.) cut to size and filed for smoothness, ¼ inch nylon machine screws, Viton fluoroelastomer O-ring gaskets (screw size #6) and Viton flat washers size #6 (all parts from McMaster-Carr, Atlanta, Ga., U.S.A.). Oil bath temperature was monitored using a type J-K-T microprocessor thermometer thermocouple (HH23A, Omega, Stamford, Conn., U.S.A.) and type T 1/8" diameter insertion probe (50335-T; Atkins Technical, Inc.; Gainesville, FL, U.S.A.). Come up time (CUT) for TDTs was determined for all target temperatures by inserting and sealing a 0.0095 inch diameter probe into the vessels (30 s). In each TDT, 100 μL of 1:4 diluted puree or 100 μL of *G. stearothermophilus* spores (ca. 10^8 CFU/mL) were inserted and sealed. For *G. stearothermophilus* spores, samples were heated for 0, 4, 8, and 12 min at 116 °C; 0, 0.5, 1, 2, 4, 8, 16, and 20 min at 121 °C; 0, 1, 2, and 4 min at 123 °C; 0, 0.5, 1, 2, and 4 min at 126 °C, all heat treatments beginning after CUT. Samples for diluted purees were heated for 0, 12, 24, 48, and 60 min at 116 °C and 0, 4, 8, 12, and 18 min at 121, 123, and 126 °C, also taking into account the CUT. Three replicates were run per time point: 3 TDTs were placed in a metal tea strainer to facilitate removal of samples from hot oil. Strainers containing TDTs were taken out of oil bath and

Table 2–Universality of primers (174 bp amplicon) and demonstration of mtDNA fragmentation (increase in Ct value) across different plant materials after autoclave treatment.

Sample	Mean uncooked Ct	Mean autoclaved Ct	Difference	Slope
Vegetables				
White potato	19.73 ± 0.45**	32.77 ± 0.40	13.04	0.65
Sweet potato puree	24.06 ± 0.18	33.00 ± 0.07	8.90	0.45
Tomato	18.86 ± 0.12	32.27 ± 0.75	13.41	0.67
Green pepper	19.45 ± 0.28	35.66 ± 1.89	16.21	0.81
Red pepper	18.99 ± 0.20	34.63 ± 1.17	15.65	0.78
Jalapeno pepper	19.96 ± 0.23	35.66 ± 0.45	15.70	0.79
Carrot	15.71 ± 0.07	32.45 ± 0.14	16.74	0.84
Green bean	22.45 ± 0.17	32.38 ± 0.27	9.93	0.50
Corn	22.40 ± 0.30	27.24 ± 0.31	4.84	0.24
Cucumber	18.47 ± 0.12	29.88 ± 0.15	11.40	0.57
Biofuels				
Switch grass	28.26 ± 0.16	34.69 ± 0.23	6.43	0.32
Fruits				
Apple	22.95 ± 0.73	36.27 ± 1.95	13.32	0.67
Blueberry	25.88 ± 2.18	35.51 ± 3.60	9.63	0.48
Peach	20.93 ± 0.17	37.52 ± 0.49	16.60	0.83
Strawberry	23.27 ± 0.18	33.17 ± 0.80	9.90	0.50
Pineapple	22.97 ± 0.70	33.31 ± 2.72	10.35	0.52
Grape	27.95 ± 0.18	32.11 ± 1.11	4.16	0.21
Nuts				
Peanut*	17.00 ± 0.21	23.10 ± 0.92	6.10	0.32
Almond	18.31 ± 0.18	27.25 ± 0.17	8.94	0.45
Pecan	25.86 ± 0.27	31.43 ± 0.28	5.57	0.28

*Roasted at 167 °C for 19 min. All others autoclaved at 121 °C for 20 min. **Standard deviation.

immediately placed in an ice slurry for 30 s to quickly cool them. Strainers were stored at room temperature until ready for DNA extraction or culture plating. Total amount of sweet potato puree recovered from hot oil bath treatment was determined from an initial sample of 100 μL.

The *D*-value (decimal reduction time) is defined as the time in minutes at a given temperature that results in a one log reduction in microbial count (Sandeep, personal communication; Pflug 1990). Given the equation:

$$N = N_0 10^{-t/D_T},$$

where N_0 and N are the initial and final number of microorganisms, respectively, the *D*-value at a given temperature (D_T) is calculated by graphing the $\log_{10}$ number of microorganisms over time (min) and determining the slope: slope $= -1/D_T$.

The *z*-value is the temperature change required for a one log change in the *D*-value of a microorganism (Sandeep, personal communication; Pflug 1990). Given the equation

$$D_T = D_{\text{ref}} 10^{\text{Tref}-T/z}$$

the *z*-value is calculated by graphing log *D*-value (s) versus temperature and determining the slope: slope $= -1/z$.

G. stearothermophilus spores were serially diluted and plated with a spiral plater (Spiral Biotech Inc., Norwood, Mass., U.S.A.) or a simplified agar plate technique (Jett and others 1997); both on BHI agar (Becton Dickinson). After 24 h incubation at 55 °C, colonies were enumerated with an automated spiral plate counter (Q-count, Spiral Biotech Inc) or counted manually. The lower detection limits were 4×10^2 and 1×10^3 CFU/mL for the spiral plate and simplified agar technique, respectively.

Ct values were converted to $\log_{10}$ copy numbers using the linear relationship determined empirically from the standard curve of the 174 bp amplicon (Figure 1):

$$y = -3.1909x + 38.091,$$

where y is the Ct value and x is the $\log_{10}$ copy number. A flow chart (Figure 2), illustrates the steps to determine and compare the *D*- and *z*-values of the *G. stearothermophilus* spores with mtDNA fragmentation (ΔCt converted to $\log_{10}$ copy number) of the low-acid purees.

Retort trials

Sweet potato puree was produced as before and placed in 68.3 × 101.6 mm cans outfitted with T-type C-2 tube and rod thermocouples (Ecklund-Harrison Technologies, Fort Myers, Fla., U.S.A.). Colorimetric *G. stearothermophilus* ampoules (Raven ProSpore; Mesa Laboratories, Inc., Lakewood, Colo., U.S.A.) were placed in the center of each can, adjacent to the thermocouple probes. Cans were sealed with a double seam using an automated can sealer (Dixie Canner Co., Athens, Ga., U.S.A.). Total weights of puree and size of head space were similar between all cans in each run. Canned sweet potato puree was loaded into a Model PR-900 pilot retort (Stock sterilisationstechnik, Hermanstock Maschf.; Neumunster, West Germany) with thermocouples attached to a recording device and run in one of 2 full water immersion protocols listed below. Protocol 00 was a substandard treatment not meant to kill spores (Appendix A: Supporting Information). Protocol 01 was a > 6D protocol meant to eliminate all *G. stearothermophilus* test spores (Appendix B: Supporting Information). Each protocol was run in triplicate using 3 cans per run. Puree was sampled from the center of each can by carefully removing the top layers with a spatula and taking a 500 μL aliquot next to the thermocouple probe. DNA was extracted from this centrally located aliquot and atp1 qPCR protocol run as before.

ProSpore (Mesa Laboratories) ampoules were incubated at 55 °C for 48 h as recommended by the supplier, and then assessed for colorimetric change. *F*-values were determined from the time-temperature data collected. *F*-value was calculated as follows:

$$F = 10^{((T-121.1)/10)}\Delta t,$$

where T is temperature in °C and t is time in minutes. Ct values were correlated to *F*-values of all runs.

Retort data statistics

To determine the cumulative *F*-value that corresponds to a given Ct value, the method of inverse prediction was used. This methodology enables the assessment of uncertainty associated with estimation of an unknown cumulative *F*-value. A linear regression of $Y = \text{CT}$ on $X = \text{cum } F$ was fit (with $R^2 = 0.875$) to $m = 21$ bivariate measurements, $(y_1; x_1), \ldots, (y_{21}; x_{21})$. The estimated value of $X = X_0$ that corresponds to a given measurement of $y = y_0$ is given by

$$\hat{X}_0 = \frac{y_0 - \hat{\beta}_0}{\hat{\beta}_1},$$

where $(\hat{\beta}_0, \hat{\beta}_1)$ denote the estimated intercept and slope from the regression. The standard error for this nonlinear function of regression coefficients may be approximated by

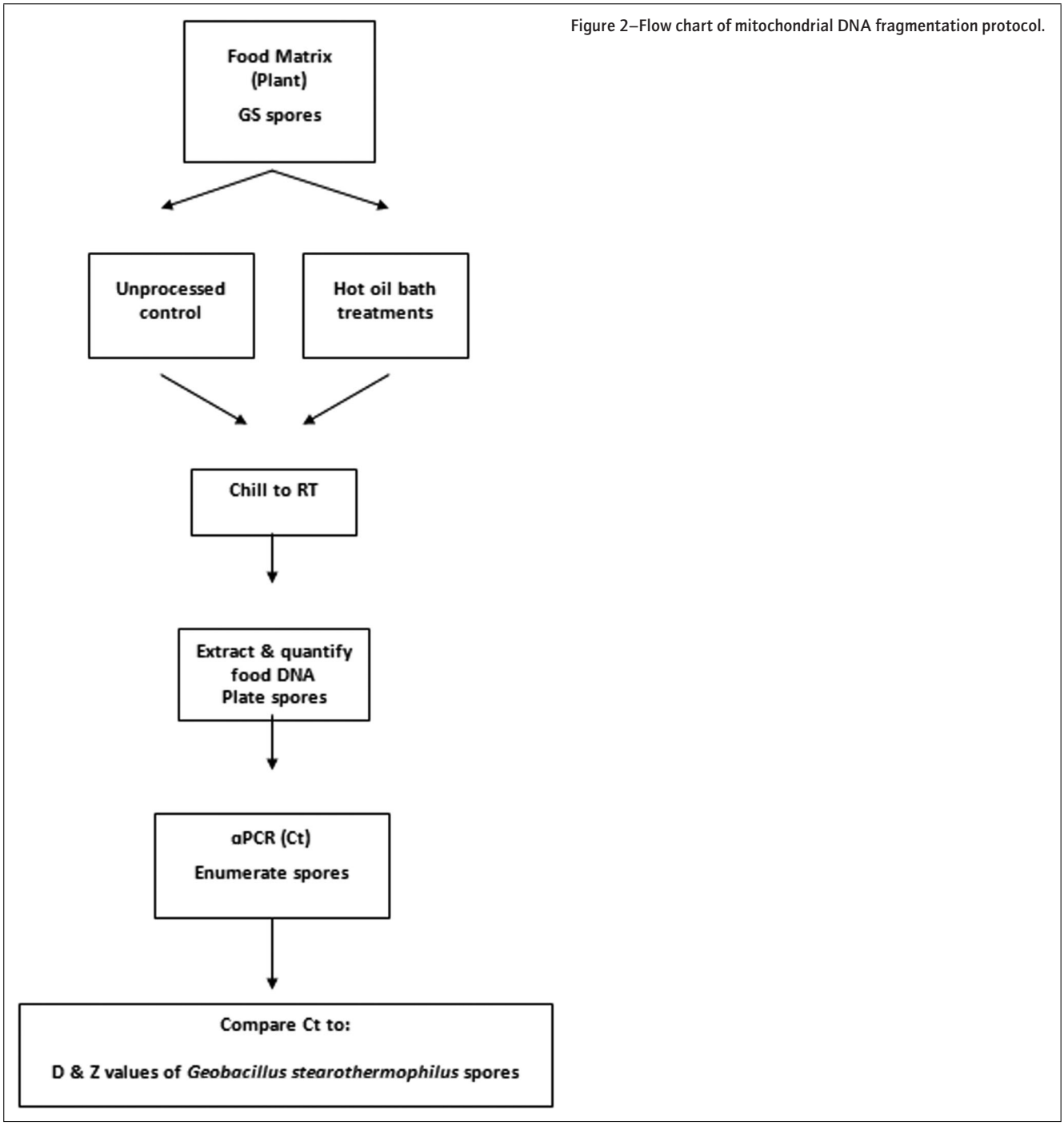

Figure 2–Flow chart of mitochondrial DNA fragmentation protocol.

Table 3–Inverse prediction statistics for retort process validation showing samples needed for low standard errors.

		99% Confidence levels	
Samples needed	Standard error (%)	Upper	Lower
32	2.3	4.66	5.33
64	1.6	4.76	5.23
128	1.2	4.83	5.16
256	0.8	4.88	5.11

$$SE(\hat{X}_0) = \sqrt{\frac{\text{MS(E)}}{\hat{\beta}_1^2}\left(\frac{1}{m} + \frac{(\hat{X}_0 - \bar{X})^2}{\sum (X_i - \bar{X})^2}\right)},$$

where MS(E) denotes the error mean square from the regression. This error mean square is an estimate of the error variance in a single measurement of Y. An approximate, symmetric 100(1 − alpha)% confidence interval (Neter and others 1983) for the average X_0 that would give rise to Y_0, is given simply by

$$\hat{X}_0 \pm t(\alpha/2, m-2)\text{SE}(\hat{X}_0).$$

An alternative procedure is to find the values of X which satisfy the inequality

$$\left(\frac{y_0 - (\hat{\beta}_0 + \hat{\beta}_1 x_0)}{\text{SE}(\hat{\beta}_0 + \hat{\beta}_0 + \hat{\beta}_1 x_0)}\right)^2 \leq t(\alpha/2, m-2)^2.$$

These values may be obtained using the quadratic formula and constitute an asymmetric confidence interval. This is the method used by the JMP statistical software package (SAS; Cary, NC, U.S.A.). For the pilot data, with $m = 21$, the computations were carried out using SAS (and checked for agreement with JMP), and then different numbers of subsamples N at a given value of $y_0 = 26.2$ were considered (corresponding to a cumulative F-value of 5 min). For N (subsample), the MS(E) term in the formulas above was replaced by MS(E)/N, and 99% confidence intervals are given in Table 3.

DNA extraction

Total DNA from treated sweet potato puree was extracted by either a MoBioPowerSoil® DNA isolation kit (Carlsbad, Calif., U.S.A.) or MasterPure DNA extraction kit (Epicentre, Madison, Wis., U.S.A.), both used according to manufacturer's recommendations. DNA samples were analyzed by spectrophotometer (Nanodrop, Wilmington, Del., U.S.A.) for quantity and quality (260 & 280 nm). For qPCR, DNA was normalized by concentration: between 5 and 10 ng/μL per reaction.

qPCR

qPCR was run in 25 μL total volume with 2X IQ SYBR Green supermix (SYBR Green I dye, 50 U/mL iTaq DNA polymerase, 0.4 mM each of dATP, dCTP, dGTP, and dTTP, 6 mM $MgCl_2$, 40 mMTris-HCl, pH 8.4, 100 mM KCl, 20 nM fluorescein; Bio-Rad, Hercules, Calif., U.S.A.), 300 nM final concentration each for forward and reverse primers (F1 *atp*1), vegetable puree DNA (5 to 10 ng/reaction) and RT-PCR water (Ambion, Austin, Tex., U.S.A.) to final volume. Amplifications were performed in a MyiQ (BioRad) thermal cycler with the following conditions: 95.0 °C for 3 min; 40 cycles of 95.0 °C for 30 s, 60.0 °C for 30 s, 72.0 °C for 30 s; with FAM channel optics on during extension stage. MIQE standards were employed for the optimization and validation of the qPCR assay (Bustin and others 2009). No template (NTC) and positive controls were used for all assays. For a sample to be considered positive, its Ct value must be less than all negative control reactions and its corresponding amplification curve had to exhibit the 3 distinct phases of real-time PCR: lag, linear, and plateau. Internal amplification controls were not employed as no PCR inhibition was apparent. The positive control was used to normalize data between assays. Ct values were used to create D- and z-values across target temperatures indicated. These values were compared to similar values for spore death.

Results and Discussion

Universal primers

More than 3 million mtDNA sequences are available at the National Center for Biotechnology Information genome web page (3,181,082 sequences as of June 2014) (www.ncbi.nlm.nih.gov) including partial mtDNA genomes for sweet potato, carrot, potato, green beans, strawberry, apple, and other common fruits and vegetables. The results of the comparative analysis between the rapeseed and *Arabidopsis* mitochondrial genomes suggest that sequences among higher plants are highly conserved (Handa 2003), thus having many similar sequences to target for consensus qPCR. The goal was to create a set of primers based on consensus sequences which would allow universal amplification of plant mtDNA. The ATP synthase F1 alpha sequence (GenBank AY596672.1) was targeted. This gene's enzyme catalyzes the final step during oxidative phosphorylation and is highly conserved in eukaryotes (Millar and others 2011).

Primer sets were tested empirically using qPCR with melt curve analysis. Each primer set produced amplicons of expected lengths when run in 1% agarose gels (data not shown). All amplicons were sequenced and exhibited 100% identity to the *atp*1 mitochondrial gene under NCBI BLAST analysis. A test comparing autoclaved (121 °C for 20 min) versus nonautoclaved sweet potato puree DNA was run with each primer set. Primer set 174 (Table 1) was chosen as the preferable pair because it exhibited the greatest difference in Ct values between the 2 samples (9 Ct difference versus 8, 5, & 5 for amplicon lengths of 141, 108, and 81 base pairs, respectively). This was expected as longer amplicons would be statistically more likely to experience degradation and fragmentation than shorter ones.

Universal primers for plant products were created using consensus sequences in the *atp1* mitochondrial gene creating a 174 bp amplicon. Therefore, this qPCR protocol could be used for all fruits and vegetable without the necessity of creating a new set of primers for each product. The universality of these primers was confirmed by surveying a variety of vegetables, fruits, nuts, and biofuel precursor (Table 2). The Ct values were determined for fresh and autoclaved product to assure a significant increase and a measurable outcome for each. The qPCR assay utilizes a scale of 0 to 40 for Ct values. The lower the Ct value, the greater numbers of the sequence of interest and the more robust the assay. A Ct value in the teens and low twenties is deemed robust. For uncooked plant products, Ct values were in this range. The exceptions were switch grass, which has a high cellulose content and was milled to 3 mm, and grapes (both Ct = 28). The drying procedure, followed by milling probably fragmented the mtDNA in the switch grass. Many of the fruits, including grapes have high sugar and pectin contents. Lower, more robust, Ct values were

obtained with pectin-containing fruits at a later date, using a different DNA extraction kit which eliminated pectin (MasterPure Plant leaf DNA purification kit; Epicentre) (data not shown). Autoclaved Ct values were obtained for all plant products tested and were well below the maximum of 40; the cut off target cited in the literature and the lower sensitivity of the generated standard curve was *ca.* 35 cycles. Differences between uncooked and autoclaved Cts ranged from 4 to 17 for grape and carrot, respectively. The Ct value represents copy number of gene fragments as illustrated by the standard curve (Figure 1). In an optimized qPCR assay near 100% efficiency, an increase in Ct of 2.5 represents a 1-log reduction in copy number. Therefore, the mtDNA gene copies were fragmented and copy number available for amplification reduced by autoclave treatment (121 °C for 20 min). This 4 to 17 increase in Ct values represents a 1.6- to 6.8-log copy number reduction of the *atp1* gene due to fragmentation.

In commercial applications, DNA extraction methods and time-temperature correlations would have to be determined for each plant product and its thermal process. However, this is relatively simple to determine empirically.

MIQE standards

A standard curve for the universal primers was developed as required by MIQE standards (Bustin and others 2009) (Figure 1). The PCR efficiency was 106%, goodness-of-fit linear regression correlation coefficient (R^2 = 0.9884), linear range of detection from $\log_{10}$ 8.0 to $\log_{10}$ 1.0 copy numbers with 10 copies (corresponding to Ct *ca.* 35 cycles) the limit of detection. These parameters were all within acceptable ranges in MIQE standards.

Correlation between Ct values and thermal death time

To compare the Ct values directly to time-temperature and spore destruction, a hot oil bath was used to reach temperatures above the boiling point of water and to give the operator complete control of hold and cooling times, having determined the CUT for the system. The Ct value of sweet potato puree had a high correlation to time (min) at 121 °C in simple linear regression (R^2 = 0.87) (Figure 3). Variability at each time point was due to DNA extraction efficiency, operator error in pipetting and small sample size. The largest factor in variation would be pipetting error due to dilutions needed to normalize each sample to 5 to 10 ng/μL. While DNA can be solubilized in water, it is a long, sticky molecule and tends to form microscopy clumps resulting in a nonhomogeneous solution. Buffers such as Tris-EDTA (pH 7.5 to 8.0) are used to create a more homogeneous DNA solution. However, these diluents interfere with downstream applications of DNA such as qPCR. In these assays, RT-PCR grade water (Ambion) was used for all dilutions. Final DNA concentrations from extractions ranged from 5 to 500 ng/L. This necessitated the use of different dilutions to normalize DNA sample concentrations prior to qPCR. Less operator handling after DNA extraction would reduce variability. Using a 96-well format with automated DNA and qPCR systems, operator error would be reduced and mean *N* values could be increased to reduce standard errors.

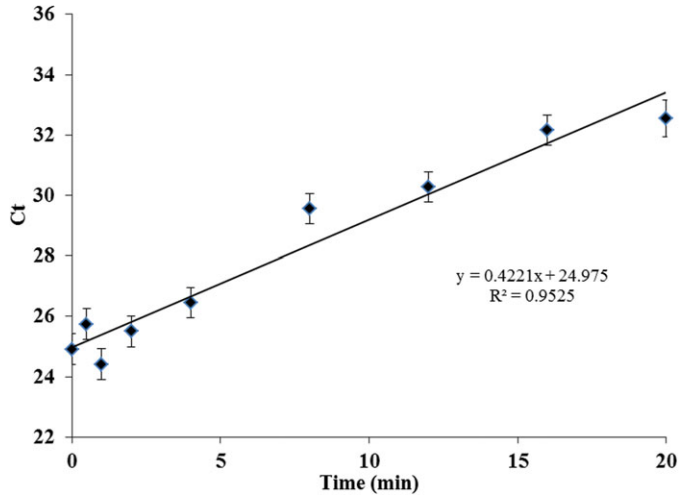

Figure 3–Sweet potato puree mtDNA fragmentation as measured by Ct value of 174 bp amplicon in hot oil bath (121 °C).

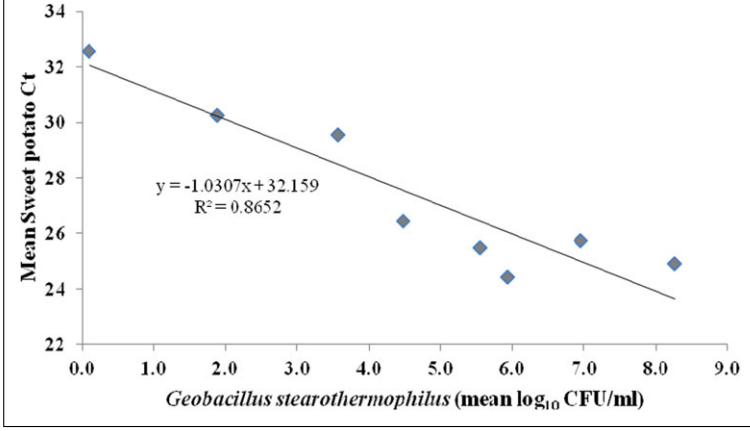

Figure 4–Mitochondrial DNA fragmentation in hot oil bath (121 °C) versus spore counts.

G. stearothermophilus was used as a spore surrogate for *C. botulinum*in a reduction curve. The mean Ct values and log CFU/mL were analyzed by simple linear regression (R^2= 0.87). This study shows that a linear response exists when spores were in the range log 8.0 to 2.0, with log 2.0 being the lowest level of detection (Figure 4). Because it correlates with spore death, Ct could be used as a rapid, presumptive test to assay a product before it is shipped from the factory.

Retort data

Pilot-scale retort data show a high correlation between Ct and cumulative *F*-values of low acid sweet potato puree (R^2 = 0.88) (Figure 5). Data points below the cumulative *F*-value of 3 min tested positive by Prospore GS ampoules (Mesa Laboratories) and above this value tested negative (>6D reduction of GS process). Retort processes are notorious for long CUT and cool down times. Since this was a non-continuous process, cumulative

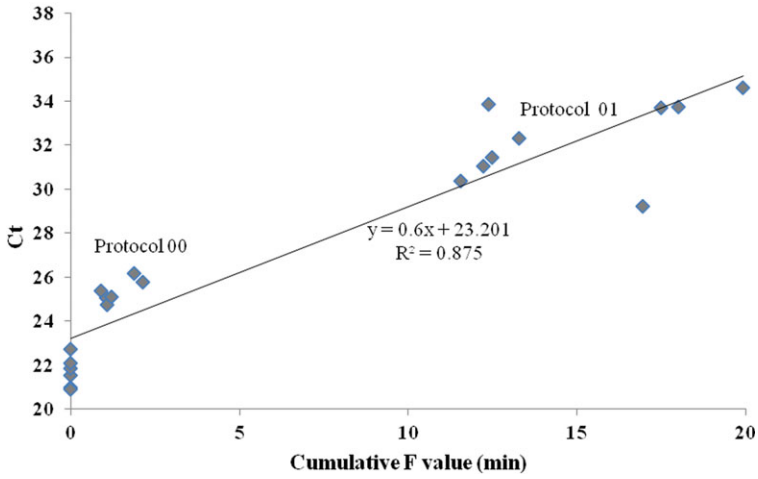

Figure 5–Comparison of mitochondrial DNA fragmentation with cumulative F values with two retort processes using canned sweet potato puree.

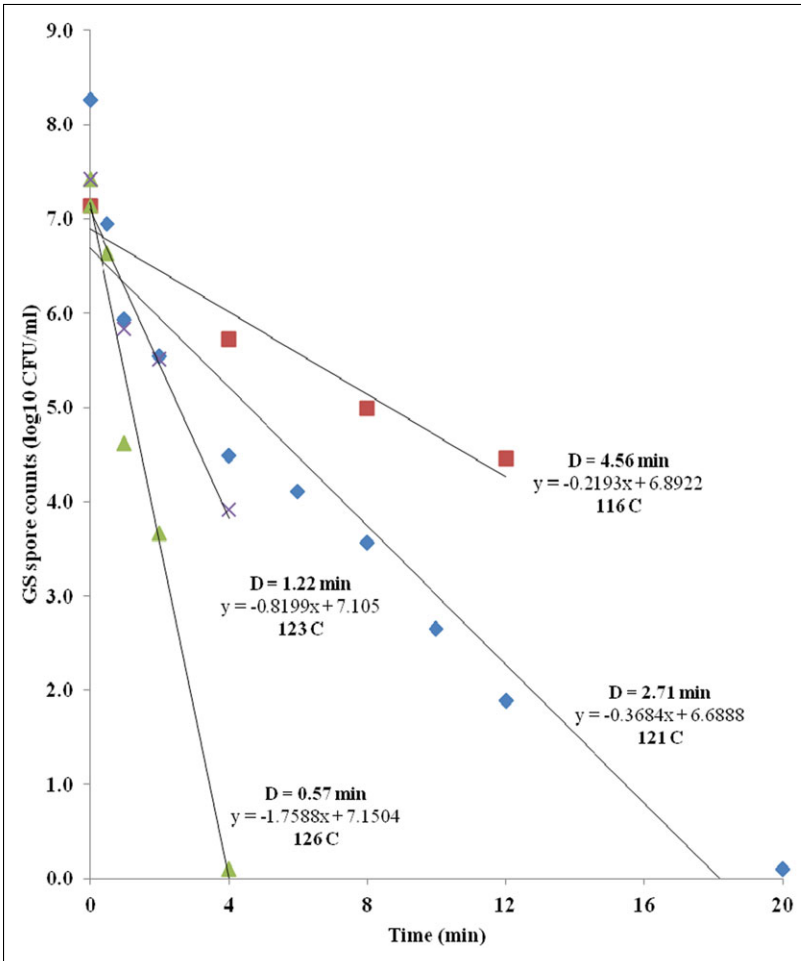

Figure 6–Calculation of *D*-values for timed oil bath treatments.

F-values are permitted. This is a total system approach that uses not just the hold time at target temperature, but the total heating profile. Using inverse prediction statistics, it was determined that for retort process validation, 32 samples would be required for a 2.3% standard error with a cumulative *F* value between 4.66 and 5.33 min for a 99% confidence interval (Table 3). This range of cumulative *F*-values corresponds to the FDA recommended time (5 min) to eliminate biological indicator spores at 121 °C (http://www.fda.gov/RegulatoryInformation/Guidances/ucm071261.htm).

Comparison of *D*- and *z*-values

D_{121} and *z*-values determined in hot oil bath for *G. stearothermophilus* ATCC 7953 (GS) spores were 2.71 min and 11.0 °C (Figure 6 and 7), respectively. These values were slightly higher than a commercial product using the same ATCC strain for autoclave validation (Prospore, Mesa Laboratories) which cited a D_{121} of 1.8 min and a *z*-value of 7.4 °C under saturated steam. Other *D*- and *z*-values for *G. stearothermophilus* spores cited in the literature and on corporate spore supplier web pages are D_{120} from 1.5 to 3 min with *z*-value of greater than or equal to 6 °C (Namsa, Northwood, Ohio, U.S.A.) and D_{121} of ca. 2 min in water (Lundahl 2003). Both of these cited values were based on an initial population of 10^6 spores. Head and others (2007) found that *D*- and *z*-values varied widely based on the initial concentration of spores (10^3 versus 10^6) when treated with superheated steam. While the TDT employed in our assay is a pressurized container, one would not expect the same time-temperature treatment in a

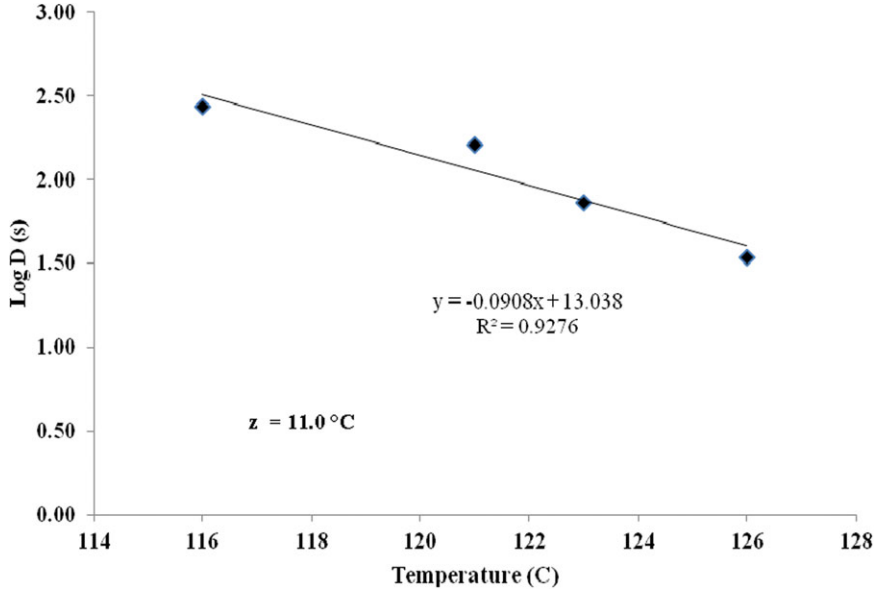

Figure 7–Calculation of z-value for *Geobacillus stearothermophilus* using *D*-values from.

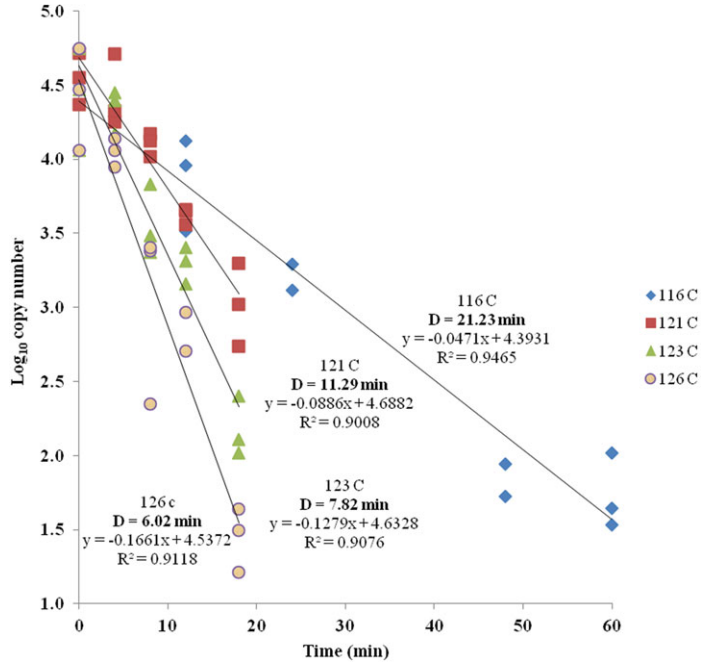

Figure 8–Sweet potato puree *D*-values in hot oil bath based on mtDNA fragmentation using $\log_{10}$ copy number.

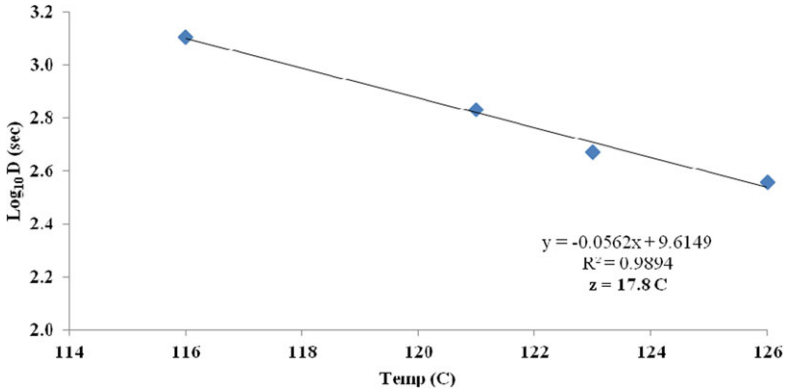

Figure 9–Calculation of *z*-value for sweet potato puree mtDNA fragmentation using *D*-values from Figure 8.

hot oil bath as pressurized, saturated stream in an autoclave. Based on precautionary notes in commercial spore technical data sheets (Prospore, Namsa) and values in the literature, spore *D*- and *z*-values can vary widely due to type of heat treatment (wet versus dry), initial concentration of spores, and spore carrier or media (Head and others 2007). As an added precaution, a safety factor is added to empirically derived data, that is, total death time is rounded up, to ensure complete destruction of spores (Tucker and others 2008).

D_{121} and *z*-values for Ct values from a 174-bp universal plant amplicon were 11.3 min and 17.8 °C (Figure 8 and 9), respectively, for mtDNA from sweet potato puree heated in a hot oil bath. Variability at each time point was due to DNA extraction efficiency, operator error in pipetting during dilutions and small sample size. Variability was analyzed in depth in the hot oil bath discussion. Due to the conversion of Ct to $\log_{10}$ copy number of amplicon the Ct-D_{121} value (11.3 min) was much higher than the *G. stearothermophilus* D_{121} (2.71 min). *G. stearothermophilus* spores have a D_{121} value approximately 10× greater than *C. botulinum* (D_{121} = 0.21 min; Esty and Meyer 1922; Townsend and others 1938; Stumbo 1965), the spore of concern in low acid, canned or aseptically packaged foods. The Ct-D_{121} value of sweet potato puree mtDNA is approximately 4× greater than the *G. stearothermophilus* indicator spore. Because of its higher D_{121} value, it might be difficult to predict the FDA recommended *F*-value for sterilization (F_0 = 5 min) using a log function of Ct value. However, sterilization in the pharmaceutical industry requires higher values (F_0 > 12 min) where *G. stearothermophilus* spores leave no measurable outcome (Lundahl 2003).

When compared directly, the increase in Ct value had nearly a 1:1 ratio with *G. stearothermophilus* destruction at 121 °C in hot oil bath treatments (ratio = 0.97) (Figure 4). A one-unit increase in Ct was calculated as 3.5 min at 121 °C (data not shown) compared to a 1-log reduction of *G. stearothermophilus* at 2.71 min. The destruction of mtDNA as measured by $\log_{10}$ copy number was not a first-order relationship but a simple inverse relationship with time-temperature. Therefore, the use of Ct values directly will have greater utility than conversion to log values.

Conclusions

Fragmentation of mtDNA, as measured by Ct, of low-acid foods at high temperature has a high correlation to time-temperature (Figure 3); cumulative *F*-values (Figure 5) and reduction curves of spore surrogates (Figure 4). This assay represents a rapid, inexpensive, quantitative method that can be used to test low-acid foods in continuous-flow and batch thermal systems for heating efficacy and microbial safety. It is especially useful for thermal processes over 100 °C, since temperatures above boiling can rapidly damage DNA. Its advantages over enzymatic assays are that mtDNA is highly stable and can be stored at freezing temperatures for long periods. Because of the stability of DNA in a food matrix, the product can be stored for many months at −20 °C and assayed later if questions arise. This is an advantage over enzymes and can be used as a process library if a past production needs to be re-analyzed. Unlike endogenous enzymes, mtDNA is universally found in all foodstuffs. mtDNA can be used for all plant products tested. Processors will be able to track process deviations using rapid molecular methods. Processors can use this presumptive test prior to shipping out a product. This method uses no probes, devices or other additives to the continuous-flow or batch systems for monitoring purposes. It utilizes equipment already available in an industrial microbiological testing lab. This method will meet MIQE standards and will be operator-friendly, requires minimal training, and has a 4 h turn-around time. This method can be standardized for consistency between labs and results are operator-independent. Quantitative PCR is a protocol approved by USDA and FDA for detection of bacteria; therefore process authorities are familiar with the technique and are more apt to approve a new use. Both DNA extraction and qPCR have been combined and automated commercially, lending this analysis to high throughput.

This approach to monitoring food safety represents a paradigm shift in the use of qPCR. The fastest moving food particles or a cold spot in a thermal process can be assayed directly, by intrinsic mtDNA, providing a rapid test for thermal efficacy. We propose monitoring and validating the efficacy of thermal processes of low-acid plant foods by using mitochondrial DNA fragmentation detection by qPCR.

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References

Anderson S, Bankier AT, Barrell BG, deBruijn MHL, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier PH, Smith AJH, Staden R, Young IG. 1981. Sequence and organization of the human mitochondrial genome. Nature 290:457–65.

Andreasson H, Gyllensten U, Allen M. 2002. Real-time DNA quantification of nuclear and mitochondrial DNA in forensic analysis. Biotechniques 33(2):402–11.

Bauer T, Weller P, Hammes WP, Hertel C. 2003. The effect of processing parameters on DNA degradation in food. Eur Food Res Technol 217:338–43.

Bogenhagen D, Clayton DA. 1974. The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells. J BiolChem 249:7991–95.

Brinley TA, Dock CN, Truong VD, Coronel P, Kumar P, Simunovic J, Sandeep KP, Cartwright GD, Swartzel KR, Jaykus LA. 2007. Feasibility of utilizing bioindicators for testing microbial inactivation in sweetpotato purees processed with a continuous-flow microwave system. J Food Sci 72:E235–42.

Budowle B, Allard MW, Wilson MR, Chakraborty R. 2003. Forensics and mitochondrial DNA: applications, debates, and foundations. Annu Rev Genomics Hum Genet 4:119–41.

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. 2009. The MIQE Guidelines: minimum information for publication of quantitative real-time pcr *e*xperiments. Clin Chem 55:611–22.

Caldwell JM, Levine JF. 2009. Domestic wastewater influent profiling using mitochondrial real-time PCR for source tracking animal contamination. J Microbiol Methods 77:17–22.

Caldwell JM, Raley ME, Levine JF. 2007. Mitochondrial multiplex real-time PCR as a source tracking method in fecal-contaminated effluents. Environ SciTechnol 41:3277–83.

Caldwell JM, Villemur R, Payment P. 2011. Mitochondrial DNA as source tracking markers of fecal contamination, In Harwood V, Hagedorn C, and Blanch A, editors. Microbial source tracking: methods, applications, and case studies. New York: Springer Science and Business Media. p 229–50.

Claeys WL, Smout C, VanLoey AM, Hendrick ME. 2004. From time temperature integrator kinetics to time temperature integrator tolerance levels: heat-treated milk. BiotechnolProg 20:1–12.

DeCordt SF, Hendrick ME, Maesmans GJ, Tobback PP. 1994. Convenience of immobilized *Bacillus licheniformis* alpha-amylase as time-temperature-integrator (TTI). J Chem Technol Biotechnol 59:193–9.

Esty JR, Meyer KF. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes XI. J Infectious Dis 31:650–63.

Foegeding PM, Busta FF. 1983. Hypochlorite injury of *Clostridium botulinum* spores alters germination responses. Appl Environ Microbiol 45:1360–68.

Foury F, Roganti T, Lecrenier N, Purnelle B. 1998. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. FEBS Lett 440:325–31.

Fujimura T, Matsumoto T, Tanabe S, Morimatsu F. 2008. Specific discrimination of chicken DNA from other poultry DNA in processed foods using the polymerase chain reaction. Biosci Biotechnol Biochem 72:909–13.

Gerber AS, Loggins R, Kumar S, Dowling TE. 2001. Does nonneutral evolution shape observed patterns of DNA variation in animal mitochondrial genomes? Annu Rev Genet 35:539–66.

Gryson N. 2010. Effect of food processing on plant DNA degradation and PCR-based GMO analysis: a review. Anal Bioanal Chem 396:2003–22.

Guan D, Gray P, Kang DH, tang J, Shafer B, Ito K, Younce F, Tang TCS. 2003. Microbiological validation of microwave-circulated water combination heating technology by inoculated pack studies. J Food Sci 63:1428–32.

Guiavarch Y, Van Loey A, Zuber F, Hendrick M. 2004. Development characterization and use of a high-performance enzymatic time-temperature integrator for the control of sterilization process' impacts. Biotechnol Bioneng 88(1):15–25.

Guiavarch Y, Zuber F, Van Loey A, Hendrick M. 2005. Combined use of two single-component enzymatic time-temperature integrators: application to industrial continuous rotary processing of canned ravioli. J Food Prot 68:375–83.

Handa H. 2003. The complete nucleotide sequence and RNA editing content of the mitochondrial genome of rapeseed (*Brassica napus* L.): comparative analysis of the mitochondrial genomes of rapeseed and *Arabidopsis thaliana*. Nuc Acids Res 31(20):5907–16.

Head DS, Cenkowski S, Holley R, Blank G. 2007. Effects of superheated steam on *Geobacillus stearothermophilus* spore viability. J Appl Microbiol 104:1213–20.

Hird H, Chisholm J, Sanchez A, Hernandez M, Goodier R, Schneede K, Boltz C, Popping B. 2006. Effect of heat and pressure processing on DNA fragmentation and implications for the detection of meat using a real-time polymerase chain reaction. Food Addit Contam 23:645–50.

Hopwood AJ, Mannucci A, Sullivan KM. 1996. DNA typing from human faeces. Intl J Legal Med 108(5):237–43.

Hrncirova Z, Bergerova E, Siekel P. 2008. Effects of technological treatment on DNA degradation in selected food matrices of plant origin. J Food Nutr Res 47:23–28.

Jett BD, Hatter KL, Huycker MM, Gilmore MS. 1997. Simplified agar plate method for quantifying viable bacteria. BioTechniques 23:648–50.

Kharazmi M, Bauer T, Hammes WP, Hertel C. 2003. Effect of food processing on the fate of DNA with regard to degradation and transformation capability of *Bacillus subtilis*. Syst Appl Microbiol 26:495–501.

Lahiff S, Glennon M, O'Brien L, Lyng J, Smith T, Maher M, Shilton N. 2001. Species-specific PCR for the identification of ovine, porcine and chicken species in meta and bone meal (MBM). Mol Cell Probes 15(1):27–35.

Lipp M, Brodmann P, Pietsch P, Pauwels J, Anklam E, Börchers T, Braunschweiger G, Busch U, Eklund E, Eriksen FD, Fagan J, Fellinger A, Gaugitsch H, Hayes D, Hertel C, Hörtner H, Joudrier P, Kruse L, Meyer R, Miraglia M, Müller W, Phillipp P, Pöpping B, Rentsch R, Wurtz A. 1999. IUPAC collaborative trial study of a method to detect genetically modified soy beans and maize in dried powder. J AOAC Intl 82(4):923–8.

Lundahl G. 2003. A method of increasing test range and accuracy of bioindicators: *Geobacillus stearothermophilus* spores. PDA J Pharm Sci Technol 57:249–62.

Marcy JE. 1997. Biological validation. Food Technol 51:48–52.

Meyer R, Candrian U. 1996. PCR-based DNA analysis for the identification and characterization of food components. Food Sci Technol-Lebensm-WissTechnol 29:1–9.

Millar AH, Whelan J, Soole KL, Day DA. 2011. Organization and regulation of mitochondrial respiration in plants. Annu Rev Plant Biol 62:79–104.

Moreano F, Busch U, Engel KH. 2005. Distortion of genetically modified organism quantification in processed foods: influence of particle size compositions and heat-induced DNA degradation. J Agric Food Chem 53:9971–79.

Murray SR, Butler RC, Hardacre AK, Timmerman-Vaughan GM. 2007. Use of quantitative real-time PCR to estimate maize endogenous DNA degradation after cooking and extrusion or in food products. J Agric Food Chem 55:2231–39.

Neter J, Wasserman W, Kutner M. 1983. Simultaneous inferences and other topics in regression analysis-1. Appl Linear Regression Models 150–53.

Orta-Ramierez A, Merrill JE, Smith DM. 2001. Sucrose, sodium dodecyl sulfate, urea and 2-mercaptoethanol affect the thermal inactivation of R-phycoerythrin. J Food Prot 64:1806–11.

Pflug IJ. 1990. Microbiology and engineering of sterilization processes. Environmental Sterilization Laboratory; Minneapolis, MN, 7th ed.

Pflug IJ, Odlaug TE, Christensen R. 1985. Computing a minimum public health sterilizing value for food and pH values from 4.6 to 6.0. J Food Prot 48:848–50.

Pflug IJ, Smith G, Holcomb R, Blanchett R. 1980. Measuring sterilizing values in containers of food using thermocouples and biological indicators units. J Food Prot 43:119–23.

Reyes-de-Corcuera JI, Cavalieri RP, Powers JR, Tang J, Kang DH. 2005. Enzyme-electropolymer-based amperometric biosensors: an innovative platform for time-temperature integrators. J Agric Food Chem 53:8866–73.

Sakalar E, Abasiyanik MF, Bektik E, Tayyrov A. 2012. Effect of heat processing on DNA quantification of meat species. J Food Sci 77:N40–44.

Smith GM, Kopelman M. 1982. Effect of environmental conditions during heating on commercial spore strip performance. Appl Environ Microbiol 44:12–18.

Smith SE, Orta-Ramirez A, Ofoli RY, Ryser ET, Smith DM. 2002. R-phycoerythrin as a time-temperature integrator to verify the thermal processing adequacy of beef patties. J Food Prot 65:814–19.

Stam CN. 2008. Development of novel biological indicators to evaluate the efficacy of microwave processing. Doctoral Disseration. NCSU Food Science; Raleigh, NC.

Steed LE. 2010. Development and validation of processes for continuous flow microwave processing of foods containing sweet potato particulates. Doctoral Disseration. NCSU Food Science; Raleigh, NC.

Stumbo CR. 1965. Thermobacteriology in food processing. 1st ed. 111 Fifth Ave, New York, Academic Press.

Townsend CT, Esty JR, Baselt FC. 1938. Heat-resistance studies on spores of putrefactive anaerobes in relation to determination of safe processes for canned foods. Food Res 3: 323–46.

Truong VD, Walter VM, Jr. 1994. Physical and sensory properties of sweetpotato puree texturized with cellulose derivatives. J Food Sci 59:1175–80.

Tucker G, Shaw H, Christian G, Gaze J. 2008. History of the minimum botulinum cook for low-acid canned foods. Campden & Chorleywood Food Research Association Group. R & D report no. 260. Doc Ref:FMT/REP/90194/1.

van Melis CCJ, Nierop Groot MN, Tempelaars MH, Moezelaar R, Abee T. 2011. Characterization of germination and outgrowth of sorbic acid-stressed Bacillus cereus ATCC 14579 spores: phenotype and transcriptome analysis. Food Microbiol 28:275–83.

Veeramuhu GJ, Price JF, Davis CE, Booren AM, Smith DM. 1998. Thermal inactivation of *Escherichia coli* O157:H7, *Salmonella senftenberg*, and enzymes with potential as time-temperature indicators in ground turkey thigh meat. J Food Prot 61:171–75.

Wang JP, Deng L, Li Y, Xu XM, Gao Y, Nahimana H, Chen HQ, Jin ZY, Kim JM, He LF. 2010. Konjacglucomannan as a carrier material for time-temperature integrator. Food SciTechnolInt 16:127–34.

Yen CY. 2009. Development and testing of enzymatic time-temperature integrator devices under isothermal and non-isothermal conditions. Masters Thesis. NCSU Food Science; Raleigh, NC.

Zhang CL, Fowler MR, Scott NW, Lawson G, Slater A. 2007. A TaqMan real-time PCR system for the identification and quantification of bovine DNA in meats, milks and cheeses. Food Control 18:1149–58.

Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Appendix A. Protocol 00: Full water immersion

Appendix B. Protocol 01: Full water immersion