ABSTRACT

CALDWELL, JANE MARY. Mitochondrial DNA Fragmentation: A Molecular Tool to Monitor Food Processing Safety and Quality. (Under the direction of Dr. Ilenys Pérez-Díaz and Dr. KP Sandeep.)

In the food industry and scientific literature, there are molecular methods using polymerase chain reaction (PCR) for detection of contaminants in meats and plant products, identification and authentication of food ingredients, and detection of transgenic species or genetically-modified organisms (GMOs). Mitochondrial DNA, from the energy-producing organelles in eukaryotic cells, is used in many disciplines which deal with degraded samples because it has more copies than nuclear genes and gives a greater sensitivity in quantitative PCR (qPCR). Instead of using qPCR to detect pathogens, identify adulterants or authenticate ingredients, this rapid technique was used in this study to quantify the fragmentation of intrinsic plant mitochondrial DNA (mtDNA). The degradation of such high copy number DNA fragments is specifically used as a time-temperature integrator (TTI) to validate thermal processing in fresh plant materials, and to forensically determine fermentation times and shelf-life in acid foods. Universal primers were developed which amplified a mitochondrial gene common to plants (atp1). These consensus primers produced a robust qPCR signal in ten vegetables, six fruits, three types of nuts and a biofuel precursor. Using sweet potato (Ipomoea batatas) puree as a model low-acid product and simple linear regression, threshold cycle (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.
Mitochondrial DNA fragmentation was assessed in several cucumber-to-pickle processes including fermentation, pasteurization, and storage time at room temperature. Cucumber pickle mtDNA fragmentation, as measured by Ct value, was significantly different based on shelf-life alone (p<0.05). The fermented, pasteurized pickle had a similar Ct value to the autoclaved cucumber. This indicates that lower temperature thermal processes (hot fill at 75 °C for 15 minutes) under acidified conditions (pH = 3.8) yield similar mtDNA fragmentation results when compared to more elevated conditions (121 °C) in low-acid foods. In a second study, cucumbers fermented in brine using the same protocol for 7, 8, 9, 11 and 12 months. The Ct value of intrinsic pickle mtDNA increased linearly and were significantly different (p>0.05) when compared to fermentation time in months. Mitochondrial DNA fragmentation was shown to be a potential new tool to monitor low-temperature (<100 °C) high acid processes (pH<4.6), and non-thermal processes such as vegetable fermentation times and shelf-life of acidified products. Finally, dry roasted peanuts were assayed using the same novel molecular TTI. Despite low moisture content, low water activity and non-animal origins, peanuts and peanut butter from roasted peanuts, have recently been implicated in *Salmonella* outbreaks. *Enterococcus faecium* was evaluated as a *Salmonella* surrogate for process validation and compared to fragmentation of intrinsic peanut mtDNA and Hunter L color, a quality indicator, for dry roasting. Mitochondrial DNA fragmentation was not linear compared to time at a given temperature, but exhibited a long lag time. Dissection of peanuts exhibited a differential heating effect depending on the part of the peanut used for DNA extraction and the type of tissue assayed. MtDNA fragmentation as measured by Ct value was deemed too variable for thermal process or quality validation of dry, solid foods such as
peanuts. However, it could be used to evaluate penetration of heat through a solid food matrix. We present these data as proof-of-concept for a molecular tool which can be used as a rapid, presumptive method for monitoring thermal efficacy, food safety, fermentation and shelf life of plant foods.
DEDICATION

For my husband, Paul Demming Hubert II.
BIOGRAPHY

Jane Mary Caldwell is an inventor, scientist, explorer, writer, teacher, musician, singer, dancer, wife, mother, confidante and bon vivant. She lives with her husband, rat terriers, goldfish and Tennessee Walking horses in Wake County, North Carolina. She is finishing her Ph.D. after a 25-year hiatus.
ACKNOWLEDGMENTS

The author would like to acknowledge Janet Hayes for lab management, ordering supplies and troubleshooting equipment; Sandra Parker and Beth King for secretarial, grant and travel assistance; Bernard Eckhardt for IT support; Michael Bumgardner, Jack Canady, and Karl Hedrick for their expert engineering acumen; Joy Smith and Dr. Jason Osborne for statistical analysis; Michelle Borges for summer internship in pickle fermentation; and Kaitlyn Casulli for collaboration with high pressure processing. The author would also like to thank Mara Massel, Jim Lockhart, Juliebeth Briseno, Gary Cartwright, Debra Clare, Evelyn Durmaz, Keith Hendrix, Fred Jimenez, Sarah O’Flaherty, Rong Reynolds, Rosemary Sanozky-Dawes, and Allison Smathers. It takes a village to make a Ph.D.
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Abstract

Mitochondrial DNA is used in many disciplines which deal with degraded samples because it has more copies than nuclear genes and gives a greater sensitivity in conventional polymerase chain reaction (PCR) and quantitative PCR (qPCR). In this review, we describe molecular methods using mtDNA for detection of contaminants in meats and plant products, identification and authentication of food ingredients, detection of transgenic species or genetically-modified organisms (GMOs), examining the effects of processing on the sensitivity of molecular assays. A new paradigm in mtDNA-qPCR is explored: using fragmentation of mtDNA to validate thermal processing in fresh plant materials, fermentation and shelf-life in acid foods.

Introduction

Consumers and processors need to be able to verify and validate food ingredients for a variety of nutritional, personal, religious, safety and ethical reasons. They also demand wholesome foods with truthful labels. Questions of authenticity include stated geographic origins, species identification and verification, Halal/Kosher requirements, and inclusion of genetically modified foods (GMOs). Safety considerations include traceability, microbial pathogens or chemical contaminants, hidden allergens, and contamination by unintended ingredients. Contamination by unintended ingredients could include cereals in gluten-free foods, allergens such as nuts, beef or pork in vegetarian or vegan foods. The development of molecular methods utilizing DNA from foodstuffs directly, increases the sensitivity, power
of the test, reduces time and increases confidence in food ingredients when compared to enzymatic and chemical assays. Molecular methods developed in the late twentieth century, conventional PCR, real-time PCR (qPCR), random amplification of polymorphic DNA (RAPD), DNA “fingerprinting”, and DNA sequencing, have begun to spread to commercial use in the food industry as they increase accuracy of test and decrease the time needed for ingredient validation. Many of these molecular techniques require highly trained personnel, standardized protocols and databases. Therefore, third-party validation is a growth industry in food testing. Protocols requiring gels, restriction digests and sequencing are not rapid enough for inspection purposes. However, many commercial kits relying on rapid qPCR methods are available for authentication, especially for identification of meat species.

**Mitochondrial DNA as Target**

DNA is targeted for identification of many plant and animal products due to its relatively high heat stability compared to proteins and other cell components which denature during processing. (Doktycz, 1997) All eukaryotes, both plant and animal, contain in their cells, multiple copies of non-nuclear DNA, mitochondrial DNA (mtDNA). Mitochondria are energy-producing organelles found in multiple copies in all cells of eukaryotes, and each mitochondrion possesses its own genome in multiple copies (Figure 1). These properties make mtDNA sequences excellent targets for amplification in terms of specificity, sensitivity and robustness due to multiple copies per cell (>1,000) (Gerber et al. 2001; Andreasson et al. 2002). Because of high copy numbers in cells, mitochondrial DNA offers an advantage over nuclear DNA for molecular detection methods and is used in disciplines such as archaeology and forensics where highly degraded samples are found. The food industry uses mtDNA for
detection because processing methods such as baking, boiling, frying and microwaving degrade DNA and make it more difficult to detect (Arslan et al. 2006). Because they produce energy in eukaryotic systems, mitochondrial genes are highly conserved (Andreasson et al. 2002). Therefore, mtDNA genes are suitable for universal primers, creating an assay which can be useful for a wide variety of foods, or conversely finding species-specific targets in non-coding regions where variations in sequence occur.

**Verification of Meats**

Meat from different species are often ground and combined for hamburger patties at a certain price point and fat content. Many consumers have taboos and religious laws concerning species allowed for consumption. Vegetarian diets, excluding meats of all kinds, are the norm for many. In the United States, Europe and other western cultures, consumers view horses and dogs as pets and companions and do not want to eat them. Consumers want to know if exotic meats such as kangaroo, rat or snake have been added to ground meats as these species are not traditionally consumed in certain cultures. Concerns over bovine spongiform encephalopathy (BSE) and variant Creutzfeld Jacob Disease (vCJD) from beef products spurred development of detection methods in both human food and cattle feeds containing meat and bone meals. Halal/Kosher laws forbid consumption of pork products. Therefore many researchers have developed meat species detection assays based on mitochondrial genes (Table 1).

While PCR detection methods using the bovine growth hormone gene in beef (Brodmann & Moor et al. 2003) and the actin gene in chicken (Hopwood et al. 1999) were found in a survey of the literature, the majority of the meat species detection assays (>24)
used mitochondrial genes (Table 1). Genes most commonly used were cytochrome b (cytb), 12S and 16S ribosomal RNA (rRNA), NADH dehydrogenase subunits (ND2, ND4, ND5), ATP synthase subunits (atp6-8), the D-loop region, and transfer RNA (tRNA-valine). Two articles cited only the mitochondrial coding region (Arslan et al. 2006; Goncalves et al. 2012) and one laboratory tested the efficacy of a commercial kit which had proprietary primers and unknown amplicon (Demirhan et al. 2012). Most PCR targets are chosen based on the amount of sequence available in public databases such as NCBI Genbank. Amplicons were larger for conventional PCR, while qPCR assays utilized amplicons below the recommended maximum length of 250 bp, due to the kinetics of the protocol. One article developed a multiplex PCR assay (Dalmasso et al. 2004) to differentiate between ruminants, poultry, fish and pork. Most articles combined PCR with sequencing, restriction fragment length polymorphism (RFLP) or RAPD and used single nucleotide polymorphisms (SNPs) or gel band sizes to distinguish between species (Table 1).

Molecular methods can utilize hyper-variable regions of mtDNA sequence and single nucleotide polymorphisms (SNPs) to differentiate between animals, even closely related species (van Asch et al. 2011). Scientists have created conventional PCR assays for the identification of partridge meat sources; discriminating between red-legged, chukar, barbary and gray partridge species using mitochondrial genes and genomic rhodopsin genes (Rojas et al. 2001). These assays would be used in inspection programs to verify labeling of partridge meat products. Others have used mitochondrial genes and the actin gene to identify species in meat products to ensure reliable labeling (Rastogi et al. 2007). This group was able to differentiate between buffalo, goat, cow, chicken and pig samples using mtDNA-based PCR
and RAPD. A library of reference fingerprint patterns for the meat species would be needed to quickly identify meats in foods. Horreo and others (2011) created universal 16S rRNA primers for identification of different meats including various types of fish, pork, turkey, beef, sheep and poultry. This protocol is not rapid as it requires sequencing of PCR products. Some researchers have used mtDNA single nucleotide polymorphisms (SNPs) as molecular diagnostic tools to differentiate between Asian and European *Sus scrofa* (pig) species (van Asch et al. 2011). These researchers recommended mtDNA as it is more abundant than genomic DNA and therefore, a more reliable target after heating and processing which degrades DNA.

Speciation is also critical in non-meat products created from animal derivatives. In Muslim and Jewish households, the presence of porcine products is not allowed. Gelatine and gelatin-containing foods can be made from animal skin, seaweed or fish. It is important for adherents of Halal and Kosher practices to avoid gelatine made with pigskin. Demirhan and others (2012) validated a commercially-available real-time PCR detection assay using the mitochondrial cytochrome b gene. The limit of detection of the assay was cited as 1.0% adulteration on a weight by weight basis in two products: marshmallows and gum drops (Table 1).

Many of the assays listed in Table 1 did not meet Minimum Information needed for Quantitative Experimentation (MIQE) standards for qPCR (Bustin, 2009) which features a quality control checklist on sample processing, nucleic acid extraction, target amplicon specifications, reaction optimization, specificity of reaction, internal amplification controls (IAC), calibration curves with calculated PCR efficiency, linear dynamic range, limits of
detection and data analysis including repeatability and statistical methods. For an assay to gain widespread use and acceptance, it must be operator- and laboratory-independent. Conformity to MIQE standards allows comparisons between labs and standardizes results.

Commercial kits are available for rapid detection of meat species by qPCR. One corporation specializing in food safety, Neogen ([www.neogen.com](http://www.neogen.com)), provides qPCR detection for beef, chicken, goat, horse, pork, rabbit, sheep, turkey and seven fish species. Its closest competitor, Nimagen ([www.nimagen.com](http://www.nimagen.com)), detects the usual suspects plus dog, donkey, buffalo and warthog. The targets used are proprietary, but mitochondrial genes are probably employed.

**Detection of GMOs in Plant Ingredients**

Authentication of plant foodstuffs has been recently driven by the inclusion of transgenic products in formulations. Public distrust of new plant breeding methods has caused hysteria over foods containing Genetically Modified Organisms (GMOs), organisms modified by non-breeding methods such as transfection, gene insertion or mutation. Many consumers refuse to buy foods that contain GMOs, so tests are needed to assure consumers and increase confidence in products. They fear recombination events due to manipulation of genetic materials directly, preferring the use of slower, traditional methods such as plant breeding or random mutation protocols. Few studies have been conducted which quantify the likelihood of “Franken-foods” horizontally-transferring genetic materials in the gut. Kharazmi and others (2003) calculated the hypothetical frequency of transformation of transgenic DNA from a cooked potato to a model microorganism, *Bacillus subtilis*. Taking into account the effects of thermal and mechanical processing on DNA fragmentation, they
cited the frequency as $8.5 \times 10^{-19}$ for homologous recombination. In real terms, an individual would have to consume 130 g of cooked potato daily for $10^8$ years for this recombination to occur once. Stated another way, the entire world population would have to consume cooked transgenic potato daily for 15 days before one individual would be exposed to a transformant generated by an homologous recombination.

Despite acceptance of transgenic foods by the FDA, World Health Organization, the American Medical Association and the United Stated National Academy of Science, molecular techniques have been developed for detection of GMOs in processed foods. Mitochondrial DNA were included in some assays as the positive control, a test for amplification inhibitors in PCR and DNA extraction efficiency (Meyer, 1999). Targeted products include grits, cornmeal, corn flour, soybean flour, formulations containing tomatoes and potatoes. Researchers developed methods to detect and quantify approved transgenics such as genetically-modified potatoes, FlavrSavr™ tomatoes, Roundup Ready™ Soya, and Bt-maize (Meyer, 1999; Debode et al. 2010; Lipp et al. 1999; Debode et al. 2007; Moreano et al. 2005; Beletsiotis et al. 2011). Detection relies on amplification by PCR of modified or inserted genes such as the 35S promoter and NOS terminator in genetically modified soy beans and maize (Lipp et al. 1999). Researchers were able to correctly identify samples containing 2% GMOs. False-positives were low (2 out of 105 samples) and attributed to contamination of laboratory equipment, an on-going problem with PCR detection assays.

Commercial kits for the quantification of Bt corn are available. Moreano and others used the GMOQuant Maximizer Bt176 Corn kit (GeneScan Europe; Bremen, Germany) to evaluate the effects of milling and heat on the quantification of GMOs in corn products such
as grits, meal and flour. They found that mechanical processes such as milling caused over- and under-estimates of GMO products when comparing products with different particle size distributions. Heat-induced DNA degradation was blamed as the probable factor causing reduced detection in heat-treated corn products (Moreano et al. 2005). Gryson, in a 2010 review article, gives an excellent summation of the effects of food processing on plant DNA degradation and its effects on the efficacy of PCR-based GMO detection. Laboratories have also added new molecular technologies to PCR to increase its sensitivity. For example, Sforza and others (2011) described the use of peptide nucleic acid probes (PNAs) to increase the sensitivity of PCR by increasing the binding capacity of primers and using shorter primers for the same effect. Debode and others (2010) designed plasmids for the purpose of multiplex calibration in GMO detection by qPCR.

**Plant Food Authentication and Geographic Origin**

Food authentication is concerned with the verification and validation of geographical, species and ingredient claims. It proves that foods are consistent with ingredient labels, wholesome and unadulterated with lower quality or less costly ingredients or fillers. Polymerase chain reaction has also been applied to authentication of plant ingredients. Mitochondrial coding sequences in plants are highly conserved (Handa, 2003). This may be the principal reason scientists use nuclear non-coding regions, microsatellite DNA or sequence from another organelle, the chloroplast, to differentiate between plant species. Two food additives, locust bean gum (LBG) and guar gum, are used as stabilizing agents in the food industry (Urdiaien et al. 2005). Guar gum is less expensive than LBG and a suspected adulterant in commercial products. Molecular certification of commercial gums (Urdiaien et
al. 2005) was developed to differentiate between the two. Although polysaccharides, these gums contained enough contaminating plant DNA for gene detection and differentiation by PCR. Researchers had to find a DNA extraction method which eliminated the sticky and viscous polysaccharide co-precipitants. Using the nuclear ribosomal intergenic spacer (ITS) sequences as targets, they created a rapid, simple molecular detection method to discriminate between the two gums via conventional PCR. Lee et al (2009) used chloroplast DNA to authenticate 21 Artemisia (Wormwood herb) species. They combined PCR with restriction fragment length polymorphisms (RFLP) to correctly identify different herb species.

Molecular methods other than PCR have been successfully validated. Faria and others (2000) used microsatellite DNA analysis (SSR) to differentiate between grape varietals. Discrimination between Robusta and Arabica coffee was facilitated by sequencing and discovering a unique SNP in the chloroplastic intraspacer region, resulting in different ‘chlorotypes’ for each species (Trantakis et al. 2011).

Geographic origin is important for labeling of regional food which have added value because of their origins. Researchers in Chile (Martinez et al. 2007) discovered a high correlation between molecular polymorphisms and geographic origins of wine yeast strains when using mitochondrial DNA restriction analysis followed by random amplification of polymorphic DNA (RAPD) for construction of phylogenetic trees. They were able to confirm the geographic origin of 64 wine yeast strains using mtDNA and molecular methods.

**Effects of Processing on DNA Fragmentation**

Molecular methods are highly dependent on the quantity and quality of DNA available from the foodstuff. There are many articles which discuss the effects of processing
on the sensitivity and reliability of the PCR assays. Heat, physical processes like milling which reduce particle size, composting, pressure, drying, smoking, salting and extrusion all have the potential to fragment cellular components including DNA. These processes can affect the primary and secondary structure of the molecule through hydrolysis and oxidation (Meyer, 1999; Kharazmi et al. 2003). Fragmentation of DNA reduces the sensitivity of molecular methods, hinders quantification and increases the limits of detection (Kharazmi et al. 2003).

**Basic Mechanisms of DNA Fragmentation**

DNA destruction by heat occurs due to depurination or deamination (Gryson, 2010). The most labile linkage in the DNA structure is the N-glycosyl bond, which is susceptible to hydrolysis (Lindahl 1993). Heat increases the rate of many reactions, including the production of reactive oxygen species which cleave the phosphodiester bond when scavenging electrons. Unlike the nucleus which is sequestered from oxygen, the mitochondria participate in oxidative phosphorylation to provide energy to the cell, so mtDNA should be more prone to oxidative damage (Lindahl, 1993). Above 100 °C, helical strands are broken causing an irreversible loss of secondary structure (Lindahl, 1993). Acidic pH conditions also cause depurination of DNA, leading to strand cleavage (Sambrook & Russel, 2001). However, DNA is relatively stable at alkaline pH values up to 9.5 (Gryson, 2010).

The effects of temperature, pH and the combination of the two parameters on DNA fragmentation were analyzed by Bauer and others (2003) using plasmid DNA (pSG100, a derivative of pUC18). They were able to monitor the first strand single break in the plasmid
by HPLC, expressing the results as percentage of covalently-closed circular plasmid (CCC). With un-nicked plasmid DNA or CCC expressed as 100%, they detected a decrease of CCC to 85% after 90 min of incubation at 85 °C/pH 8.4. Under acidic conditions at lower temperature (37 °C/pH 4.0), the CCC was reduced to 64% after 90 min. The greatest percentage of single-nicked plasmid occurred when temperature and acid were combined (65 °C/pH 4.0), resulting in 1% of CCC remaining after 90 min of incubation. However, since temperatures and pH values were not consistently paired, definitive conclusions concerning the relative effects of treatments could not be reached. Two more closely paired treatments looked at the effect of temperature and low pH versus food matrix (65 °C/pH 4.0 in buffer versus 65 °C/pH 4.3 in tomato serum) and were found to have identical results at 25, 60 and 90 min, implying that there were no protective or accelerating effects from components of the tomato serum. This article looked at damage of the DNA phosphodiester bond (single-strand nicking) only. Since mitochondrial DNA are also circular and relatively small when compared to nuclear DNA, the analogy to plasmid nicking would be appropriate. Cleavage of the phosphodiester bond would be the initiating step in DNA fragmentation, then oxidation and hydrolysis of nucleotides could follow due to increased conformational availability to enzymes and reactive oxygen species.

**Effect of Heat**

DNA can be brought up to temperatures approaching the boiling point of water (95 °C), where it will denature and unravel, then reform into its original configuration when brought down to the annealing temperature of its base pairs. This describes the process of polymerase chain reaction, which works due to the stability and fidelity of nucleic acid
chains at temperatures above physiological conditions. A temperature of 100 °C does not degrade DNA very severely (Gryson, 2010). However, above 100 °C, DNA can become destabilized and permanently damaged. When the effect of thermal treatment on DNA degradation in legumes and maize was studied (Hrncirova et al. 2008), researchers found treatment at 80 °C/360 min had no effect on amplification of amplicons ranging in size from 874 to 1371 bp as measured by conventional PCR. Treatments at 100 and 200 °C did exhibit a drop in amplification after 60 and 30 minutes, respectively; when amplicons longer than 1 kb were no longer detectable after PCR and gel electrophoresis.

Despite high temperature (121 °C) and pressure, it was reported that autoclaving for 15 to 20 min did not completely fragment and deactivate DNA found on samples, plastic and glassware (Suyama & Kawaharasaki, 2013). Using a 2682 bp DNA oligonucleotide and an 84 bp nested amplicon for detection, these researchers were able to amplify residual DNA from the bottom water of the autoclave pan after autoclaving for standard conditions for 20 min. Amplifiable DNA was destroyed only after autoclaving at 121 °C for 80 min. Because molecular methods are extremely sensitive, they recommended using other methods to dispose of DNA to reduce laboratory contamination.

**Relationship Between Amplicon Length and Heat**

Sakalar and others (2012) studied the effects of several cooking regimes, including baking and boiling, on the identification and quantification of meat species. They concluded that the results of qPCR detection of mtDNA were influenced by time-temperature and the amplicon size. The baking of beef, pork and chicken (amplicon sizes 374, 290 & 183 bp, respectively) caused an increase in Ct value within 10 min at 200 °C. They recommended
employing small amplicons (<100 bp) to lessen the effect of heat on the sensitivity of the qPCR assay. Others studying the effects on heat and pressure on DNA fragmentation in turkey meat, found baking and boiling had little effect when measured by qPCR using TaqMan probe, small amplicon size (<200 bp) and targeting the mitochondrial cytochrome b gene (Hird et al. 2006). These researchers measured the effect of amplicon length by designing amplicons which had the same reverse primer and TaqMan probe, but different forward primers. The forward primers were used to increase the length of the amplicons and ranged in size from 81, 116, 240, 317, 351, 427, 470 to 565 bp. They observed an increase in Ct value with increasing amplicon size. Amplicons up to 240 bp had similar Ct values, but over that value, the increases in Ct value were more drastic. Canned and autoclaved turkey gave numerically higher Ct values than raw, boiled and baked product when using longer amplicons (<300 bp). They concluded that designing longer amplicons might improve specificity while still providing sufficient measurable signal after harsh thermal treatments of meats. However, this article did not provide standard curves for each amplicon size, showing PCR efficiency, linear range of detection and lower detection limits. The increases in Ct value may have been partly or fully due to lack of assay optimization.

Other researchers contend that thermal processing results in not only increased DNA fragmentation but changes in DNA extraction efficiencies which could affect downstream applications such as PCR (Murray et al. 2007)

**Effects of Other Processing Methods**

Besides heat, other processes degrade intrinsic food DNA. Bauer and others (2004) studied the effects of processing parameters on potato products and found that drying exerted
the strongest effect when measured by qPCR (increase in Ct value). However, certain food components reduced DNA degradation by DNase I when measured by plasmid transformation frequency in *E. coli*. Plasmids in the presence of maltol or putrescine had DNA degradation reduced by one order of magnitude. Degradation was completely inhibited by the presence of slightly higher levels of maltol, octyl gallate and spermine (Bauer et al. 2004). Processes which result in the disruption of cell walls and membranes result in the release of endonucleases which fragment DNA (Kharazmi et al. 2003). However, under acidic pH conditions, the DNA-degrading enzymes are destroyed more rapidly than the DNA itself and their effect is minimized (Herman, 1997). Endonucleases may also cleave DNA during storage of fresh foods (Chen et al. 2007).

DNA was also degraded at environmental temperatures. Bovine mtDNA, as measured by qPCR, was reduced 93% due to composting of cattle carcasses under ambient environmental conditions for 147 days (Xu et al. 2009). The goal of Xu and others (2009) was to determine the end point of the mortality compost prior to its use as fertilizer on crop land. This article did not speculate on the biochemical and physical mechanisms of DNA degradation.

In a review article describing the effect of food processing on DNA degradation, Gryson (2010) provides a comprehensive list of processes which increase Ct value in qPCR: cooking, microwaving, fermentation, grinding, filtering, sterilization, acidification, salting, bleaching, degumming, centrifugation, drying, wet-milling, grinding, ensiling and sonication. The article asserts the difficulty in obtaining good quality DNA from a highly processed food like corn flakes. Furthermore, of all the shear forces, grinding has the greatest effect on
DNA fragmentation. This could impact the use of certain DNA extraction methods upstream from qPCR. The review concludes by saying that high temperature (>100 °C) and low pH are the most important factors in DNA degradation or fragmentation.

**Future Directions: Validation and Monitoring of Thermal Processing, Fermentation and Shelf-Life**

Many researchers recognize the effect of thermal processing of plant or animal food matrix as reducing amplification of target in conventional and qPCR assays (Murray et al. 2007; Kharazmi et al. 2003; Hrncirova et al. 2008; Bauer et al. 2003; Sakalar et al. 2012; Gryson, 2010). Thermal processing can result in the elimination of detectable amplicon bands in electrophoretic gels and increase the Ct values for the respective methods. To reduce the effects of heat and other processing, researchers suggest the use of very small amplicons (<100 bp) (Aslan et al. 2009; Sakalar et al. 2012). However, the fragmentation of DNA might have a practical use as a time-temperature integrator (TTI), due to its widely cited correlation with both parameters while still providing a measurable outcome after treatment. Dozens of researchers have reported on the effects of processing, with special attention to the detrimental effects of heat, on copy number of amplicons as described by increase in Ct value (Gryson, 2010). None of these laboratories has harnessed this trend for quantification of process efficacy. The goal of this dissertation is to explore the use qPCR of mitochondrial genes, foodstuff intrinsic DNA, as TTIs in thermal processing. We will monitor the destruction of mtDNA over time-temperature and compare these values to D and z-values of indicator microorganisms such as *Geobacillus stearothermophilus* and *E. coli* O157:H7.
Other researchers (Xu et al. 2009) have looked at DNA as an indicator of biodegradation, albeit with carcasses and not food. Endonucleases may fragment DNA during storage of foods (Chen et al 2007). Therefore, we will also study the fragmentation of mtDNA as measured by qPCR to monitor fermentation and shelf-life in high acid products such as pickles.
References


Brodmann PD, Moor D. 2003. Sensitive and semi-quantitative TaqMan real-time polymerase chain reaction systems for the detection of beef (Bos Taurus) and the detection of the family Mammalia in food and feed. Meat Sci 65:599-607.


detection of meat using a real-time polymerase chain reaction. Food Addit Contam 23:645-650.


<http://en.wikipedia.org/wiki/Mitochondrial_DNA>

Figure 1. Mitochondrial genome
<table>
<thead>
<tr>
<th>Target</th>
<th>Gene</th>
<th>Amplicon size (bp)</th>
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Table 1 Continued

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Abbreviations: cyt b = cytochrome b, rRNA = ribosomal RNA, RFLP = restriction fragment length polymorphism, RAPD = random amplification of polymorphic DNA, RE = restriction enzymes, tRNA = transfer RNA, ND = NADH dehydrogenase subunits, ATP = ATP synthase subunits, Val = valine
MITOCHONDRIAL DNA FRAGMENTATION AS A MOLECULAR TOOL TO MONITOR PLANT FOOD THERMAL AND HIGH-PRESSURE PROCESSING

Abstract

Cycle threshold (Ct) increase, quantifying plant-derived DNA fragmentation, was evaluated for its utility as a time-temperature integrator (TTI). 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 (atp1). These consensus primers produced a robust qPCR signal in ten vegetables, six fruits, three types of nuts and a biofuel precursor. There was a significant increase in Ct value after autoclave treatment of all plant materials tested (5 to 17 ΔCt).

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 Geobacillus 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. To consider this protocol 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. High pressure processing at room temperature (400 mPa) was evaluated for mtDNA fragmentation and found to have no effect. We present these data as proof-of-concept for a molecular tool which can be used as a rapid, presumptive method for monitoring thermal processing in low-acid plant products.

**Introduction**

**The Role of Mitochondrial DNA in Research and Industry**

Foodstuffs such as vegetables, nuts and fruits contain in their cells, multiple copies of non-nuclear DNA, mitochondrial DNA (mtDNA), which can be amplified via quantitative polymerase chain reaction (qPCR). Mitochondria are organelles which supply power in the form of ATP to eukaryotic cells. Mitochondria are found in multiple copies in all cells of eukaryotes, such as plants, and each mitochondrion possesses its own genome in multiple copies (Fig. 1). These properties make mitochondrial DNA sequences (mtDNA) excellent targets for amplification in terms of specificity, sensitivity and robustness due to multiple copies per cell (>1,000) (Gerber et al. 2001; Andreasson et al. 2002). Therefore, the advantages of targeting mtDNA with qPCR are substantial.

Mitochondrial DNA (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/). Mitochondrial DNA is also used in human typing for forensic analysis (Hopwood et al. 1996; Andreasson et al. 2002; Budowle et al. 2003) using tissues such as bones, teeth, and hair shafts for DNA extraction. Mitochondrial DNA primers/probes have been developed for source tracking fecal contaminates in wastewater
influents and effluents using multiplex qPCR (Caldwell et al. 2007; Caldwell and Levine, 2009; Caldwell et al. 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 et al. 2001; Zhang et al. 2007; Fujimura et al. 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 Effects of Heat on Cellular DNA**

The effect of high temperature on DNA degradation is drastic. Above 100 °C, denaturation, depurination, deamination and loss of secondary structure occurs (Gryson, 2010). However, autoclaving a foodstuff at 121 °C for 15 minutes does not destroy all DNA available for PCR (Lipp et al. 1999). Other researchers have reported reduced recovery of DNA via qPCR from cornmeal boiled for 60 min at 100 °C (Murray, 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 (GMO) (Moreano, 2005). In a study by Stam (2008), *Clostridium sporogenes* spores were heat-treated (121 °C in 2 min intervals for 18 min) to determine degradation of bacterial DNA over time. It was noted that heat-treating the spores for only 2 minutes resulted in the absence of DNA bands using agarose gel electrophoresis. However, the autoclaved spore DNA was still detectable by qPCR, having a reduced Ct value of 35 compared to a Ct of 12 for viable spores (Stam, 2008). Therefore, DNA is degraded
but still detectable by qPCR, when using thermal processing techniques such as heat or microwave suitable for preserving vegetables and fruits. Cellular DNA, both nuclear and mitochondrial, is degraded during heating or microwaving as detected by conventional PCR or qPCR (Stam, 2008; Gryson, 2010; Murray et al. 2007; Moreano et al. 2005).

**Relationship Between Amplicon Size and Detection**

The effect of heat treatments on the quantification and detection of meat DNA by qPCR was found to be dependent on the duration and temperature of the treatment, plus the size of the amplicon (Sakalar et al. 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 et al. 2012). A relationship between amplicon size and detection after heat treatment or mechanical processing was cited in other meat assays (Hird et al. 2006) and plant products such as soybeans, maize, peas and white potatoes (Hrncirova et al. 2008; Bauer et al. 2003; Kharazmi et al. 2003).

**Standards of Safety and Biological TTIs for Low-Acid Thermal Processing**

Thermal validation and monitoring can employ microbial culture methods for verification of sterility (Pflug et al. 1980; Smith & Kopelman, 1982; Marcy, 1997; Guan et al. 2003). For low-acid products (pH 4.6-6.0) the target organism is the *Clostridium botulinum* spore (Pflug et al. 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 minutes at 121 °C (Esty & Meyer, 1922; Townsend et al. 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 F₀ of 3 minutes (Tucker et al. 2008), F being the cumulative
time-temperature treatment at 121 °C. Destruction of spore-forming *Clostridium botulinum* surrogates such as *Geobacillus stearothermophilus* have been used to monitor heat processes in low-acid foods such as sweet potato puree (Smith & Kopelman, 1982; Brinley et al. 2007, Steed 2010). Problems with using a culture approach include tracking and recovering surrogate spores, and time required to culture (48 hrs). 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 TTIs. Beta-glucosidase from *Pyrococcus furiosus* (Yen 2009), alpha-amylase from *B. licheniformis* (DeCordt et al. 1994; Guiavarch et al. 2004) or *B. subtilis* (Guiavarch et al 2005), algal R-phycoerythrin (Smith et al. 2002; Orta-Ramirez et al. 2001), glucose oxidase (Reyes-De-Corcuera et al. 2005) and endogenous muscle proteins such as lactate dehydrogenase in meats (Veeramuthu et al. 1998) and alkaline phosphatase and lactoperoxidase in milk (Claeys et al 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 (Wang et al 2010; Reyes-De-Corcuera et al. 2005; De Cordt et al. 1994, Guiavarch et al. 2004). Endogenous enzymes are specific to the product and not universal like DNA.
Advantages of Molecular Techniques

Quantitative PCR (qPCR) has several advantages over culture techniques and conventional PCR: it is rapid (4-6 hr), does not require gels or plates and is quantitative. For this study, all mtDNA qPCR assays developed met Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) Guidelines (Bustin et al. 2009) which features a quality control checklist on sample processing, nucleic acid extraction, target amplicon specifications, reaction optimization, specificity of reaction, internal amplification controls (IAC), calibration curves with calculated PCR efficiency, linear dynamic range and data analysis including repeatability and statistical methods. 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 (*atp1*) mitochondrial gene (GenBank AY596672.1). Amplicons for primer sets ranged from 81 to 174 base pairs. 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 *atp1* gene with 100% identity, not only in *Ipomoea batatas*, but 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 *atp1* gene (sequence = table 1). Ten-fold serially dilutions of *atp1* copies (10^8-10^1) were performed and PCR amplification efficiency (E) was determined using the slope of the standard curve:

\[ E = (10^{-1/slope}) - 1 \]

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

**Assessing Universality of Primers**

The *atp1* gene was found to be highly conserved among plant species. When BLAST analysis was performed for the 174 forward and reverse primers, they exhibited 100% identity with a wide variety of fruits, nuts and vegetables. Our lab wanted to create primers which could be used universally to test plant-based foods, both singly and in mixtures such as soups. To this end we purchased fresh, uncooked fruits, vegetables and nuts from a retail grocery store. The samples were 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, three uncooked controls and three autoclave treatments (20 min at 121 °C). The tissue culture protocol of the MasterPure DNA purification kit (Epicentre, Madison, WI) was used. DNA was quantified and qualified using a spectrophotometer (Nanodrop, Wilmington, DE). DNA was normalized to 5-10 ng/well using the qPCR assay as above. 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 two values (Table 1).

**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 – 16 °C and 80 – 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 thermoscrew cooker (Rietz Manufacturing co., Santa Rosa, CA) and minuted in a hammer mill (Model D, Fitzpatrick Co., Chicago, IL) with a 0.15 cm screen. The puree was stored in polyethylene bags at -20 °C until used in thermal trials.

**Sporulation**

Lyophilized *Geobacillus stearothermophilus* (NRRL No. B-1102, equivalent to ATCC 7953) was obtained from the ARS Culture Collection (USDA-ARS; Peoria, IL), 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)
was inoculated directly from freezer stocks or 1% volume from liquid culture and incubated overnight at 55 °C, static. Bacillus heat resistant agar (BHR) containing 13 g nutrient broth, 0.51 g MgSO₄(7H₂O), 0.97 g KCl, 0.2 g CaCl₂(2H₂O), 0.003 MnSO₄(H₂O), 0.00055 g FeSO₄(7H₂O), and 15 g agar brought up to 1L with distilled water and autoclaved (Stam, 2008), was spread with 100 ul overnight culture. Over twenty plates were spread in this fashion. Plates were incubated at 55 °C for 5 days. Spores were harvested by applying 10 ml cold, sterile, distilled H₂O containing 0.1% Tween 80 (Sigma-Aldrich; St. Louis, MO) directly to each plate and scraping with a sterile cell scraper (van Melis et al. 2011). 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 6,000 X g for 10 min at 4 °C. Enzymatic cleaning to eliminate vegetative cells (modified from Foegeding & 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 hr with occasional vortexing. Spore solutions were rinsed 10X with 25 ml cold water/1% Tween 80 (6,000 X g for10 min at 4 °C). After final spin, spores were resuspended 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 resuspended weekly to maintain and prevent germination.

**Autoclave Trials**

To see if mtDNA fragmented during the high heat and pressure of autoclaving, we concurrently tested sweet potato puree and *Geobacillus stearothermophilus* (GS) spores in a
laboratory autoclave (Amsco Eagle SG-3021 Scientific Gravity Sterilizer, Steris Corp., Mentor, OH). We programmed the autoclave to run liquid sterilizing times of 2, 4, 8 & 20 minutes at 121 °C. Come up times (initial CUT = 5 min, all others = 1 min) and come down times (exhaust = 11:53 to 13:25 min) varied slightly for each run. Three samples were included per run: 300 mg sweet potato puree, 250 ul GS spores (log 8 CFU/ml) both samples in 1.5 ml micro-centrifuge tubes in which a small hole had been placed in the top to vent water vapor, and one commercial GS vial (Prospore, Mesa Laboratories, Inc, Lakewood, CO). After autoclave treatment, sample tubes were placed on ice until they cooled to RT. Vent holes were covered with Parafilm (city, state). DNA was extracted from samples using the Power Soil Kit (MoBio Laboratories, Carlsbad, CA). DNA was quantified and qualified via spectrophotometry (Nanodrop, Wilmington, DE). GS spores were serially diluted and plated with a spiral plater (Spiral Biotech Inc. Norwood, MA) on BHI agar (Becton Dickinson, Sparks, MD). After 24 hr incubation at 55 °C, colonies were enumerated with an automated spiral plate counter (Q-count, Spiral Biotech Inc. Norwood, MA). The lower detection limit was $10^2$ CFU/ml. Prospores were incubated at 55 °C for 48 hr, and then checked for indicator colors. Quantitative PCR was run using the same methods as in oil bath trials below.

**Global DNA Fragmentation**

Global DNA fragmentation using total nuclear and organelle (including mitochondrial) DNA was assessed using cucumber as a model vegetable. Cucumber was used rather than sweet potato puree because a raw, uncooked, non-pureed control was needed. DNA was extracted from one fresh and one autoclaved (121 °C for 20 min)
cucumber sample using the MasterPure DNA extraction kit (Epicentre, Madison, WI). Both samples were diluted to ca. 200 ug/ul and total DNA fragment lengths analyzed using the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). A chromotograph was manually compiled using the generated fragment lengths (Fig. 3)

**Hot Oil Bath Trials**

In an effort to mimic and quantify values in a 12D thermal process, the reduction curve of *Geobacillus 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, NH) filled with 8 L white mineral oil (Therminol XP, Solutia, Inc, St. Louis, MO) 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, MI) 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). Temperature was monitored using a type J-K-T microprocessor thermometer thermocouple (HH23A, Omega, Stamford, CT). Come up time (CUT) for TDTs was determined for all target temperatures. In each TDT, 100 ul of 1:4 diluted puree or 100 ul of *Geobacillus stearothermophilus* (GS) spores (ca. 10^8 CFU/ml) were inserted and sealed. For GS spores, samples were heated at 0, 4, 8 and 12 minutes at 116 °C; 0, 0.5, 1, 2, 4, 8, 16 and 20 minutes at 121 °C; 0, 1, 2 and 4 minutes at 123 °C; 0, 0.5, 1, 2 and 4 minutes at 126 °C, all heat...
treatments beginning after come up time (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 reps 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 immediately placed in an ice slurry for 30 sec to quickly cool them. Strainers were stored at RT 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 ul.

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

$$D_T = D_{ref} 10^{T_{ref}-T/z}$$

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

*Geobacillus stearothermophilus* spores were serially diluted and plated with a spiral plater (Spiral Biotech Inc. Norwood, MA) or a simplified agar plate technique (Jett et al.}
both on BHI agar (Becton Dickinson, Sparks, MD). After 24 hours incubation at 55 °C, colonies were enumerated with an automated spiral plate counter (Q-count, Spiral Biotech Inc. Norwood, MA or counted manually. The lower detection limits were 4 x 10² and 1 X 10³ CFU/ml for the spiral plate and simplified agar technique, respectively.

Ct values were converted to log₁₀ copy numbers using the linear relationship determined empirically from the standard curve of the 174 bp amplicon (Chapter 2, Fig 2):

\[ y = -3.1909x + 38.091 \]

where y is the Ct value and x is the log₁₀ copy number. A flow chart (Fig. 4), illustrates the steps to determine and compare the D- and z-values of the Geobacillus stearothermophilus spores with mtDNA fragmentation (ΔCt converted to log₁₀ copy number) of the low-acid purees.

DNA Extraction

Treated sweet potato puree was removed from TDT and placed directly into a MoBio bead-beater tube. The MO BIO PowerSoil® DNA isolation kit (Carlsbad, CA) or MasterPure DNA extraction kit (Epicentre, Madison, WI), were both used according to manufacturer’s recommendations to extract sweet potato DNA from the treated puree. DNA samples were analyzed by spectrophotometer (Nanodrop, Wilmington, DE) for quantity and quality. For qPCR, DNA was normalized by concentration: between 5-10 ng/ul per reaction.

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was run in 25 ul 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 MgCl₂, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 20 nM
fluorescein; BioRad, Hercules, CA), 300 nM final concentration each for forward and reverse primers (F1 atrl), vegetable puree DNA (5-10 ng/reaction) and RT-PCR water (Ambion, Austin, TX) to final volume. Amplifications were performed in a MyiQ (BioRad, Hercules, CA) 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 sec, 72.0 °C for 30 sec; with FAM channel optics on during extension stage. MIQE standards were employed for the optimization and validation of the qPCR assay (Bustin, 2008). No template (NTC) and positive controls were used for all assays. For a sample to be considered positive, its threshold cycle (Ct) value must be less than all negative control reactions and its corresponding amplification curve had to exhibit the three distinct phases of real-time PCR: lag, linear and plateau. Internal amplification controls were not employed as no PCR inhibition was apparent. The Ct (threshold cycle) is the intersection of an amplification curve detected by fluorescent and a system-generated or manually determined threshold line (http://www.lifetechnologies.com/us/en/home/life-science/pcr/real-time-pcr/qpcr-education/pcr-understanding-ct-application-note.html). The threshold line is determined by three parameters: baseline, Rn and delta Rn. The baseline is formed in qPCR initial cycles when there is little change in the fluorescent signal. Normalized reporter (Rn) is the ratio of fluorescence between the reporter and passive reference dye. Delta Rn is the normalization of Rn by subtracting the baseline. Delta Rn is used to determine the Ct value. The threshold is set above the baseline and within the exponential region of the amplification curve. Because the position of the baseline is arbitrary, Ct values for these experiments were manually adjusted from the automated system of the thermal cycler using a normalizing
DNA sample (positive control), so that values between 96-well plates could be compared. 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.

**Retort trials**

Sweet potato puree was produced as before and placed in 68.3 X 101.6 mm cans outfitted with T-type C-2 tube and rod thermocouples (Ecklund-Harrison Technologies, Fort Myers, FL). Colorimetric *Geobacillus stearothermophilus* ampoules (Raven ProSpore; Mesa Laboratories, Inc., Lakewood, CO) 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). 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 two full water immersion protocols listed below. Protocol 00 was a substandard treatment not meant to kill spores (Appendix A). Protocol 01 was a > 6D protocol meant to eliminate all *Geobacillus stearothermophilus* test spores (Appendix B). Each protocol was run in triplicate using three cans per run. Puree was sampled from center of each can by carefully removing the top layers with a spatula and taking a 500 ul aliquot next to the thermocouple probe. DNA was extracted from this centrally-located aliquot and atp1 qPCR protocol run as before. ProSpore (Mesa Labs, Lakewood, CO) ampoules were incubated at 55 °C for 48 hr as recommended...
by the supplier, and then assessed for colorimetric change. F values were determined from the thermal couple 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 \( Y = CT \) on \( X = \text{cum F} \) was fit (with \( R^2 = 0.875 \)) to \( m = 21 \) complete 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 asymptotic standard error for this nonlinear function of regression coefficients may be approximated by

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

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

\[
\hat{X}_0 \pm t(\alpha/2, m - 2)\overline{SE}(\hat{X}_0).
\]

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

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

These values may be obtained using the quadratic formula and constitute an asymmetric interval. This is the method used by the JMP statistical software package. 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 given value of \( y_0 = 26.2 \) was 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, appear in the output on the next page.

**High Pressure Processing**

To test the effects of high pressure alone on mtDNA fragmentation, 50 ml aliquots of sweetpotato puree were pipetted into a sterile bag (Cryovac M-312 medical film, Cryovac Inc, Duncan, SC). Air was removed from each bag and the bag was sealed with a 16-inch impulse heat sealer (H-306; Uline, Chicago, IL). High pressure processing was performed with an isostatic press (Autoclave Engineers, CIP2-22-60). The pressure transmitting fluid was a solution of 1 part Hydrolubic 120 B fluid (E. F. Houghton & Co, Valley Forge, PA) to 20 parts deionized water. The pressure chamber was maintained at room temperature (20 °C)
with some adiabatic heating of the pressure transmitting fluid due to pressure buildup (3 °C per 100 MPa). Samples were loaded into the pressure chamber and processed at 400 MPa for 0, 5, 10, 15, 30, 45 and 60 minutes, in triplicate. Samples were stored at -20 °C until DNA extraction. A 10 ul aliquot was taken from each sample and DNA extracted using a MasterPure kit (Epicentre, Madison, WI). DNA was quantified and qPCR run as above. Mean Ct values were analyzed by ANOVA and significant differences between treatments determined (Table 3; p<0.05).

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. It is possible to design primers which are either species-specific or which show consensus between many different plant sequences. Developing primers and probes specific for each foodstuff of interest is performed by aligning sequences and locating unique stretches containing many single nucleotide polymorphisms (SNP), especially at 3’ primer ends. There are sufficient divergences among species to design species-specific primers for qPCR. Conversely, areas of similarity can be found by sequence alignment, and then primers with identical or degenerate bases can be used to create an assay which will target many members of vegetable or fruit families. The results of the comparative analysis between the rapeseed and Arabidopsis mitochondrial genomes suggest that higher plant mitochondria are extremely conservative.
(Handa, 2003), thus having many similar sequences to target for consensus qPCR. Our goal was to create a set of primers based on consensus sequences which would allow universal amplification of plant mtDNA. We chose the ATP synthase F1 alpha sequence (*atp1*; Fig. 1) whose enzyme catalyzes the final step during oxidative phosphorylation and whose structure is highly conserved in eukaryotes (Millar et al. 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 *atp1* mitochondrial gene under NCBI BLAST analysis. A test comparing autoclaved (121 °C for 20 min) versus non-autoclaved sweet potato puree DNA was run with each primer set. Primer set 174 was chosen as the preferable pair because it exhibited the greatest difference in Ct values between the two 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 (Table 2). 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 1). 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 early 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, Madison, WI) (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 (Fig. 2). 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 application, 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, 2008) (Fig. 2). 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.

**Global DNA Fragmentation**

Global fragmentation of cellular DNA, both nuclear and organelle-based (including mtDNA), by heat and pressure was demonstrated in this chromatograph (Fig. 3). DNA was extracted from fresh and autoclaved cucumber (MasterPure DNA extraction kit, Madison, WI), both analyzed in an Agilent Bioanalyzer 2100. The fresh, unheated cucumber DNA had fragment lengths ranging from 1,000 to 10,000 base pairs (bp) with greatest concentrations at 1000, 1500, 2000 and 6000 bp. The autoclaved cucumber DNA had fragment lengths up to 3500 bp with the vast majority of fragments below 500 bp. The effect of autoclave treatment was drastic and obvious: thermal destruction of the phosphodiester backbone. Quantitative PCR is dependent on intact DNA for amplification. If a targeted amplicon is cut, even once, it will not amplify. The Ct value is based on the threshold between lag and log amplification. This threshold value depends on the quantity of the amplicon of interest. The more amplicon, the sooner the threshold level for logarithmic amplification is reached and the lower the Ct value. When DNA is destroyed and fragmented by heat, pressure, UV light or DNases, the Ct value increases. A high Ct value is due to reduced recovery of the targeted DNA sequence. This chromatograph indicates that high temperature (121 °C for 20 minutes) combined with pressure fragments DNA. However, even after a similar autoclave treatment, sweet potato puree mtDNA is still available for
amplification and detectable by qPCR (Fig. 5). For monitoring purposes, a measurable outcome must be available.

DNA fragmentation is also influenced by extraction method. Therefore, DNA extraction methods must be evaluated, optimized and held constant between trials if one wants to compare Ct values of plant mtDNA between different time-temperature treatments.

**Autoclave Trial**

The autoclave trial was conducted by re-programming the autoclave to different treatment times at 121 °C. The Ct values were not linear to time due to differences in come-up time and cooling times which were not programmable (Fig. 5A). Better results followed in the hot oil bath treatments where come-up time (CUT), hold temperature and cooling time were all controlled by the operator (Fig. 6). These graphs using the mean of three replicates per time point, suggested a good correlation between increase in Ct value, time-temperature (Fig. 5A) and spore reduction (Fig. 5B) during autoclave treatment of 121 °C in low acid sweet potato puree. It also proved that a measurable outcome could be obtained after high-temperature/ high-pressure treatments.

**Hot Oil Bath**

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 ($R^2 = 0.87$) (Fig. 6). Variability at each time point was due to DNA extraction efficiency, operator error in pipeting and small sample size. The largest factor in variation
would be pipeting error due to dilutions needed to normalize each sample to 5 – 10 ng/ml. While DNA can solubilize in water, it is a long, sticky molecule and tends to form microscopy clumps resulting in a non-homogeneous solution. Buffers such as Tris-EDTA (pH 7.5 – 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; Austin, TX) was used for all dilutions. Final DNA concentrations from extractions ranged from 5 to 500 ng/ul. 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.

*Geobacillus 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 ($R^2 = 0.087$). 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 (Fig. 7). 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 ($R^2 = 0.88$) (Fig. 8). Data points below the cumulative F value of 3 minutes tested positive by Prospore GS ampoules (Mesa Labs; Lockwood, CO) and above this value tested negative (>6D reduction of GS process). Retort processes are notorious for
their long CUT and cool down times. Since this was a non-continuous process, cumulative F values are permitted. This is a total system approach which 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) for aseptic processing at 121 °C.

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

$D_{121}$ and z-values determined in hot oil bath for *Geobacillus stearothermophilus* ATCC 7953 (GS) spores were 2.71 min and 11.0 °C (Figs. 9 & 10), respectively. These values were slightly higher than a commercial product using the same ATCC strain for autoclave validation (Prospore, Mesa Labs, Lakewood, CO) 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 GS 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, OH) 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 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 et al. 2007). As an added precaution, a safety factor is added to empirically derived data, i.e. total death time is rounded up, to ensure complete destruction of spores (Tucker et al. 2008).

$D_{121}$ and $z$-values for Ct values from a 174-bp universal plant amplicon were 11.3 min and 17.8 °C (Figs. 11A & B), 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 pipeting 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 10X greater than C. botulinum ($D_{121} = 0.21$ min; Esty & Meyer, 1922; Townsend et al. 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 4X 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 ($F_0 = 5$ minutes) using a log function of Ct value. However, sterilization in the pharmaceutical industry requires higher values ($F_0 > 12$ min) where GS 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 = 0.875) (Fig. 7). 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.

**High Pressure Processing**

There was no significant difference between mean Ct values of controls and any time point in high pressure processing (HPP) of sweetpotato puree up to 60 min at 400 mPa (Table 4). This process was held at room temperatures (20-32 °C) as the expected temperature increase due to adiabatic pressure only was ca. 12 °C. This result was comparable to many studies which have shown that bacterial spores cannot be inactivated by pressure treatments at room temperature (Wantanabe et al. 2003; Furukawa & Hayakawa, 2000; Nakayama et al. 1996). Past studies using HPP at low temperatures have found that covalent bonds remain unbroken, limiting the effectiveness of this process against spores, gram-positive bacteria and enzymes (Balasubramaniam & Farkas, 2008). We conclude that high pressure without heat does not fragment mtDNA at a detectable level. Therefore, high pressure processing cannot be monitored by qPCR-mtDNA fragmentation.

**Conclusions**

Fragmentation of mtDNA, as measured by Ct, of low-acid foods at high temperature has a high correlation to time-temperature (Fig. 6); cumulative F values (Fig. 8) and reduction curves of spore surrogates (Fig. 7). 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 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. Mitochondrial DNA 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-hour 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 through-put.

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.
References


Esty JR, Meyer KF. 1922. The heat resistance of the spores of *B. botulinus* and allied anaerobes XI. J Infectious Dis 31:650-663.


detection of meat using a real-time polymerase chain reaction. Food Addit Contam 23:645-650.


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.


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

<http://en.wikipedia.org/wiki/Mitochondrial_DNA>

Figure 1. Mitochondrial genome
Figure 2. mtDNA qPCR standard curve for 174 bp amplicon
Figure 3. Global measurement of cucumber DNA sizes before & after autoclave treatment
Figure 4. Flow chart of DNA fragmentation protocol
Figure 5A. Sweet potato puree mtDNA fragmentation in autoclave (121 °C)

\[ y = -0.0448x^2 + 1.3433x + 23.317 \]

\[ R^2 = 0.9428 \]
Figure 5B. Timed autoclave treatments (121 °C)

Mean Ct of pureed sweet potato

GS spores counts (mean log10 CFU/ml)

\[ y = 0.0366x^3 - 0.5557x^2 + 1.1229x + 32.107 \]

\[ R^2 = 0.8664 \]
Figure 6. Sweet potato puree mtDNA fragmentation (174 bp) in hot oil bath (121 °C)

y = 0.4323X + 24.941
$R^2 = 0.8666$
Figure 7. mtDNA fragmentation in hot oil bath (121 °C) versus spore counts
Figure 8. Retort process for canned sweet potato puree

$y = 0.6x + 23.201$

$R^2 = 0.875$

Protocol 00

Protocol 01
Figure 9. Slopes to calculate D values for timed oil bath treatments
Figure 10. *Geobacillus stearothermophilus* slope to calculate z-value

\[ y = -0.0908x + 13.038 \]

\[ R^2 = 0.9276 \]

z = 11.0 °C
Figure 11A. Sweet potato puree mtDNA fragmentation D values in hot oil bath
Figure 11B. Calculation of z-value for SPP mtDNA fragmentation

\[
y = -0.0562x + 9.6149
\]

\[
R^2 = 0.9894
\]

\[
z = 17.8 \, ^{\circ}C
\]
Table 1. Universality of proprietary primers and demonstration of mtDNA fragmentation across different plant materials after autoclave treatment.

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

* Roasted at 167 °C for 19 min. All others autoclaved at 121 °C for 20 min. **Standard deviation
Table 2. qPCR universal primers for 174 bp amplicon of *atp1* gene

<table>
<thead>
<tr>
<th>Type</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward</td>
<td>5’ –TTT CCG CGA TAA TGG AAT GCA CGC-3’</td>
</tr>
<tr>
<td>Reverse</td>
<td>5’ –TCC GAT CGT TTA GCC GCT CTT TCT-3’</td>
</tr>
</tbody>
</table>
### Table 3. Inverse prediction statistics for retort process validation

<table>
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<tr>
<th>Samples needed</th>
<th>Standard error (%)</th>
<th>99% Confidence levels</th>
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</thead>
<tbody>
<tr>
<td>32</td>
<td>2.3</td>
<td>4.66</td>
</tr>
<tr>
<td>64</td>
<td>1.6</td>
<td>4.76</td>
</tr>
<tr>
<td>128</td>
<td>1.2</td>
<td>4.83</td>
</tr>
<tr>
<td>256</td>
<td>0.8</td>
<td>4.88</td>
</tr>
</tbody>
</table>
Table 4. High pressure processing (400 mPa) of sweet potato puree at room temperature

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mean Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>19.30&lt;sup&gt;ab&lt;/sup&gt;*</td>
</tr>
<tr>
<td>5</td>
<td>19.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>18.91&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>19.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>19.98&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>45</td>
<td>18.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>60</td>
<td>19.42&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*<sup>p<0.05</sup>
MITOCHONDRIAL DNA FRAGMENTATION TO MONITOR PROCESSING PARAMETERS IN HIGH ACID FOODS

Abstract

MtDNA fragmentation was assessed in several cucumber-to-pickle processes including fermentation, pasteurization, autoclaving and storage time at room temperature. MtDNA fragmentation of each process was assayed by qPCR using universal *atp1* primers. Cucumbers were sliced into hamburger dill chips, fermented and aerated in 7% NaCl for 8 mo, pasteurized at 75 °C for 15 minutes and stored at room temperature for 2 and 20 mos. Cucumber mtDNA fragmentation, as measured by Ct value, was significantly different based on shelf-life alone (p<0.05). Threshold cycle (Ct) values were significantly different between all treatments, except heat treatments. The fermented, pasteurized pickle had a similar Ct value to the fresh, autoclaved cucumber. This indicates that lower temperature thermal processes (hot fill at 75 °C for 15 minutes) under acidified conditions (pH = 3.8) yield similar mtDNA fragmentation results when compared to more elevated conditions (121 °C) in low-acid foods. In a second study, cucumbers (size 2B) were stored in brine and fermented using the same protocol for 7, 8, 9, 11 and 12 months. The Ct value of intrinsic pickle mtDNA increased linearly when compared to fermentation time in months. In studies modeling high acid juices, pasteurization (96 °C, 0-24 min) of tomato serum had high correlation to time-temperature and a similar D value to that of low-acid high-temperature processing of sweet potato puree (*ca. 11 min, Chapter 2*). Primers producing longer amplicons targeting the same mitochondrial gene gave greater sensitivity in heat treatments of tomato serum at 95 °C and less than 2 min in duration. An extrinsic mtDNA model was
developed using Gblocks™, synthetic double-stranded oligonucleotide fragments, in citric acid solutions at different pH values. The D values which were elucidated from this extrinsic model were similar to those of spore-forming, juice-spoilage microorganisms, B. coagulans and Alicyclobacillus spp. as cited in the literature (7.43 versus 8-9 min). Mitochondrial DNA fragmentation was shown to be a potential new tool to monitor low-temperature (<100 °C) high acid processes (pH<4.6), and non-thermal processes such as vegetable fermentation times and shelf-life of acidified products.

Introduction

High acid foods such as fruit and some vegetable juices require a 5-log reduction of the pertinent pathogen (US FDA, 2001). This is defined as the most resistant microorganism of public health significance that is likely to occur in the juice. Furthermore, this reduction should remain in effect for a period at least as long as the shelf life of the product when stored under normal and moderate abuse conditions (US FDA, 2001). Escherichia coli O157:H7 is widely considered as the target pathogen in high acid foods such as fruits and acidified vegetables, due to its acid-resistance, tolerance of organic acids and ability to survive refrigerated storage for extended periods (Koodie & Dhople, 2001; Mak et al. 2001; Miller & Kaspar, 1994; Zhao et al. 1993). Many producers utilize thermal processing as a critical control point to assure 5-log pathogen reduction. Acid and acidified foods do not require intensive heat treatment (>100 °C) since the combination of heat and low pH are lethal to target pathogens (US FDA, 2001). Here are some examples of high-acid, low temperature thermal processes for achievement of 5-log reductions in the literature: Pasteurization of apple cider at 71.1 °C for 6 sec was recommended by the New York State
Department of Agriculture and Markets (1998) for most apple varieties. Wisconsin apple grower’s cider pasteurization treatments ranged from 68.1 °C to 80.9 °C for 14 sec (Mak et al. 2001). Breidt and others (2005) determined 5-log pathogen reduction times for acidified vegetable brines and, while typical commercial processing times were between 70 and 80 °C for up to 15 min, they achieved the mandated reduction at much lower time-temperature parameters (65 °C, <1.7 min, pH 4.1). Linear models were determined for acidified vegetable products (4.1> pH >3.3) and for processing temperatures above 83 °C, less than 6 sec was required for 5-log pathogen reduction (Breidt et al. 2010).

While researchers have demonstrated that E. coli O157:H7 can survive in tomato products with increased survival due to refrigeration (Eribo & Ashenafi, 2003), other potential health hazards exist. Spoilage organisms such as Bacillus coagulans and Alicyclobacillus spp. are frequently isolated from spoiled canned vegetables (Peng et al. 2012; Bevilacqua & Corbo, 2011) and may increase the pH of acidic foods, creating a food safety hazard. Thermal processing treatments for 5-log reduction of pathogens are inadequate for elimination of spoilage spore-forming organisms such as Bacillus and Alicyclobacillus (ACB) spp. Alicyclobacillus acidoterrestris spores were able to survive hot fill protocols used to process juices (2 min at 88 - 96 °C; Pontius et al. 1998). Splittstoesser and others (1994) reported D values of 2.4 to 2.8 min at 95 °C for A. acidoterrestris. Therefore, hot fill protocols for fruit juice may produce less than 1-log reduction for these thermophilic, acid-resistant spores.

Fermented foods such as cucumber pickles use salt brine (7% NaCl) to promote growth of native lactic acid bacteria which lower pH and discourage growth of pathogens or
sporulation of toxin-producing spores. This fermentation/brining process is performed in large outdoor tanks (10,000 to 40,000 gal) and may last from several months to over one year depending on production schedule. Temperatures vary by climate and season, but pH values rapidly decline to the acidic range (pH<4.6) due to lactic acid production by native microbial flora, usually *Lactobacillus plantarum*. After hot fill pasteurization, pickle products such as hamburger dill chips are stored at room temperature and have an estimated shelf-life of 2 years if unopened. At present, fermentations are monitored by pH alone and shelf-life is estimated.

A temperature of 100 °C does not severely degrade DNA (Gryson, 2010). Polymerase chain reaction cycles DNA in the range of 60-95 °C to denature, anneal, extend and amplify the molecule with no apparent damage. However, PCR is performed at neutral pH (7.4). Above the boiling point of water, 100 °C, DNA can become destabilized and permanently damaged (Gryson, 2010). Earlier work in this lab using mtDNA fragmentation as a time-temperature integrator (TTI) for low acid, high temperature processes (121 °C) discovered a high correlation between *Geobacillus stearothermophilus* spore death in hot oil bath treatments and cumulative F value of retort processes (Chapter 2). This study will examine the use of mtDNA fragmentation as molecular TTI for high-acid, low-temperature (pH <4.6; <100 °C) processes for fruit juices and fermented vegetables. The utility of the protocol as a monitor of cucumber fermentation times and shelf-life of acidified vegetables will also be evaluated.

Finally, an extrinsic mtDNA model was created using Gblocks™, synthetic double-stranded oligonucleotide fragments, in citric acid solutions at different pH values. The
calculated D value of the model was compared to D values from spoilage microorganisms such as *Bacillus coagulans* and *Alicyclobacillus* spp. The goal of this research was to develop a novel molecular validation process to assure elimination of organisms of concern in thermal processes where intrinsic DNA was unavailable or difficult to obtain.

**Materials and Methods**

**Cucumber-to-Pickle Processing**

In order to assess mtDNA fragmentation in acidified vegetables, fresh cucumbers were autoclaved at 121 °C for 20 min in a Steris Autoclave under standard conditions. Fermented, fermented/pasteurized, and stored pickles were taken from jars from the same lot number. The jars were processed as follows: Briefly, cucumbers were, fermented and air-purged for 20 hr/day in 5.9% NaCl, sliced into hamburger dill chips, pasteurized at 75 °C for 15 minutes and stored at room temperature in jars for 2 and 20 mos. All treatments were collected in triplicate; for fresh, a rep was one cucumber; for processed pickles, a chip from one jar. Punch samples were taken from all cucumber/pickle products by inserting a 1,000 ul pipette tip into the mesocarp to obtain a 5-10 mg circular slice. DNA was extracted by the MasterPure DNA extraction kit (Epicentre, Madison, WI) and qPCR run in duplicate wells.

**Monitoring Fresh Geographical Origins and Fermentation Times in Cucumbers**

Fresh, size 2B cucumbers (no less than 2 inches in diameter and no less than 5 inches long; [http://www.ces.ncsu.edu/depts/hort/hil/ag552c.html](http://www.ces.ncsu.edu/depts/hort/hil/ag552c.html)) were taken from refrigerated lots, shipped in from three different geographic locations: Mexico, Georgia and Florida. Fermented cucumbers were taken from tanks stored in 7% NaCl brine and air-purged for 20 hr/day for 7, 8, 9, 11, and 12 mos. Comparisons were made between Ct values of fresh
cucumbers from different growing locations, fermentation times and other processes such as desalting. All treatment samples were collected from the mesicarp in triplicate and assayed via qPCR in duplicate as above.

**5-log Reduction of *E. coli* O157:H7 in Tomato Serum**

This experiment compared a reduction curve of an *E. coli* O157:H7 cocktail to Ct values from intrinsic mtDNA in tomato serum at different times (0, 0.25, 0.5, 1.0, 2.0 min) under a pasteurization-type thermal process of 95 °C. Three fresh tomatoes (Redsun, Mexico 4664) were processed in a Waring blender at medium speed until homogeneous to create tomato puree. Three aliquots of puree were frozen at -20 °C for later use. The remainder of the puree was placed in 330-micron filter bags (Nasco Whirl-pak, Fisher Thermo Scientific, Atlanta, GA) and stomached (Seward Stomacher 400; Tekmar, Cincinnati, OH) at low speed for 30 sec. Aliquots of tomato serum were obtained from filtered side of bag. The pH of the filtered tomato serum was noted (4.26; Accumet AR25, Fisher Thermo Scientific, Atlanta, GA) and product frozen at -20 °C until use. Five cultures of *E. coli* O157:H7 (FSRU culture collection B200-204) were grown separately at 37 °C, at 200 rpm overnight in LB broth (BD, Sparks, MD). Cultures were concentrated 1:10 by centrifugation (6,000 rpm, 4 °C, 10 min; Sorval RC-1, Spectrofuge, Asheville, NC) and combined the day of the trial to create an inoculum cocktail (10⁹ CFU/ml). A time-temperature trial was run using a thermal cycler (BioRad; Heracles, CA) as a constant, programmable heat source. Come up time (CUT) to 95 °C was 36 seconds, determined empirically using the thermal cycler internal sensor. Bacterial cells were added to 100 ul tomato serum at 10⁹ cells/ml and 100 ul serum was run separately to provide intrinsic mtDNA. Tests were conducted in 0.1 ml PCR tube strips with
three tubes per treatment per temperature. The entire reduction curve, from 0 to 2 min, was run in triplicate on three different days. After the target time was reached, tubes were plunged into ice water slurry and cooled to room temperature. Tomato serum was stored at 4 °C until DNA extraction and qPCR.

**Plate Counts**

*E. coli* O157:H7 cells were enumerated using a simplified agar plate technique (Jett et al. 1997) with square petri dishes and LB agar (Becton Dickinson, Sparks, MD). This simple drip-dilution method had a lower detection limit than automated spiral plating methods. After 24 hours incubation at 37 °C, colonies were enumerated manually. The lower detection limit is $10^2$ CFU/ml. Ct values from tomato serum intrinsic mtDNA were compared to plate counts at 95 °C over 2 minutes at intervals of 0, CUT, 0.25, 0.5, 1.0 and 2.0 min.

**Tomato Serum D$_{96}$ Value for Hot Fill Process**

To quantify mtDNA fragmentation in a high acid product at a relatively low temperature, we conducted a laboratory-scale hot fill process on tomato serum and calculated its D value at 96 °C. Tomato puree and serum was obtained from stored freezer aliquots from the study above. Using a thermal cycler (BioRad, Hercules, CA) as the heating element, a time-temperature trial was conducted. Two hundred ul filtered tomato serum was added to three 0.2 ml PCR tubes/time point. Come up time (CUT) to 96 °C was 30 seconds, determined empirically using the thermal cycler internal sensor. Serum samples were run for 0, 2, 4, 8, 16, 24, and 48 minute reps using 3 tubes per rep. After the target time was reached, tubes were plunged into ice water slurry and cooled to room temperature. Tomato
serum was stored at 4 °C until DNA extraction and qPCR. Quantitative PCR was run and Ct values converted to $\log_{10}$ copy number to determine D value of tomato serum at 96 °C.

**Extrinsic mtDNA Model**

An extrinsic DNA source as a TTI was explored using *atp1* 174 base pair Gblock™ double-stranded oligonucleotides ($10^6$ copies/ul final concentration) diluted in 0.5% sterile citric acid solutions at three pH levels: 3.6, 4.0, 4.4. A thermal cycler (BioRad, Hercules, CA) was used at a heat source. Domed PCR tubes containing 20 ul citric acid/ Gblock solutions were added manually to the thermal cycler after it reached the target temperature of 96 °C. The number of tubes added during one rep was limited to reduce variation. Time points were 0, 4, 8, 16 and 24 min with three reps at each time. Samples were placed in ice slurry after treatment to rapidly cool. DNA extraction was not necessary. The qPCR assay was performed using the *atp1* 174 bp amplicon protocol. $\log_{10}$ copy numbers were calculated using the linear relationship determined empirically from the standard curve of the 174 bp amplicon (Chapter 2, Fig. 2). Data were segregated by pH treatment and analyzed by simple linear regression for goodness-of-fit ($R^2$) (Fig 6).

**DNA Extraction**

DNA from cucumber and pickle mesocarp samples or tomato serum (5-10 mg or ml) was extracted using the MasterPure DNA purification kit (Epicentre Biotechnologies; Madison, WI) according to manufacturer’s recommendations for plant tissues. DNA samples were analyzed by spectrophotometer (Nanodrop, Wilmington, DE) for quantity and quality. For qPCR, DNA was normalized by concentration, diluted to deliver 5-10 ng/ul per reaction.
**Standard Curve**

A standard curve for the 1,016-bp amplicon was generated using gBlocks™ Gene Fragments (https://www.idtdna.com/pages/products/genes/gblocks-gene-fragments) which are double-stranded, sequence-verified oligonucleotides of the *atp1* gene from ATP synthase subunits. Ten-fold serially dilutions of *atp1* copies (10⁹-10¹) were performed and PCR amplification efficiency (E) was determined using the slope of the standard curve:

\[ E = \left(10^{-1/\text{slope}}\right) - 1 \]

Data analysis of the qPCR standard curve was performed using goodness-of-fit linear regression correlation coefficient (R²). The amplification efficiency was calculated as 88%, R² = 0.996 for the 1,016 bp amplicon (Fig. 4). Previously, a standard curve was developed for the 174 bp amplicon (Chapter 2, Fig. 2), using the same methodology.

**Measurement of mtDNA Fragmentation (qPCR)**

Quantitative PCR was performed 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 MgCl₂, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, and 20 nM fluorescein (BioRad, Hercules, CA)), 300 nM final concentration each for 174F and 174R primers or 500 nM final concentration each for 1016F and 1016R primers, cucumber, pickle or tomato serum DNA (5-10 ng/reaction) or 1 ul Gblock™/citric acid solution and qPCR water (Ambion, Austin, TX) to final volume. qPCR amplifications were performed in a MyiQ (BioRad, Hercules, CA) thermal cycler with the following conditions for 174 bp amplicons and Gblock™/citric acid solutions: 95.0 °C for 3 minutes; 40 cycles of 95.0 °C for 30 seconds, 60.0 °C for 30 seconds, 72.0 °C for 30 seconds; with FAM channel optics “on”
during annealing stage. For longer amplicon at 1,016 bp, qPCR amplifications were performed with slightly different conditions: 95.0 °C for 10 minutes; 40 cycles of 95.0 °C for 30 seconds, 55.0 °C for 30 seconds, 72.0 °C for 120 seconds; with FAM channel optics “on” during annealing stage. No template control (NTC) and positive controls were used for all assays. A positive control was used to normalize data between assays and manually adjust threshold baseline. For a sample to be considered positive, its threshold cycle (Ct) value must be less than all negative control reactions, and the corresponding amplification curve has to exhibit the three distinct phases of real-time PCR: lag, linear and plateau.

Statistical Analyses

All ANOVA statistical analyses were performed using SAS (Version 9.3, SAS Institute, Inc., Cary, NC). For the tomato serum versus *E. coli* reduction study, the MIXED procedure was used to analyze this completely random study with 18 reps and 6 treatment times. The treatments were statistically different, *p*<0.001. Other statistical analyses cite predetermined *p* values. Simple linear regressions were performed and goodness-of-fit regression correlations determined using Excel graphing software.

Results and Discussion

Monitoring of Cucumber-to-Pickle Processes and Shelf-Life

Fragmentation of intrinsic cucumber mtDNA was evaluated as a method to monitor commercial cucumber-to-pickle processes. Fermented, fermented/pasteurized, and stored finished pickles were taken from jars having the same lot number. DNA was extracted and compared to fresh and fresh/autoclaved cucumber DNA (not from the same lot) by analyzing Ct values of *atp1* gene. The Cts of four processes were compared to fresh cucumber values.
All treatments were significantly different from the fresh cucumber control (p<0.05). The fermented cucumber had an increase of 4 Ct compared to the fresh, indicating significant mtDNA fragmentation caused by acidification of the brine and possibly other factors inherent in the growth of lactic acid bacteria. Process 3 contained pickles which were fermented, pasteurized and stored for 2 mo at room temperature in 16 oz glass jars. Process 3 had Ct values which were significantly higher than fresh and fermented values. However, process 3 had almost the identical Ct value (ca. 30) as compared to process 5, the fresh autoclaved cucumber. This indicates parity between two very different thermal processes: an acidic hot-fill process (75 °C for 15 min) and autoclaving under pressure (121 °C). Because processes 3 and 4 differ by only time of storage, mtDNA fragmentation may be useful as a measure of shelf-life in jarred, finished, acidic vegetables. Process 4 at 20 months storage had significantly higher Ct values than process 3 at 2 months storage, a difference of 18 months. Twenty months is near the projected end of shelf-life (2 yr) for this particular product. Only one study was found in the literature which considered mtDNA as a tool to quantify decomposition over time. The goal of Xu and others (2009) was to determine the end point of carcass composting prior to its use as fertilizer on crop land. A gruesome shelf-life determination, they quantified bovine mtDNA by qPCR and found a reduction of 93% due to composting of cattle carcasses under ambient environmental conditions for 147 days. Future studies in our laboratory will examine smaller gradations in storage time of acidic vegetables to determine the usefulness of this protocol for shelf-life monitoring and end-of-shelf-life projections.
Effect of Fermentation Times and Geographical Origins

Comparisons were made between Ct values of fresh cucumbers from different growing locations, fermentation times and other processes such as desalting. The Ct value of intrinsic pickle mtDNA increased linearly when compared to fermentation time in months (Table 1A). There was a significant difference between mean Ct values of pickles fermented at 8, 9, 11 and 12 months (p<0.022). None of the fermented treatments was desalted prior to mtDNA fragmentation analysis. One desalted treatment was available and included in the assay. It had a mean Ct value of 21.38, which was lower than all the fermented values. Further investigation revealed that the desalted treatment was fermented at a different commercial facility in Michigan. Records showed that the desalted treatment had been fermented for 7 months in a very different climate from North Carolina (data not shown). The desalted treatment was not included in the ANOVA since it was confounded by location. However, it did follow the same trend as other local, commercial fermentations.

Fragmentation of intrinsic mtDNA appears to be a novel method to monitor cucumber fermentations. It may be linked to shelf-life and other quality parameters such as crispness and rancidity in future studies. Applications may extend to the brewing industry.

Geographical origin of 2B cucumbers had no significant effect on mean Ct value (Table 1B). Cucumbers from Florida, Georgia and Michigan were not significantly different, having mean Ct values spanning only 0.76 units. This was counter-intuitive since the three regions have very different climates, growing conditions and shipping times. However, since fresh cucumber mtDNA will provide the baseline for all subsequent assays monitoring cucumber-to-pickle-processes, this will allow greater comparisons between processes and
produce from different geographical regions. This uniformity of produce across the United States is a testament to commercial plant breeding, Good Agricultural Practices and critical control points under HACCP shipping regulations.

**High Acid/Low Temperature Processing (Hot Fill)**

To quantify mtDNA fragmentation in a high acid product at a relatively low temperature, we conducted a laboratory-scale hot-fill process on tomato serum to calculate its D value at 96 °C. According to Pontius et al. 1998, most fruit juices are high acid (pH <4.6) and use a hot-fill and hold of 2 min at 88 to 96 °C to pasteurize. We extended the time of the process because this article (Pontius et al. 1998) reported the D value of the spoilage organisms of concern, *Alicyclobacillus* spp. (ACB), as 8-9 minutes at 97 °C. Therefore, it was noted that commercial hot-fills would not eliminate even low concentrations of this spoilage microorganism. This thermophilic, acid-resistant spore-former can not only produce off-flavors but increase the pH of infected juices. This could cause a public health hazard by allowing toxin-producing spores, such as *Clostridium botulinum*, to grow during extended storage. *Bacillus coagulans* has a similar profile and D value to ACB (Peng et al. 2012).

The goal of this experiment was to compare the D value of tomato serum mtDNA fragmentation to that of ACB and *B. coagulans* at a commercial pasteurization temperature. The tomato serum was heated at 96 °C for up to 24 min (Fig. 2). This represents ca. 3-log reduction of ACB. MtDNA fragmentation was measurable for the duration of the thermal treatment and Ct values increased numerically from 17 to 24 units. Ct values were converted to log10 copy numbers using the linear relationship determined empirically from the standard curve of the 174 bp amplicon (Chapter 2, Fig 2):
\[ y = -3.1909x + 38.091 \]

where \( y \) is the Ct value and \( x \) is the \( \log_{10} \) copy number. The \( D_{96} \) value of the tomato serum mtDNA fragmentation was determined using the reciprocal slope of the line and was calculated as 11.63 min (Fig. 3). This value is similar to the \( D_{121} \) for mtDNA fragmentation from sweet potato puree heated in a hot oil bath and using the same universal plant amplicon (11.3 min; Chapter 2, Fig. 11). Since the \( D_{96} \) value exceeds that of ACB and is still measurable after at least a 3-log reduction of the spoilage organism, this molecular approach may have a place in commercial hot-fill monitoring and validation.

**E. coli Reduction Curve for Acidic Foods**

This experiment compared a reduction curve of an *E. coli* O157:H7 cocktail to Ct values from intrinsic mtDNA in tomato serum at different times under a quick pasteurization process of 95 °C (Table 2A). A 5-log reduction of *E. coli* O157:H7 was seen in 15 seconds or less (Fig. 5). This reduction time was slightly longer than the predicted values published by Breidt et al. 2010. In their report, 5-log reductions of *E. coli* O157:H7, *Listeria monocytogenes* and *Salmonella* strains should occur in less than 6 seconds at a pH of 4.1 or below. In our study, the tomato serum pH was slightly higher, 4.26, which could account for the small discrepancy. Looking at spoilage microorganisms, yeasts were cultured serendipitously from the tomato serum and proved very hardy. There were yeast counts (log 2.0 CFU/ml) from 0.5 to 2 min at 95° C; long after pathogenic *E. coli* had been eliminated (data not shown).

When Ct values were statistically analyzed, there was no significant difference in Ct values from 0, CUT, 0.25, 0.5 and 1 min (\( p<0.05 \); Table 2A). The mean Ct values for 2 min
were significantly higher than the other times. We observed that the Ct values were not sensitive enough to enumerate the 5-log reduction of pathogens in the serum. However, it was indicative of total pathogen kill (9-log reduction) which was achieved at 2 min (Fig. 5).

The previous hot-fill assay with tomato serum exhibited a high correlation with Ct values and time-temperature treatment at the same pH at 96 °C ($R^2 = 0.95$; Figure 2). However, the time points of this previous assay were much greater than 2 min: 4, 8, 16, and 24 min.

Longer qPCR amplicons might give more sensitive results as they have a higher probability of fragmentation. The amplicon length used for the initial assay was 174 bp (Table 2A). Primers which produced a 1016 bp amplicon from the same mitochondrial gene were tested to see if increased length would also increase sensitivity. The longer amplicon did increase the sensitivity from 2 min to 0.5 min ($p<0.05$; Table 2B). However, there was no significant difference between the Ct values of unheated tomato serum and the 0.25 min heated product, nor the CUT. There was a significant difference between the Ct values of the 0.5 min and 2 min treatment, but no difference between 0.5 min and 1 min at 95 °C.

Protocols for qPCR generally use amplicons less than 200 bp due to the kinetic limitations of the rapid process. An amplicon of over 1,000 bp pushes the limits of the assay. The standard curve for the 1,016 bp amplicon had a linear range of $10^4$ to $10^9$ log$_{10}$ copy numbers, a limit of detection of $10^4$ and an amplification efficiency of 88% (Fig 4). The amplification efficiency was too low to meet MIQE standards of 90 – 110% for qPCR (Bustin, 2009), but data can be used if the Ct values are in the empirically-determined linear range. The limit of detection ($10^4$ copy numbers) was high and could limit the usefulness of this assay. Researchers in past studies have used longer qPCR amplicons in heat treatments...
and have reached conclusions without validating with MIQE standards (Hird et al. 2006). These researchers measured the effect of amplicon length during thermal treatments and observed an increase in Ct value with increasing amplicon size. They concluded that designing longer amplicons might improve specificity while still providing sufficient measurable signal after harsh thermal treatments of meats. However, this article did not provide standard curves for each amplicon size, showing PCR amplification efficiency, linear range of detection and lower detection limits. The increases in Ct value may have been partly or fully due to lack of assay optimization. This was not an isolated incident as there are many peer-reviewed articles which use qPCR without MIQE validation.

Noting the caveats in the paragraph above, a longer amplicon (1,016 bp) with a lower amplification efficiency (88%) and smaller range of detection for the same mitochondrial gene (Fig. 4) was shown to increase the sensitivity of the mtDNA time-temperature integration in thermal protocols requiring rapid heat treatments at lower temperatures such as 5-log reduction of *E. coli* O157:H7 in high acid foods (tomato serum, pH = 4.26, 95 °C, 30 sec).

**Extrinsic DNA Model**

Some processors may want to use an extrinsic, containerized source of DNA to validate thermal processes. To respond to this need, an extrinsic model for low temperature applications (<100 °C) was designed and evaluated using Gblocks™ in citric acid solutions. Three solutions at pH 3.6, 4.0 and 4.4 were evaluated. All pH treatments were between the pKa1 and pKa2 values of 3.13 and 4.76, respectively. Therefore, only one of the three carboxyl groups in citric acid would be protonated. The treatment at pH 4.4 had the only
acceptable $R^2$ value as determined by simple linear regression and goodness-of-fit ($R^2 = 0.90$; Fig. 6). The other pH treatments had low regression correlations: $R^2 = 0.63$ and 0.47 for pH 4.0 and 3.6, respectively. The lower pH solutions probably fragmented the Gblock™ oligonucleotide prior to the thermal treatment, causing increased variation of Ct values. In future studies, higher pH values will be tested.

The D value of the pH 4.4 solution (7.43 min at 96 °C) was calculated from the reciprocal slope of the linear regression of log$_{10}$ copy number versus time (Fig 6). The other pH solutions had similar D values (8.11 & 6.56, for 3.6 and 4.0, respectively; data not shown) despite their low $R^2$ values. The D value for the pH 4.4 solution is similar, though slightly greater, than the D value of Bacillus coagulans ATCC 8038 of 7.05 min at 95 °C (Peng et al. 2012). This particular combination of pH, organic acid and Gblock™ concentration would be a useful molecular monitoring tool for hot fill processes of acidic juices, where B. coagulans is the spoilage organism of concern. Besides thermophilic, acid-resistant spores, there are many other juice spoilage microorganisms such as yeasts (Saccharomyces cerevisiae, Rhodotorula mucilaginosa, Torulaspora delbrueckii, Zygosaccharomyces rouxii) molds (Penicillium citrinum, Penicillium roquefortii, Aspergillus niger) and lactic acid bacteria (Lactobacillus fermentum and L. plantarum) whose D values range from 9.4 to 32 min (Shearer et al. 2002). Extrinsic DNA models could be adapted to target D and z-values of organisms of interest by changing certain parameters: pH, organic acid used (pKa values), addition of salts such as NaCl or MgCl, sequence length of Gblock™, and insertion of nucleotide analogues which effect the melting temperatures or cleavage of oligonucleotides. Disadvantages would include the use and recovery of a
container to carry the extrinsic TTI, and the cost of Gblocks\textsuperscript{TM}. Advantages would include rapid assessment: DNA would not need to be extracted as from an intrinsic source. This would also save on costs per sample. The extrinsic samples could go directly into the qPCR assay (2 hr) for quantification. This method could be used in processes where intrinsic DNA is not available, such as thermal processes of glass, metal or packaging materials. It could be used where foods contain inhibitors to DNA extraction and qPCR such as lipids, pectins, chelators, glycoproteins, casein micelles, and chocolate. Extrinsic DNA methods could be standardized and used in processing plants where the products change, depending on the season. Future studies will examine other similar models using higher pH values, salts, different organic acids and different Gblock\textsuperscript{TM} sequences and lengths. This extrinsic method could be developed into a series of sophisticated DNA thermometers useful for thermal validation in the food and pharmaceutical industries.

\textbf{Conclusions}

Mitochondrial DNA fragmentation as a molecular TTI was assessed in high-acid, low-temperature thermal processes, cucumber fermentations, and storage time of acidified vegetables. Kinetic studies indicate that D values for these thermal processes are very similar to those in low-acid, high-temperature processes. Furthermore, D values from tomato serum studies are similar to those from spoilage microorganisms of concern and could be used for monitoring of thermal process efficacy. Fermentation times in cucumbers could be monitored and optimized using the same technique. Shelf-life evaluation and ultimately, prediction, could rely on quantification of decomposition of mtDNA at room temperatures. Finally, both intrinsic and extrinsic TTI methods could be adjusted and optimized to target
temperatures with desired D and z-values by changes in oligonucleotide and chemical parameters in both protocols.
References


U.S. Food and Drug Administration. 2001. hazard analysis and critical control point (HACCP); procedures for the safe and sanitary processing and importing of juice: final rule (21CFR part 120) Fed. Regist. 66:6137-6202


1 = fresh cucumber
2 = fermented cucumber
3 = fermented, pasteurized, 2 mo storage
4 = fermented, pasteurized 20 mo storage
5 = fresh, autoclaved cucumber

p<0.05

Figure 1. Comparison of mtDNA fragmentation during pickle processing and storage
Table 1A. Effect of fermentation times on cucumber mtDNA fragmentation

<table>
<thead>
<tr>
<th>Months</th>
<th>Mean Ct</th>
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</thead>
<tbody>
<tr>
<td>8</td>
<td>22.30</td>
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<tr>
<td>9</td>
<td>23.56</td>
</tr>
<tr>
<td>11</td>
<td>27.07</td>
</tr>
<tr>
<td>12</td>
<td>28.22</td>
</tr>
</tbody>
</table>

*p<0.022

Table 1B. Effect of geographical origin on cucumber mtDNA fragmentation

<table>
<thead>
<tr>
<th>Location</th>
<th>Mean Ct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florida</td>
<td>20.66</td>
</tr>
<tr>
<td>Georgia</td>
<td>20.30</td>
</tr>
<tr>
<td>Michigan</td>
<td>21.09</td>
</tr>
</tbody>
</table>

*no sig dif
Figure 2. Tomato Juice mtDNA fragmentation at 96 °C

Figure 3. Calculation of tomato serum $D_{96}$ value

$y = 0.2743x + 17.504$
$R^2 = 0.9533$

$y = -0.086x + 6.4516$
$R^2 = 0.9533$

$D = 11.63$ min
Table 2A. Tomato juice mtDNA detection at 95 °C with 174 bp amplicon

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mean Ct</th>
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<tbody>
<tr>
<td>2.0</td>
<td>21.20a</td>
</tr>
<tr>
<td>1.0</td>
<td>20.01b</td>
</tr>
<tr>
<td>0.25</td>
<td>20.00b</td>
</tr>
<tr>
<td>CUT</td>
<td>19.92b</td>
</tr>
<tr>
<td>0</td>
<td>19.72b</td>
</tr>
<tr>
<td>0.5</td>
<td>19.65b</td>
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</table>

Table 2B. Tomato juice mtDNA detection at 95 °C with 1,016 bp amplicon

<table>
<thead>
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</tr>
<tr>
<td>1.0</td>
<td>21.28b</td>
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<tr>
<td>0.5</td>
<td>21.17b</td>
</tr>
<tr>
<td>CUT</td>
<td>21.04bc</td>
</tr>
<tr>
<td>0.25</td>
<td>20.82bc</td>
</tr>
<tr>
<td>0</td>
<td>20.25c*</td>
</tr>
</tbody>
</table>

*p<0.05
Figure 4. mtDNA standard curve for 1,016 bp amplicon

\[ y = -3.6489x + 45.699 \]
\[ R^2 = 0.996 \]
**Figure 5.** *E. coli* O157:H7 reduction at 95 °C in tomato serum
Figure 6. Extrinsic DNA in citric acid (pH 4.4) at 96 °C

y = -0.1346x + 5.61
R² = 0.9014

D = 7.43 min
MITOCHONDRIAL DNA FRAGMENTATION TO MONITOR SAFETY AND QUALITY IN ROASTED PEANUTS

Abstract

Mitochondrial DNA (mtDNA) fragmentation has been proposed as a time-temperature integrator (TTI) for high-moisture thermal processes using low-acid, high-temperature and high-acid, low-temperature protocols. In this study, dry roasted peanuts were assayed using the same novel molecular TTI. Despite low moisture content, low water activity and non-animal origins, peanuts and peanut butter from roasted peanuts, have recently been implicated in *Salmonella* outbreaks. *Enterococcus faecium* was evaluated as a *Salmonella* surrogate for process validation and compared to fragmentation of intrinsic peanut mtDNA and Hunter L color, a quality indicator, for dry roasting. Reduction curve data for *E. faecium* were highly repeatable as similar kinetics were observed when compared to another study which used a commercial, contract laboratory to validate this same surrogate for use with dry roasted peanuts processes (4-log reduction after 10 min at 167 °C). Mitochondrial DNA fragmentation was not linear compared to time at a given temperature, but exhibited a long lag time. D and z-values were calculated using *E. faecium*, Ct and Hunter L color values. D values for *E. faecium* were 2.68, 2.06 and 1.89 min for 138, 153 and 167 °C roasting temperatures, respectively. The *E. faecium* z-value was very large (192 °C) as microbial destruction in dry, roasted nuts does not exhibit true first-order kinetics, but has significant tailing. Mitochondrial DNA fragmentation as measured by Ct had a D value of 12.3 min at a roasting temperature of 167 °C which was slightly higher than “wet” processes (ca. 11.5
Hunter L values had an inverse, linear relationship with time at a given temperature. Hunter L color, if applied to individual peanuts and empirically validated, could be used as an inexpensive visible method to troubleshoot continuous flow systems. Ct values were not linear or highly correlated to Hunter L values. It was difficult to compare Ct values to Hunter L values since the former used DNA from individual peanuts and the latter looked at the mean values of many units. Three different peanut DNA extraction methods were compared. Grinding by mortar or commercial grinding tube gave significantly lower Ct values but similar DNA quantities and 260/280 ratios, when compared to a non-grinding protocol. No qPCR inhibition was seen due to fats, oils or other peanut components. Dissection of peanuts exhibited a differential heating effect depending on the part of the peanut used for DNA extraction and the type of tissue assayed. MtDNA fragmentation as measured by Ct value was deemed too variable for thermal process or quality validation of dry, solid foods such as peanuts. However, it could be used to evaluate penetration of heat through a solid food matrix, to find the coldest spot and test the worst case scenario.

**Introduction**

*Salmonella Outbreaks in Peanuts*

Because of inherent low moisture content, low water activity ($A_w$) and non-animal origins, roasted peanuts, peanut butter and other nuts were considered low risk for *Salmonella* spp. contamination (Sheth et al. 2011). However, infective doses of *Salmonella* have been reported in fatty, low-moisture foods such as chocolate and cheese (Gill et al. 1983; D’Aoust, 1985). The first reported outbreak of *Salmonella* in peanut
butter occurred in Australia in 1996 (Scheil et al. 1998). The source of the implicated *Salmonella* Mbandaka was traced back to a peanut roasting factory. Other outbreaks were associated with peanut-containing snacks in Israel and North American (Killalea et al. 1996) and Asian-style peanuts still in their shell (Kirk et al. 2004). From 2006 to 2007, *Salmonella* Tennessee in peanut butter was responsible for a major food-borne disease outbreak in the United States (Deng et al. 2013; MMWR, 2007). An epidemiological study identified 715 cases from 48 states, resulting in 93 hospitalizations (Sheth et al. 2011). In September 2012, Sunland Inc. of Portales, New Mexico announced a voluntary recall of almond and peanut butter due to a rare outbreak of *Salmonella* Bredeney associated with the products (MMWR, 2013). These reoccurring outbreaks of *Salmonella* from peanut products in recent years have caused processors to install new equipment, make roof repairs, sanitize facilities, ensure separation of raw and finished product and hastened implementation of new Hazard Analysis and Critical Control Points plans (HACCP) to assure the safety of peanuts (Sheth et al. 2011).

**Salmonella Heat Tolerance and Persistence**

New research indicates that *Salmonella* spp. are able to persist in foods under a variety of conditions. Burnett and others (2000) found that *Salmonella* could survive for at least 24 weeks in low-moisture foods such as peanut butter. It was able to habituate in high-fat, low water activity environments and survive high temperatures (Mattick et al. 2000). Aviles and others (2013) reported that exposures to high fat content and low A$_w$ in peanut butter caused a protective effect with increased survival of *Salmonella* Tennessee in gastric models. Stressed *S. enterica* were found to have significantly more
heat resistance than naïve bacteria (He et al. 2011) and outbreak strains were shown to be more thermo-tolerant than other strains tested (Ma et al. 2009). Heat resistance can be influenced by many factors including previous growth and storage conditions with lower moisture levels generally produce greater heat resistance (Podolak et al. 2010). Likewise, increased $A_w$ can reduce thermal resistance (He et al. 2013). Addition of 7% moisture to cocoa and hazelnut shells significantly reduced D values of two Salmonella strains (Izurieta & Komitopoulou, 2012). Corn syrup, soy protein, vitamin and mineral supplements in peanut butter formulations may increase the viability of Salmonella during the first weeks of storage (Burnett et al. 2000). The survival and heat resistance of S. enteric was significantly affected by peanut butter formulation and storage temperature. Due to its persistence, Salmonella contamination is especially troubling when it survives in products such as peanuts and peanut butter that have a long shelf life (Sobel et al. 2001).

**Salmonella Detection Methods**

Samples of raw, shelled peanuts from 2008-2010 were examined for Salmonella contamination (Calhoun et al. 2013). Out of 944 samples, 2.33% were positive for Salmonella using the VIDAS assay, an automated enzyme-linked fluorescent immunoassay (Crowley et al. 2011). Using a two-step enrichment, the VIDAS assay was deemed comparable to older, culture methods (Crowley et al. 2011). Another similar method using enrichment plus a lateral flow immunoassay, the Reveal Salmonella 2.0 test provided accurate results regardless of initial sample volume (Hoerner et al. 2011). Quantitative polymerase chain reaction (qPCR) kits have been evaluated (Li et al. 2012;
Balachandran et al. (2011) and performed well against FDA/BAM methods. Other researchers have detected the *Salmonella invA* gene using isothermal methods which require only a water bath and could be used on-site or in the field (Kim et al. 2011). While all these molecular detection methods are rapid, they fail to quantitate *Salmonella* cells and therefore have limited use in thermal process validation.

**mtDNA Fragmentation in Solid Foodstuffs**

The destruction and fragmentation of mitochondrial DNA (mtDNA) from high- and low-acid foods was highly correlated to spore surrogate destruction, cumulative F-values and time-temperature treatments in earlier studies (Chapters 2 & 3). The goal with roasting peanuts was to evaluate mtDNA fragmentation as a TTI in a dry thermal process as opposed to previous “wet” processes. To this end, the effectiveness of mtDNA fragmentation, as determined by Ct value in qPCR, was examined using a solid, high-fat, low moisture and low A_w product. The Almond Board of California has recently recommended the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella enterica* in validation of dry roasting processes in almonds (ABC, October 2007). *E. faecium* was also validated in oil and dry roasting of peanuts at various temperatures and found to have similar kinetics but proved more resistant to heat processing than *Salmonella* spp. (Sanders & Calhoun, 2014). These researchers provided documentation for the use of this microorganism as an acceptable surrogate for *Salmonella* on peanut thermal processes. Additionally, fragmentation of intrinsic peanut mtDNA was compared to reduction curves of this newly validated *Salmonella* surrogate.
To assess its usefulness as a quality parameter, intrinsic mtDNA fragmentation was compared to Hunter L color value, a quality indicator.

**Materials and Methods**

**Inoculation**

*Enterococcus faecium* (ATCC 8459) was inoculated into BHI broth (Remel, Lenexa, Kansas) from freshly plated colonies and incubated statically overnight at 35 °C. Cultures were concentrated 2X by centrifugation (5810R, Eppendorf, Hamburg, Germany) at 6,000 rpm for 10 minutes at 4 °C and resuspended in 0.5 X initial volume with sterile 0.9% saline. Initial culture concentration was determined by A$_{600}$ and a simplified agar plate technique (Jett et al. 1997) utilizing square Petri plates and the track-dilution method. *Enterococcus*-inoculated saline was added to the total weight of peanuts to be tested in a large plastic zipper bag, diluting the culture 1:20. The bag was closed and secured. Contents were mixed thoroughly, and then allowed to sit for 5 minutes to absorb the liquid. Target concentration was 10$^8$ CFU/ml. Inoculated peanuts were poured onto wire racks in 100 g aliquots and allowed to air dry for 20 minutes.

**Roasting**

A convection oven (Despatch Model LXD1-42-2; Despatch Industries; Minneapolis, MN) was set at 138, 153 or 167 °C and allowed to equilibrate for 30 minutes. A metal rack was inserted in the oven and brought to temperature. This rack was designed to hold smaller racks made of hardware cloth through which peanuts would not pass. Each batch consisted of 100g lots laid out on the small hardware cloth racks. Once the smaller racks are inserted into the larger rack, the peanuts were essentially
suspended in the moving air inside the oven. For each roasting batch, the oven door was quickly opened, the tray with the peanuts slid into the large rack and the door of the oven quickly closed. There was a drop in the oven temperature due to opening the door. The lowest temperature reached and the number of seconds required for the oven to return to set point was recorded for each batch. At the appropriate time point, the oven was opened quickly and the peanuts removed in the small rack. This rack was placed over a box fan with sufficient flow to cool the peanuts to room temperature in 30 seconds. To prevent cross contamination between reps, a new pair of gloves was used for loading each batch into the small rack and into the oven and a separate pair used to remove each batch. Between runs, the small rack and the cooler were sprayed with 70% EtOH and allowed to dry before the next batch came into contact with them. Cooled peanuts were placed in plastic bags for mitochondrial DNA and Enterococcus faecium plate count analysis.

**Culture Enumeration**

Three replicate samples were taken from the following time points: 0, 3, 6, 9, 12, 15, 18, and 21 minutes. Ten grams were taken from each 100 g replicate and placed in a stomacher bag (Filtra-bag, Fisher, Pittsburg, PA) with 10 ml sterile 0.9% saline (1:1 dilution). Peanuts were stomached in a Seward Stomacher 400 (Tekmar, Cincinnati, Ohio) for 2 minutes at normal speed. Filtrate was aseptically removed from the stomacher bag, serially diluted and plated as above using the simplified agar plate technique (Jett et al. 1997) with BHI agar (BD, Sparks, Maryland). Plates were incubated
at 35 °C over night. Plates were counted manually and CFU/g peanuts were calculated, taking into account concentration and dilution factors.

Peanut Dissection

To determine if there were significant positional differences in mtDNA fragmentation after roasting, we dissected and separated the following parts of single peanuts: testa (skin), embryo, outer and inner endosperm. Georgia Green medium runner peanuts were roasted at 167 °C as previously and samples given the following time treatments: 0, 12 and 18 minutes. Three peanuts were chosen at random from each time point and dissected used a razor blade and cutting board, both of which were decontaminated between samples with 70% ethanol. The skin and embryo were removed and placed in micro-centrifuge tubes. The endosperm was dissected in the following manner to differentiate between inner and outer portions: skin was removed and peanut cut in half along natural break, with the outer portion facing upward, two longitudinal cuts were made, dividing the nut half into three long sections, the middle section was turned on its side and cut in half, dividing the inner from the outer portion, the middle of each inner and outer portion was used for DNA extraction (~ 5 to 10 mg). All samples were frozen at -80 °C until DNA extraction with MasterPure DNA purification kit (Epicentre, Madison, Wisconsin) using the tissue sample portion of the protocol. Tissues were not ground but placed directly into first enzyme procedure.

DNA Extraction

Three peanuts from each replicate were ground under liquid nitrogen in a mortar and pestle. The mortar and pestle were thoroughly cleaned between samples with 70%
ethanol to prevent cross contamination. DNA was extracted using *ca.* 2.5 mg or one inoculation loop of ground peanut in the MasterPure DNA purification kit (Epicentre, Madison, Wisconsin) using the tissue sample portion of the protocol. DNA was quantified and qualified with a spectrophotometer (Nanodrop, Wilmington, Delaware).

**Quantitative PCR (qPCR)**

Quantitative PCR (qPCR) was run in 25 ul 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 MgCl₂, 40 mM Tris-HCl, pH 8.4, 100 mM KCl, 20 nM fluorescein; BioRad, Hercules, CA), 300 nM final concentration each for forward and reverse primers, peanut DNA (5-10 ng/reaction) and RT-PCR water (Ambion, Austin, TX) to final volume.

Amplifications were performed in a MyiQ (BioRad, Hercules, CA) 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 sec, 72.0 °C for 30 sec; with FAM channel optics on during annealing stage. No template (NTC) and positive controls were used for all assays. The positive control was used to normalize data between assays. For a sample to be considered positive, its threshold cycle (Ct) value must be less than all negative control reactions and its corresponding amplification curve had to exhibit the three distinct phases of real-time PCR: lag, linear and plateau.

**Hunter L Color Determination**

The Hunter L color value was determined for each time point and rep. Testa were removed and 80 g of sample were placed in a glass petri dish and inserted above a
calibrated HunterLab DP 9000 with D25 sensor (Hunter Associates Laboratory; Reston, VA) utilizing the Lab scale. Readout was recorded three times per sample with the peanuts removed and resorted in the petri dish between readings with nuts placed outer-side down if broken. The color was expressed as the mean of 3 replications/sample presented to the colorimeter. The scale of the readings ranged from 1-100 with 1 representing black and 100 representing white.

**Comparison of Processing and DNA Extraction Methods**

Three different grinding techniques were evaluated for DNA extraction and qPCR efficacy: mortar and pestle, commercial micro-centrifuge grinder tubes, no grinding but use of removed embryo only. All were processed with Master Pure DNA extraction kit. Mortar and pestle procedure was used as before. Grinder tubes utilized frozen peanut samples which were chopped into small pieces (5 -10 mg) with sterile razor blades and placed in micro-centrifuge grinding tubes (GE Healthcare, USA) then ground in 100 ul Master Pure tissue and lysis solution. Recovered solution from grinder tube centrifugation (60-70 ul) was brought up to 300 ul total tissue and lysis solution and proteinase K was added as the first step of the Master Pure DNA extraction kit. The no-grind protocol simply required the user to dissect out the peanut embryo and add it in its entirety to the first proteinase K enzymatic step of the Master Pure DNA extraction. After extraction, all DNA samples were quantified and quality evaluated by Nanodrop spectrophotometer. Quantitative PCR was run for mtDNA as before.
Results and Discussion

mtDNA Fragmentation in Dry Roasting

Peanut mtDNA fragmentation was assayed during a dry roast using a lab-scale convection oven. While this oven had convective airflow it was not comparable to industrial-scale, commercial ovens which utilize a conveyor belt, multi-directional air flows and more than one layer of peanuts. Ct values were initially steady, then increased after 12 minutes of roasting at 167 °C to a mean value ca. 22 units after 21 minutes (Fig. 1). Ct values did not increase in a linear fashion over time, perhaps due to low peanut A_w (0.40; Lee & Resurreccion; 2004) where water was sequestered in the solid matrix of the nut. It was theorized that after ca. 10 min at 167 °C, peanut cell membranes were broken and water was released, promoting mtDNA fragmentation and Ct values to increase rapidly in the last two minutes of the trial. The D value of mtDNA fragmentation was calculated using the log_{10} copy number as determined by the qPCR standard curve for the 174 bp amplicon (Chapter 2, Fig. 2). The D value was 12.3 min at 167 °C (Fig. 2), which was slightly higher than values obtained for low- and high-acid thermal processes (11.29 & 11.63 min, respectively). However, the correlation between log_{10} copy number and time (min) was much lower (R^2 = 0.65) than for these wet processes (low acid, R^2 = 0.87; high-acid, R^2 = 0.95). Peanut mtDNA fragmentation was correlated to surrogate death at 167 °C (Fig. 3, R^2 = 0.64) by quadratic equation. Dry roasting at 167 °C exhibited similar trends, such as initial Ct lag, to the other lower temperature treatments of 138 and 153 °C (data not shown). All three temperature treatments were compared to *E. faecium* reduction (Fig. 4), with the 153 °C treatment having the highest correlation (R^2 = 0.89),
possibly due to fewer time points. The 167 and 138 °C treatments were only weakly correlated to *E. faecium* reduction ($R^2 = 0.54$ and 0.22, respectively).

**Peanut Salmonella Surrogate**

*Enterococcus faecium* D values were determined (Fig. 5) and decreased only slightly with significant increases in temperature. D values for *E. faecium* were 2.68, 2.06 and 1.89 min for 138, 153 and 167 °C dry roasting temperatures, respectively. The *E. faecium* z-value was very large (192 °C) as microbial destruction in dry, roasted nuts does not exhibit true first-order kinetics, but has significant tailing (Fig. 6; Harris presentation, 2009). The Almond Board of California cautions against extrapolation of D values in dry roasting due to this phenomena. Podolak and others (2010) state that care must be taken when applying D and z-values from the literature in processes involving low-moisture foods and *Salmonella* contamination. The kinetics of *E. faecium* reduction in this study (4-log reduction after 10 min at 167 °C) were similar to an earlier study (Sanders and Calhoun, 2014) which evaluated the usefulness of this organism as a *Salmonella* surrogate in peanut roasting.

**Hunter L Color Values**

With this particular variety and lot of peanuts, one Hunter L color value (56 units) corresponded to the complete die off of *E. faecium* regardless of time/temperature treatment (data not shown). Other researchers (McDaniel et al. 2012) have also noted the achievement of equivalent surface colors using different roast time-temperature combinations. They related Hunter L values to quality and nutrient parameters such as moisture content, sugars, amino acids, tocopherols and antioxidants. They concluded that
peanuts roasted to equivalent Hunter L values using different time-temperature treatments were not equivalent in quality parameters. Further research with Hunter L values versus safety parameters may reveal a similar trend.

Hunter L values had an inverse linear relationship with high correlations ($R^2 > 0.96$) to time-temperature in the three treatments studied (Fig 7). Peanut mean Ct values were compared to Hunter L values and the 153 °C treatment had the highest correlation ($R^2 = 0.89$) using simple linear regression (Fig 8). MtDNA fragmentation was more closely related to color change than *E. faecium* reduction.

**Peanut Dissection**

While peanut mtDNA Ct values increased with time of dry roast, there was wide variation at each time point (>1.0 Ct, data not shown). This was probably due to the part of the peanut was chosen for extraction and small sample size (n = 3). Our peanut dissection assay indicated large variation between individual peanuts (data not shown). A visual examination of individual peanuts also revealed large differences in roast color. Since Hunter L values use an average of 80 g, individual peanuts are not assessed. Ct values between different parts of the same peanut also varied (Table 1). Significant differences were seen between the log$_{10}$ copy numbers of the testa or skin and the embryo and both the inner and outer endosperm at all time points in the 167 °C dry roast. Since the testa was desiccated compared to the other peanut parts, this was not unexpected. The log$_{10}$ copy number of the embryo and the inner and outer endosperm were not significantly different prior to roasting at time 0. After roasting for 12 min, the inner endosperm has a significantly higher log$_{10}$ copy number compared to the embryo and
outer endosperm, since the temperature inside the peanut was probably lower than outside the nut, resulting in less mtDNA fragmentation. After 18 min of dry roasting, the inner and outer endosperm had equilibrated, exhibiting no significant difference in log$_{10}$ copy number. However, at this time point, the embryo log$_{10}$ copy number was significantly lower than either part of the endosperm. The embryo has a smaller mass than the endosperm and sits along an inner channel which bisects the peanut and could transport hot air through the midline of the peanut. Also, the embryo is composed of germ plasm, a different material from the endosperm, whose function is to provide nutrients to the growing embryo. Since it is easy to remove and continues to fragment after 18 min of dry roast, peanut dissection confirms the usefulness of the embryo as a reliable sample site from the nut.

**Peanut Processing Prior to DNA Extraction**

Many articles in the literature use dry ice and grinding in a mortar and pestle to process the nut samples prior to DNA extraction. However, prevention of DNA cross-contamination between samples in the mortar requires frequent cleaning. Different methods of peanut grinding and processing prior to DNA extraction did not significantly affect the quality of DNA (Table 2). The 260/280 ratio, indicative of DNA quality, across all treatments was between 1.7 to 2.1 with a mean and standard deviation of 1.92 +/- 0.13. Ratios above 1.8 are considered acceptable for downstream processes like qPCR. No qPCR inhibition was seen due to fats, oils or other peanut components as tested by serial dilution (data not shown). DNA concentrations varied with grinding process. The mortar system had the most consistent DNA concentrations while both the
grinding micro-centrifuge tube and no-grind embryo process varied 1.5- to 3-fold. However, when DNA concentrations were normalized to 5-10 ng/well, Ct values were similar within and between grinding treatments, although Ct values for the no-grind procedure were ca. 0.8 units higher and significantly different. The no-grind process, while producing somewhat higher Ct values, was rapid, required no special equipment such as dry ice and reduced cross-contamination of samples while still providing robust Ct values.

**Conclusions**

Intrinsic mtDNA fragmentation, as measured by increase of Ct value in qPCR, occurs during dry roasting of peanuts. D values are slightly higher than in low-acid, high-temperature and high-acid, low temperature processes (Chapters 2 & 3). However, correlations to safety (*E. faecium* reduction) and quality (Hunter L value) are low, due primarily to initial lag time of DNA destruction. Because it involves individual solid units having low moisture content and movement in a continuous process, peanut roasting efficacy is difficult to quantify. D and z-values are difficult to extrapolate because of significant tailing at both ends of the reduction curve. However, most heat treatments for peanuts are several minutes longer than needed to kill *Salmonella*, or its surrogates, regardless of temperature used. Contamination with *Salmonella* probably occurs after roasting during handling and further processing. This means that indicators of thermal process efficacy are useless until equipment and employee cross-contamination after roasting are eliminated.
*E. faecium* reduction in peanut dry roast was found to be highly repeatable, with similar kinetics found between a commercial and academic lab. Sanders and Calhoun (2014) found *E. faecium* to have similar kinetics but a slightly higher heat resistance than *Salmonella* during peanut roasting temperatures. The Almond Board of California endorses this surrogate for almond roast and this study confirms and strengthens its usefulness in peanut dry roasts.

Looking at quality indicators, Hunter L color could be used as a quick, presumptive indicator of adequate time-temperature to kill pathogens. However, the final Hunter L color corresponding to a safe roast must be determined empirically and compared to surrogate reduction with each type of peanut, roaster, and other variable parameters in the thermal process. Despite using pooled samples as compared to individual nuts, Hunter L value correlated to mtDNA fragmentation more highly than *E. faecium* reduction.

Ct values from intrinsic mtDNA were highly variable at each time point and exhibited non-linear destruction due to initial lag. Future trials should increase sample numbers or pool and homogenize many samples to reduce variation within time-temperature treatments and between individual units. Use of the embryo only for DNA extraction would serve to standardize peanut sampling procedures. Grinding of the peanut prior to DNA extraction was not necessary to get robust Ct values and no inhibition of qPCR by peanut components was found. The most promising aspect of this study is the use of mtDNA fragmentation with dissection of a low-moisture, glassy, solid matrix, to quantify heat penetration over time. Pathogens can also penetrate nuts, fruits, and
vegetables; therefore, heat treatments should monitor the center of these solids or different physiological parts directly. It is difficult to place a probe inside a solid without changing its thermal kinetics. Intrinsic mtDNA fragmentation with dissection can be utilized to pinpoint the coldest spot in a solid food such as a peanut, providing a worst case scenario for testing.
References

Almond Board of California (ABC) Guidelines for validation of dry roasting processes. October, 2007. <AlmondBoard.com>


Figure 1. Peanut mean Ct versus dry roasting time at 167 °C

\[ y = 0.0011x^3 - 0.011x^2 + 0.0255x + 16.314 \]
\[ R^2 = 0.8882 \]
Figure 2. Log₁₀ copy number of mtDNA versus time of roasting at 167 °C
Figure 3. Mean peanut Ct versus *E. faecium* reduction at 167 °C

\[ y = 0.04x^2 - 0.6046x + 18.526 \]

\[ R^2 = 0.643 \]
Figure 4. Ct versus *E. faecium* reduction during peanut dry roasting

138 C
\[ y = -0.172x + 21.452 \]
\[ R^2 = 0.2164 \]

153 C
\[ y = -0.717x + 25.089 \]
\[ R^2 = 0.892 \]

167 C
\[ y = -0.2694x + 18.142 \]
\[ R^2 = 0.5407 \]
Figure 5. *E. faecium* reduction on dry roasted peanuts at three temperatures
Figure 6. Calculation of z-value for *E. faecium* in dry roasted peanuts

\[ y = -0.0052x + 2.9172 \]

\[ R^2 = 0.9296 \]

z-value = 192 °C
Figure 7. Hunter L values at three dry roasting temperatures
Figure 8. Peanut mtDNA fragmentation versus Hunter L color under three dry roast temperatures

$y = -0.5495x + 53.314$
$R^2 = 0.6613$

$138\,^\circ C$

$y = -0.7969x + 68.377$
$R^2 = 0.8912$

$153\,^\circ C$

$y = -0.5013x + 46.48$
$R^2 = 0.5993$

$167\,^\circ C$

Peanut mean Ct

Hunter L color
Table 1. Differences in mtDNA qPCR amplification in various peanut parts during dry roasting at 167 °C

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*Denotes significant differences between means (p<0.05).
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<sup>a</sup>Denotes significant differences between means (p<0.05).
SUMMARY AND FUTURE RESEARCH PROJECTIONS

This dissertation examined the use of plant-derived, intrinsic, mitochondrial DNA (mtDNA) as a time-temperature or time-process indicator by quantifying its fragmentation by qPCR after thermal processing, high-pressure processing, fermentation and room-temperature storage. When analyzed by simple linear regression, Ct value exhibited a high correlation to time-temperature treatments using low acid/high temperature, and high acid/low temperature protocols. D- and z-values were calculated for low acid/high temperature protocols and exhibited zero-order kinetics. There was a ~1:1 ratio between Ct increase and 1-log reduction of spore surrogate. Under high acid/low temperature protocols, increase in mtDNA amplicon size could improve the sensitivity of the assay.

Future research using this new qPCR paradigm would include creating synthetic DNA double-stranded oligos for use as extrinsic, containerized, time-temperature integrators. D- and z-values of these assays could be engineered to hit desired processing targets. Future studies have been funded by the Center for Advanced Processing and Packaging Systems (CAPPS). These include rapid shelf-life evaluations using oxidation reactions and an intrinsic, global DNA fragmentation, quantified by the Agilent TapeStation and the DNA integrity number (DIN). The USDA-ARS Food Science Research Unit has included this assay in their upcoming 5-year plan to evaluate cucumber fermentation and pickle shelf-life. The USDA Office of Technology Transfer (OTT) in partnership with the NCSU OTT, has applied for both a United States and
International patent on all materials stated in this dissertation. They are presently soliciting licensing partners to develop and validate these processes for industry.
APPENDIX
Appendix A

Protocol 00: Full water immersion

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### Appendix B

**Protocol 01: Full water immersion**

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