Comparative Study of Ancient DNA Extraction Methods for Archaeological Plant Remains

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Abstract

Despite the potential for plant ancient DNA (aDNA) to address important archaeological questions, there are significantly fewer studies of plant aDNA compared to human and animal aDNA, partially due to a lack of research on DNA extraction methods for ancient plant remains.

The current study uses heat to degrade modern corn, pea, and squash seeds to simulate degraded DNA associated with archaeological macro-botanical remains. I then compare DNA recovery efficiencies of three common DNA extraction methods using these artificially degraded samples. Standard and quantitative PCR are used to assess the quality and quantity of recovered DNA.

We have determined that the silica-spin column method is superior for degraded DNA recovery from all three plant species. Additionally, DNA recovery rates of the three methods differ across all plant species tested. We recommend that selection of extraction techniques be carefully considered to optimize recovery of DNA from ancient macro-botanical remains.

Keywords: ancient plant DNA; artificial DNA degradation; silica-spin column; heat treatment

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Glossary

Ancient DNA	DNA from archaeological remains, generally characterized as limited quantities of degraded Deoxyribonucleic Acid (DNA).
Amplicon	DNA fragment produced by PCR amplification using sequence-specific primers.
Cotyledon	Part of the embryo of a plant disseminule; upon germination, the cotyledon becomes the first leaf of the seedling; angiosperms are classified as monocots (single cotyledon) or dicots (two cotelydons).
Extract	Solution containing isolated DNA from samples following the DNA extraction process.
Expected Recovery	A calculation from the data generated by the IPC qPCR assay. It attempts to predict the success of future PCR reactions using the same sample extract based on the amount of inhibition and the sample dilution used. The expected recovery is calculated using the formula: $ER = 100\ \% \times (\text{dilution factor} \times 2^{\Delta Cq})^{-1}$
Gel Electrophoresis	A method of separating molecules, including DNA, based on their size:charge ratio; electricity is run through an agarose gel causing DNA to migrate in the direction of the current with shorter fragments migrating faster (and thus farther down the gel) than larger fragments.
Hydrolysis probe	Also known as Taqman probe; a dual-labelled specific probe designed to bind to a target DNA sequence with a fluorescent reporter dye at one end and a quencher at the other end; the quenching molecule suppresses fluorescence of the reporter molecule until the probe anneals to a DNA template and is excised as a result of DNA polymerase activity; used in quantitative PCR as the amount of fluorescence can be compared to a known standard for sample quantification.
Internal Positive Control	IPC; a control reaction in which an artificial DNA standard is amplified; sample extracts are added to the control reaction to determine whether inhibitory complexes are present in the sample, which interfere with amplification of the control reaction.

Maillard products	Cross-linked compound resulting from the chemical reaction between an amino group (part of a protein or DNA strand) and a sugar molecule; prevents bound DNA from being amplifiable by PCR; this cross-linking reaction is promoted by heat and thus is particularly problematic for the recovery of DNA from heat treated samples (*e.g.,* artificially degraded DNA or charred ancient plant remains).
PCR	Polymerase chain reaction; method of amplifying specific fragments of DNA in the laboratory; characterized by repeated cycles of temperature changes to facilitate the denaturing, annealing, and extension phases of DNA replication; also known as standard PCR or Sanger PCR.
Polyphenols	Aromatic metabolite found in mature plant seeds and dried fruits; cause brown colouration when oxidized; released from vacuoles and bind to DNA following cell disruption, leading to PCR inhibition.
Primers	Short fragment of DNA (generally 18-30 basepairs) used during PCR to target a specific sequence of template DNA for amplification; binds to template DNA and serves as the starting location for the replication enzyme, DNA polymerase.
Quantification cycle	C_q; reflects the cycle number at which the fluorescence generated within a reaction crosses an assigned threshold value (*i.e.,* the point at which a sufficient number of amplicons has accumulated). Previously referred to as cycle threshold (C_t) prior to the MIQE guidelines related to qPCR.
Quantitative PCR	qPCR; continuous collection of fluorescent signal from different types of dyes (*e.g.,* hydrolysis probes) during PCR to permit quantification of samples during the exponential phase of DNA replication; DNA samples are compared to known DNA standards.
ΔRn	ΔRn is a measure of the amount of amplification present in a quantitative PCR sample. The magnitude of the ΔRn is determined by subtracting the background fluorescence from the reporter dye fluorescence signal at multiple time points during PCR.

List of Abbreviations

aDNA	Ancient DNA
bp	Basepairs
BSA	Bovine serum albumin
Ct	Cycle threshold
CTAB	cetyltrimethylammonium bromide
cpDNA	Chloroplast DNA
C_q	Quantification cycle
DNA	Deoxyribonucleic acid
DTAB	Dodecyltrimethylammonium bromide
ER	Expected recovery
IPC	Internal Positive Control
mtDNA	Mitochondrial DNA
nDNA	Nuclear DNA
NMWL	Nominal molecular weight limit
PCR	Polymerase chain reaction
qPCR	Quantitative PCR

Chapter 1: Introduction

The recovery and analysis of DNA from ancient plant remains has enormous potential to shed light on a number of fields including paleoecology and archaeology. In particular, ancient DNA (aDNA) from archaeological macro-botanical remains can be used to reconstruct plant use in the past and shed light on issues including plant domestication, regional vegetation, migration, trade, and diet (Schlumbaum *et al.* 2008a). Archaeobotanists frequently use plant morphology to identify and classify ancient macro-botanical remains, providing insight into the subsistence of early civilizations, their social structure, and even historical climate conditions (Hastorf and Popper, ed. 1988; Pearsall 2000; Zohary and Hopf 2000). Unfortunately, many macro-botanical remains are fragmented, charred or otherwise processed, which can make morphological identifications problematic. In some of these challenging cases, plant aDNA has proven particularly useful due to its ability to identify plant remains to the species level in the absence of distinct morphological features (Allaby *et al.* 1994; Allaby *et al.* 1999; Brown *et al.* 1998; Schlumbaum *et al.* 1998).

Despite the potential applications of plant aDNA research, there have been far fewer plant aDNA studies than human and other vertebrate aDNA studies (Gugerli *et al.* 2005). A survey of approximately 500 aDNA studies found that only seven percent were focused on plant aDNA (Gugerli *et al.* 2005). There are several possible reasons for this imbalance: a greater general interest in humans and other vertebrates than in plants; limited understanding of DNA preservation in archaeobotanical remains compared to

animal remains; the inherent limitations associated with plant DNA markers compared to DNA markers in vertebrates; and technical difficulties including co-extraction of plant components (*e.g.,* Maillard products, polyphenols, and polysaccharides) that inhibit the polymerase chain reaction (PCR), an essential step in DNA analysis (Evershed *et al.* 1997; Gugerli *et al.* 2005; Schlumbaum *et al.* 2008a; Xin and Chen 2006). Regardless of the reason(s), this imbalance has contributed to the overall slower progress in the field of plant compared to animal aDNA.

One area in particular in which the field of plant aDNA has lagged behind animal aDNA research is the comparison and optimization of DNA extraction methods. As Schlumbaum *et al.* (2008a:236) note, the plant aDNA field has "no preferred extraction methods for ancient plant material, nor is there any comprehensive comparison of protocol, such as exists for animals." While several comprehensive comparisons of the various DNA extraction methods have been conducted using human and faunal bones (Bouwman and Brown 2002; Lee *et al.* 2010; MacHugh *et al.* 2000; Prado *et al.* 2002; Rohland and Hofreiter 2007; Yang *et al.* 1998), only one assessment of two common plant aDNA extraction methods has been carried out, and this study focused on a single plant species, wheat (Giles and Brown 2008). Consequently, current studies in the field of plant aDNA use different extraction techniques without knowing the efficiency of the chosen method for the type of plant remains being studied. This is particularly problematic because, unlike bone structure, which is very similar across a wide spectrum of vertebrate species and thus allows a universal extraction method to be used, the internal structures (*e.g.,* polysaccharides, storage proteins, and secondary products) of plants from different families and species are considerably more heterogeneous. Weising

et al. (2005:88) predict that because "the biochemical composition of plant tissues and species varies considerably, it [will be] virtually impossible to supply a single [DNA] isolation protocol that is optimally suited for each plant species. Even closely related species may require quite different procedures."

The three most commonly used DNA extraction methods for archaeobotanical remains include: [1] a cetyltrimethylammonium bromide (CTAB) extraction with a phenol-chloroform step (Anderson-Carpenter *et al.* 2011; Gyulai *et al.* 2006; Manen *et al.* 2003; Pollmann *et al.* 2005; Schlumbaum *et al.* 2008b); [2] a silica-based extraction method (Brown *et al.* 1998; Giles and Brown 2008; Poinar *et al.* 1998, Hofreiter *et al.* 2000; Pollmann *et al.* 2005; Schlumbaum *et al.* 1998); and [3] a commercially available plant extraction kit, DNeasy Plant Mini Kit by Qiagen (Dumolin-Lapegue *et al.* 1999; Elbaum *et al.* 2006; Erickson *et al.* 2005; Liepelt *et al.* 2006; Speirs *et al.* 2009; Schlumbaum *et al.* 2008b). In addition, some studies involve the extraction of DNA by two different methods to support the authentication of results (Blatter *et al.* 2002a; Elbaum *et al.* 2006; Pollmann *et al.* 2005; Schlumbaum *et al.* 2008b) or employ different extraction methods for modern samples than those that are used for the ancient remains (Elbaum *et al.* 2006; Schlumbaum *et al.* 1998). Thus, there is a lack of consensus on which, if any, extraction method is best and many researchers assume that the most appropriate methods for modern and ancient plant remains may be different.

To further compound the issue, plant aDNA studies tend to have a significantly lower PCR amplification success rate compared to human and faunal aDNA studies (Blatter *et al.* 2002b; Brown *et al.* 1998; Rohland and Hofreiter 2007; Schlumbaum *et al.* 1998; Yang *et al.* 2004, Yang *et al.* 2008). The most commonly recovered

archaeobotanical specimens are macro-botanical remains preserved by charring (Zohary and Hopf 2000). However, the DNA within these samples can be highly fragmented and unsuitable for DNA analysis. The resulting low DNA amplification success rates emphasize the importance of using the most efficient DNA extraction method possible when extracting DNA from ancient plant remains to increase the likelihood of recovering the very limited and degraded DNA. Use of inefficient extraction methods could potentially mislead researchers to conclude that the DNA is completely degraded in their samples when in actuality it is preserved but not recoverable with the selected extraction method. A study by Threadgold and Brown (2003) in which wheat grains were subjected to heat treatment to artificially degrade the DNA demonstrated that low amplification rates could be due to rapid degradation of DNA during the heat treatment process. Building upon this research, Giles and Brown (2008) used heat treatment to test the efficiency of DNA recovery using two different extraction methods from single wheat grains. They concluded that the silica-spin column method yielded more consistent results and stronger amplification than a modified CTAB method with dodecyltrimethylammonium bromide (DTAB) known as DTAB/CTAB (Giles and Brown 2008). These studies serve as the solid foundation for more extensive comparisons of DNA extraction methods on a variety of plant species, which is the focus of the current study.

Research Objective and Hypotheses

The primary objective of this study is to assess the effectiveness of three DNA extraction methods currently used in the field of plant aDNA by conducting a comprehensive comparison using multiple plant species selected to represent three

different plant families. It was particularly important in this study to include multiple plant species and families because, unlike animal bones, which are fairly homogeneous in structure and composition, plant remains are highly heterogeneous in terms of internal composition. Thus, we hypothesize that no single extraction method will be optimal for all plant specimens tested, and consequently different extraction methods should be used for different macro-botanical remains. Alternatively, it is possible that, as is the case for ancient faunal remains, one extraction method will be the most efficient at recovering artificially degraded DNA from multiple plant species, and therefore it has the potential to be used as a universal extraction method in future studies focused on ancient macro-botanical remains.

For the purposes of this study, the effectiveness of the extraction methods will be determined by measuring both how much DNA each extraction method recovers (extraction efficiency) and how clean the sample extracts are in terms of the extent of PCR inhibition. Additionally, to simulate the quantity and quality of DNA expected to be present in macro-botanical remains, which contain limited amounts of degraded DNA, modern plant samples will be artificially degraded using heat treatment. This approach will allow us to conduct a much-needed laboratory-controlled comprehensive comparison of methods without consuming precious archaeological samples. The outcomes of this research may result in identification of avenues for future improvements in DNA extraction methodology, more appropriate and careful selection of extraction methods for individual plant aDNA studies, and ultimately lead to increased success rates of DNA recovery in such studies.

Preservation of Plants in the Archaeological Record

There are many forms of plant preservation in the archaeological record including charring, desiccation, waterlogging, mineralization, and preservation in coprolites (Pearsall 2000; Zohary and Hopf 2000). All of these forms of preservation prevent bacterial and fungal growth and decomposition of the macro-botanical remains. This is due to the conversion of plant material into charcoal for charred plant remains, the extremely dry conditions in desiccated plant remains, and anaerobic conditions present in waterlogged plant samples (Zohary and Hopf 2000). While charred macro-botanical remains have a wide global distribution, not surprisingly, recovery of desiccated or waterlogged plant remains is geographically restricted to arid or temperate locations, respectively (Zohary and Hopf 2000). Charring of plant remains can occur under a variety of conditions and is affected by factors including the type of heat source, the degree of oxygen present, and the proximity of the plant remains to the heat source. Seeds[1] that are close to an open fire experience higher temperatures and aerobic conditions, resulting in faster charring, drastic morphological changes, and likely no DNA preservation (Guarino and Sciarrillo 2004; Threadgold and Brown 2003; Zohary and Hopf 2000). However, seeds that are located further away from an open fire or in a smouldering pit can be exposed to lower temperatures and limited oxygen conditions, and thus may char with minimal morphological changes and preserve limited amounts of DNA (Schlumbaum *et al.* 2008a; Zohary and Hopf 2000). Guarino and Sciarrillo (2004) investigated a number of variables that they hypothesized may affect seed charring including the type of seed and the distance from the heat source. They observed that

[1] The term "seed" is used throughout this manuscript to describe both seeds and seed-like fruits.

cereals were much more sensitive to heat than legumes, possibly due to the greater protection provided by thick seed coats; that the temperature to which a seed is exposed is related to the depth at which it is buried; and that the temperature at a particular location changes over the life of a fire (Guarino and Sciarrillo 2004). In addition, they further observed that seeds exposed to extreme heat were completely disintegrated, and therefore would not be expected to preserve archaeologically (Guarino and Sciarrillo 2004). Popper and Hastorf (1988) noted that size and density of macro-botanical remains can affect their preservation in the archaeological record. Additionally, specific variables related to site formation including the type of soil, amount of moisture in the soil, and depth at which the plant remains are deposited can all play an important role in whether macro-botanical remains will be preserved (Popper and Hastorf 1988).

DNA Degradation in Ancient Samples

DNA (deoxyribose nucleic acid) is the hereditary material or genetic code found in living organisms. While most DNA is contained within the nucleus of a cell (nDNA), DNA can also be found in the mitochondria (mtDNA) and chloroplasts (cpDNA) in plants. DNA is composed of two anti-parallel strands that form a double helix structure consisting of multiple nucleotides in succession, forming the DNA backbone. A nucleotide is made of a sugar (deoxyribose), a phosphate group, and a nitrogen base. Phosphodiester bonds between the sugar and phosphate molecules bind these nucleotides together. There are four nitrogen bases: adenine (A), guanine (G), cytosine (C), and thymine (T). DNA follows strict base pair matching where adenine and thymine, and cytosine and guanine each form complimentary base pairings.

DNA degradation in ancient samples can occur *via* multiple mechanisms. Following cell death, DNA repair mechanisms cease to occur, shifting the balance to favour DNA degradation by endogenous nucleases, enzymes that break down DNA (Alaeddini *et al.* 2010; Hofreiter *et al.* 2001; O'Rourke *et al.* 2000; Paabo *et al.* 2004). In addition to endogenous nucleases, DNA is also degraded by both bacterial and fungal nucleases (Paabo *et al.* 2004). As a result of this enzymatic degradation of DNA, which generally occurs directly after cell death, the DNA backbone is broken down into small fragments (Paabo *et al.* 2004). Because PCR amplification requires an intact DNA template, aDNA researchers target short DNA fragments when designing primers in an effort to increase their chances of a successful amplification. Under certain conditions including the presence of high salt, extreme dryness leading to rapid desiccation, or low temperatures, enzymatic DNA degradation is reduced increasing the chances of DNA preservation (Hofreiter *et al.* 2001).

Another form of DNA degradation in ancient samples occurs as a result of the interaction of DNA with water, a process referred to as hydrolysis. The hydrolysis reaction breaks down the bond between the deoxyribose and the nitrogen base, resulting in the creation of an abasic site (a location within the DNA strand missing a nitrogen base) (Alaeddini *et al.* 2010; Lindahl 1993). This can then trigger additional damaging reactions including: strand breakage due to further disruption of phosphodiester bonds; cross-linking of DNA strands (which will prevent PCR amplification of that DNA strand); and incorrect incorporation of a base during PCR amplification, a process known as miscoding lesions (Alaeddini *et al.* 2010). A third form of DNA damage called oxidation involves the interaction of oxygen radicals with DNA, and can result in abasic

sites, DNA cross-links, sugar modifications, and conversion of nitrogen bases to hydantoins, which inhibit PCR (Alaeddini *et al.* 2010; Lindahl 1993; Hofreiter *et al.* 2001; Paabo *et al.* 2004).

As discussed above, archaeological plant remains are often preserved charred from heating in a hearth or drying kiln, parched in dry, arid conditions, or waterlogged in lakes or bogs (Zohary and Hopf 2000). While all of these processes allow for morphological preservation of plant remains in the archaeological record, they can be detrimental to the preservation of plant aDNA. Heat significantly degrades DNA (Lindahl 1993) either *via* direct heating during the charring process (Threadgold and Brown 1993) or as a result of hot and arid conditions over extended time periods in warm climate environments such as Egypt (Marota *et al.* 2002). While an arid environment may reduce the level of hydrolytic and oxidative damage that normally degrades ancient plant DNA over time, this can be countered by accelerated DNA degradation caused by the hot temperatures often associated with such arid climates (MacHugh *et al.* 2000; Marota *et al.* 2002; Schlumbaum *et al.* 2008a). Some aDNA studies on charred plant remains have reported very low amplification success rates (Allaby *et al.* 1997; Blatter *et al.* 2002a; Brown *et al.* 1998; Schlumbaum *et al.* 1998), which may be explained by the rapid DNA degradation that results from the charring process.

Similarly, waterlogged ancient plant remains can present a challenge for aDNA researchers because hydrolytic damage to DNA can be extensive (Schlumbaum *et al.* 2008a). However, some ancient aDNA studies (Manen *et al.* 2003; Elbaum *et al.* 2006; Pollmann *et al.* 2005; Schlumbaum *et al.* 2008b) have successfully amplified DNA from pits and seeds of waterlogged remains, which contain hard outer shells that are

hypothesized to limit the amount of DNA degradation via hydrolysis (Pollmann *et al.* 2005; Schlumbaum *et al.* 2008a). While others have successfully analyzed plant aDNA from desiccated remains such as ground sloth coprolites, these remains were found in cool cave environments that would likely limit the extent of DNA degradation (Hofreiter *et al.* 2000; Poinar *et al.* 1998).

Extraction Techniques used to Recover DNA from Ancient Plant Remains

The three most commonly used DNA extraction methods for archaeobotanical remains include: 1) a surfactant-based method known as CTAB; 2) a silica-based method; and 3) a commercially available plant extraction kit, DNeasy Plant Mini Kit by Qiagen. Murray and Thompson (1980) originally developed the CTAB method, which is still widely used for DNA extraction from modern plants. This method is easily modified based on the plant tissue type and the plant species being tested, and a number of modified protocols have been published (Kasem *et al.* 2008; Xin and Chen 2006). The DTAB/CTAB method is one example of a modification from the original CTAB protocol in which an additional surfactant, DTAB is also used. Giles and Brown (2008) effectively describe the DTAB/CTAB extraction method:

> CTAB (cetyltrimethylammonium bromide) and DTAB (dodecyltri-methylammonium bromide) are detergents that form insoluble complexes with nucleic acids, leaving carbohydrate, protein and many other contaminants in solution. The insoluble precipitate is collected by centrifugation and resuspended in a salt solution, which causes the complex to break down, releasing the purified DNA. [Giles and Brown 2008:2586]

The disadvantage of the general CTAB method is that it can yield a lower quality of DNA compared to alternative methods, but is generally sufficient for most researchers' modern plant applications (Xin and Chen 2006).

The silica method, although originally designed for faunal remains (Boom *et al.* 1990; Hoss and Paabo 1993), has also been used successfully for both ancient plant remains and artificially degraded plant remains (Brown *et al.* 1998; Giles and Brown 2008; Poinar *et al.* 1998; Hofreiter *et al.* 2000; Schlumbaum *et al.* 1998). The silica binds DNA at high salt concentrations, inhibitors are washed away, and the purified DNA is eluted from the silica at low salt concentrations (Giles and Brown 2008; Yang *et al.* 1998). While DNA extraction using silica can occur either in a slurry form (Boom *et al.* 1990; Hoss and Paabo 1993) or as a spin column (Yang *et al.* 1998), the silica-based spin column method has been recommended over the silica slurry method by Bouwman and Brown (2002), MacHugh *et al.* (2000), and Yang *et al.* (1998). The second phase of the silica-spin column method involves the use of a commercially available kit from Qiagen, which allows for a portion of the method to be standardized. Furthermore, because of this standardization and use of a commercial kit, no additional reagent preparation is required, decreasing the variability and risk of contamination compared to the silica slurry method (Kemp *et al.* 2006; Yang *et al.* 1998).

The third extraction method commonly utilized for ancient plant materials is the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany), which effectively removes proteins and polysaccharides while the DNA remains bound to a silica-based spin column (DNeasy Plant Handbook 2006). Commercial extraction kits have been used successfully for both ancient plant remains and modern plant samples, producing consistent results

and high quality DNA for modern plant samples (Xin and Chen 2006). One of the limitations of this extraction method is the manufacturers' recommendation that only 20 mg of dried plant tissue or 100 mg of wet plant tissue be used for each extraction (DNeasy Plant Handbook 2006). An additional disadvantage of these commercial extraction kits (used for both the silica-spin column method and the DNeasy Plant Mini Kit) is their relatively high cost per sample compared to other methods including the DTAB/CTAB extraction method where numerous buffers and reagents are prepared by the researcher (Xin and Chen 2006).

Of these three techniques, both the DTAB/CTAB method and the DNeasy Plant Mini Kit were designed for use with plant samples while the silica-based spin column method was originally designed for animal remains. Thus, it is possible that the efficiency of the silica-based spin column method reported for animal remains will not translate into similar success with plant remains. Additionally, the three extraction methods may also differ in their ability to remove potential PCR inhibitors found in ancient and artificially degraded plant samples (*e.g.,* Maillard products, polysaccharides, polyphenols, and others), thereby affecting the extraction efficiency of each method. Furthermore, silica extraction methods (present as a component of both the silica-based spin column method and the DNeasy Plant Mini Kit) have been proven to be very efficient at extracting highly degraded DNA from animal remains (Rohland and Hofreiter 2007). The DTAB/CTAB method may not be as efficient at extracting highly degraded DNA because it was originally designed for use with modern plants in which the DNA is not significantly degraded.

Previous Studies involving Artificial DNA Degradation by Heat Treatment

Given the destructive nature of DNA testing, the small size of most ancient plant remains, and the quantity of sample required for methodology comparison studies, it would be irresponsible to conduct the type of comprehensive comparison of extraction methods proposed in the current work directly on ancient samples. In addition, the highly variable nature of ancient plant remains in terms of both sample preservation and PCR success rates does not lend itself well to a controlled comparison of methodology. For these reasons, we have used heat treatment to artificially degrade modern plant samples to simulate the limited amounts of degraded DNA expected to be found in ancient plant remains. This method has been used previously by a number of research groups examining a variety of sample types including bone, teeth, and plant samples (Dobberstein *et al.* 2008; McGrath 2010; Threadgold and Brown 2003). Results from these studies indicate that there is a general pattern to DNA degradation: an initial rapid degradation phase is followed by a more gradual degradation phase (Figure 1) (Dobberstein *et al.* 2008; McGrath 2010; Threadgold and Brown 2003). These degradation curves are used to identify the experimental conditions (*e.g.,* heating temperature and exposure time) under which modern samples, containing millions of copies of DNA, are degraded into samples that simulate, as closely as possible, ancient samples, which generally contain hundreds or thousands of DNA copies, if any. These degradation curves can also be used to identify heat treatment conditions beyond which point the modern DNA is likely completely degraded and is no longer useful for comparing the effectiveness of DNA extraction methods.

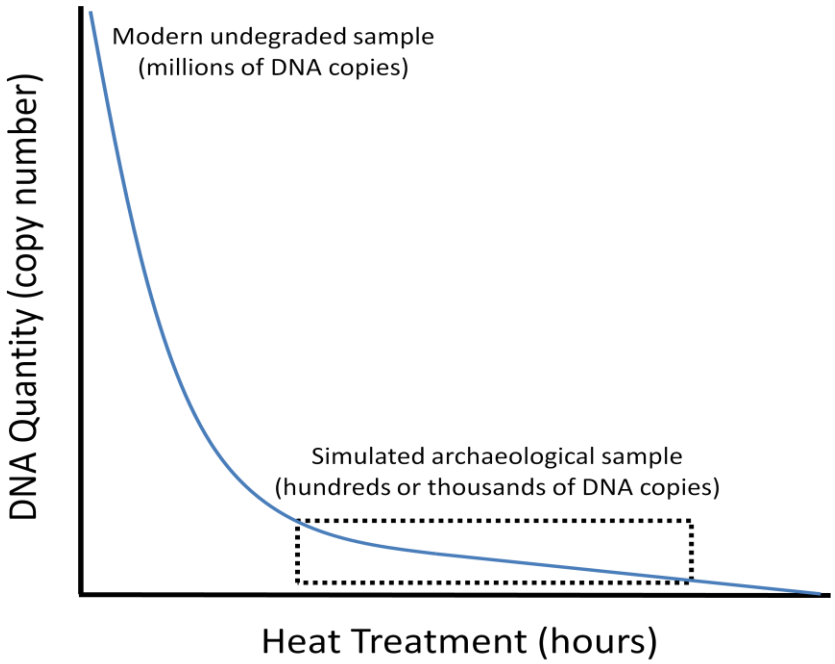

Figure 1: An example of the artificial DNA degradation process using heat treatment.
Modern plant samples, containing millions of DNA copies, are subjected to various heat treatment conditions (*e.g.,* temperature and exposure times) to simulate archaeological samples, which contain limited amounts of degraded DNA.

Numerous heat treatment experiments have been conducted in laboratory settings on various plant species. However, most of these studies have focused on the effect of heat treatment on plant morphology to determine which conditions are likely favourable for archaeological preservation of plant remains (Braadbaart 2008; Braadbaart and van Bergen 2005; Braadbaart and Wright 2007; Braadbaart *et al.* 2004a; Braadbaart *et al.* 2004b; Braadbaart *et al.* 2004c; Braadbaart *et al.* 2005; Braadbaart *et al.* 2007; Boardman and Jones 1990; D'Andrea 2008; Markle and Rosch 2008; Wright 2003). In addition, some experimental charring studies have been conducted in the field to more closely approximate archaeological conditions (Guarino and Sciarrillo 2004; Gustafsson 2000). Despite these studies investigating the effects of heat on plant morphology, relatively few

studies have tested DNA recovery from artificially degraded plant samples to determine how heating affects plant DNA during the charring process (Chalfoun and Touross 1999; Giles and Brown 2008; Threadgold and Brown 2003). The first study to address this issue was conducted by Chalfoun and Touross (1999) who heated barley seeds at various temperatures and time points, and investigated the extent of protein and DNA degradation. They extracted DNA using a CTAB method and determined that amplifiable DNA was no longer present after heating at 150 ˚C for one hour (Chalfoun and Touross 1999).

In another study, Threadgold and Brown (2003) heat treated wheat seeds to artificially degrade the DNA at temperatures ranging from 150 ˚C to 250 ˚C for up to five hours to simulate the cooking process. They then extracted and amplified DNA at various exposure times using the DTAB/CTAB method and observed an initial sharp decrease in DNA concentration followed by a plateau region at both 150 ˚C and 200 ˚C (Threadgold and Brown 2003). The authors noted, however, that at temperatures above 200 ˚C, this plateau effect was not observed and the DNA was fully degraded prior to five hours, suggesting that these higher temperatures may not be suitable for DNA preservation in ancient wheat grains (Threadgold and Brown 2003). In addition, Threadgold and Brown (2003) found that at 200 ˚C, approximately fifty percent of the wheat DNA was degraded within the first thirty minutes and eighty percent was degraded after two hours.

Only one study has used heat treatment in order to test the ability of two different DNA extraction methods to recover artificially degraded DNA (Giles and Brown 2008). The authors attempted to recover DNA from single wheat grains exposed to 200 ˚C for 1 hour and concluded that the silica-based spin column method yielded more consistent

results and stronger amplification than did the DTAB/CTAB method (Giles and Brown 2008). To date, this is the only study that has used laboratory-controlled experiments to advance the field of plant aDNA by providing much needed evidence about the effectiveness of the different extraction techniques.

Chapter Summary

The recovery and analysis of DNA from ancient macro-botanical remains has the potential to address many important archaeological questions. Despite this potential, there can be significant challenges associated with this type of research and the number of studies in the plant aDNA field has lagged behind the human and animal ancient DNA research. One of the main challenges is the lack of comprehensive studies comparing the effectiveness of the different extraction methods currently used in the field (*e.g.,* silica-based extraction, CTAB extraction with phenol-chloroform, and the DNeasy Plant Mini Kit). In addition, because macro-botanical remains have varying internal components the optimal method for individual plant species may be different due to the differential ability of each method to recover degraded DNA and remove PCR inhibitors. Conversely, it is possible that one single extraction method is optimal for a range of plant species and has the potential to be used as a universal extraction method in future ancient plant DNA studies.

Chapter 2: Background

Challenges in Plant Ancient DNA Studies

As discussed above, one potential explanation for the discrepancy in the proportion of ancient plant DNA studies compared to animal/human aDNA studies is that interests in aDNA reflect archaeological interests with the primary focus on humans followed by animals, microorganisms, and finally plants (Gugerli *et al.* 2005). Gugerli *et al.* (2005:412) also suggest other possible technical reasons for this discrepancy including "differences in (1) sample preservation, (2) DNA isolation, and (3) polymerase chain reaction (PCR) inhibition." While sample preservation has been discussed in detail above, the recovery of DNA from these preserved samples can present its own set of challenges. A number of plant components including polyphenols, polysaccharides, and Maillard products can interfere with PCR if they are not removed during the extraction process. In addition, the selection of DNA markers in plant aDNA studies can be more challenging than selection of DNA markers for animal aDNA studies. Finally, contamination control and authentication efforts can be difficult for ancient plant remains.

Challenges in DNA Extraction from Plants

Polyphenols and Polysaccharides

There are a number of different components of plant materials that may interfere with the successful recovery and amplification of DNA. Polyphenols and other secondary

metabolites including phenols, terpenes, alkaloids, and flavonoids are aromatic metabolites that are abundant in mature plant seeds and dried fruit, and cause brown colouration when oxidized (Black *et al.* 2006; Weising *et al.* 2005). Polyphenols bind to DNA after the physical disruption of plant cells, leading to PCR inhibition (Kasem *et al.* 2008). Polysaccharides including starch and cellulose are repeating carbohydrate structures that are abundant in plants and can also result in PCR inhibition if they are co-extracted with DNA (Kasem *et al.* 2008). In addition, many plant proteins, including storage proteins, must also be removed during DNA extraction to permit successful isolation and amplification of DNA. Generally, cereal grains have a higher proportion of polysaccharides; legumes have a high proportion of storage proteins; and cucurbit seeds have relatively equal proportions of storage proteins and polysaccharides (Black *et al.* 2006). Because the concentrations of polysaccharides and polyphenols also depend on the plant tissue type, many modern plant DNA researchers prefer to extract DNA from the plant leaves because they contain less of these PCR inhibitors than do seeds or dried fruit (Kasem *et al.* 2008). However, compared to seeds and dried fruits, leaves are preserved in the archaeological record only in exceptional circumstances, such as in arid environments. Thus, the efficient removal of polysaccharides, polyphenols, and secondary metabolites during plant aDNA extraction is critical to ensuring the success of PCR amplification from archaeological plant macro-remains. Plant seeds and dried fruits included in the current study are expected to have variable levels of polysaccharides and polyphenols, and the ability of each DNA extraction method to remove these inhibitory components from the plant samples is also likely to vary.

Maillard Products

"Maillard products are formed by condensation reactions between sugars and primary amino-groups in proteins and nucleic acids" (Paabo et al 2004:649). Work by Poinar *et al.* (1998) was the first study to report the removal of these inhibitory DNA-protein-sugar cross-links, allowing for successful silica extraction and amplification of ancient plant DNA from ground sloth coprolites. It should be noted that Poinar and colleagues (1998) were unable to successfully amplify ancient DNA from the ground sloth coprolites without the addition of N-phenalcylthiazolium bromide (PTB), a chemical that cleaves these DNA-protein-sugar cross-links (Vasen *et al.* 1996) to effectively free ancient DNA from interactions with Maillard products. Hofreiter *et al.* (2000) also used PTB to effectively extract and amplify ancient plant sequences from five desiccated coprolites. Conversely, while the use of PTB was systematically tested on DNA extraction from cave bear bones to determine if it had any effect on removing PCR inhibitors from animal samples, no advantage or disadvantage was detected in this study (Rohland and Hofreiter 2007). In addition, Kemp *et al.* (2006) directly compared the effectiveness of PTB by conducting a controlled experiment using ancient bone and coprolite samples and concluded that PTB did not help decrease PCR inhibition in their samples. Finally, Maillard products were also reported to negatively affect the amplification of artificially charred wheat samples (Threadgold and Brown 2003). Threadgold and Brown (2003) determined that, while the onset of Maillard products was dependent on the temperature as well as aerobic or anaerobic conditions, these inhibitory products were generally observed to appear shortly after the start of laboratory controlled heat treatment and were degraded within three hours.

Methods used to overcome PCR inhibition

In addition to polysaccharides, polyphenols, Maillard products, storage proteins and numerous other compounds are known to inhibit PCR including humic acids, fulvic acids, tannins, and hematin (Kemp *et al.* 2006). There have been many varied attempts to overcome PCR inhibition. The most frequently used approach involves the dilution of the DNA extraction sample in water, diluting both the amount of DNA and the PCR inhibitors, ideally to a point where the inhibitors no longer negatively affect PCR amplification but there is still enough DNA to yield a successful amplification (Kemp *et al.* 2006; King *et al.* 2009). Reports published by Pollman *et al.* (2005) and Schlumbaum *et al.* (2008b) provide two examples of ancient plant DNA studies in which sample dilutions were required for successful PCR amplification. More specifically, dilutions of either 1:50 or 1:100 were required in order to overcome PCR inhibition from waterlogged plum stones and apple seed extracts using CTAB, silica slurry and DNeasy Plant Mini Kit extraction methods. The disadvantage of the dilution approach for aDNA studies is that, due to the limited amount of DNA present in ancient plant samples, there is a significant risk of diluting out the DNA so that there is no longer enough DNA left for successful PCR amplification (Kemp *et al.* 2006; King *et al.* 2009).

Because some PCR inhibitors deactivate Taq polymerase, the enzyme responsible for amplifying DNA during PCR, one approach to improving PCR success is to add more Taq polymerase to overcome inhibition (Kemp *et al.* 2006). In addition, supplementing the reaction with bovine serum albumin (BSA), which is thought to either bind inhibitors or stimulate Taq polymerase activity, can also improve amplification success rates (Copper 1994; Kemp *et al.* 2006; King *et al.* 2009). King *et al.* (2009) used quantitative

PCR (qPCR) to assess the effectiveness of dilution, BSA, and increased Taq at overcoming PCR inhibition, and found that the effectiveness of each modification depended on the type of sample being tested. Kemp *et al.* (2006) observed that there was likely an association between the dark colouration of DNA extracts and the extent of PCR inhibition. They found that repeating the last step of the silica extraction (potentially multiple times) resulted in successful PCR amplification of DNA from samples that were otherwise not amplifiable even when dilution, BSA, and PTB modifications were used (Kemp *et al.* 2006).

Challenges in DNA Marker Selection

Determining which DNA marker to choose in plant aDNA studies depends on many factors including the likelihood of DNA preservation and the objective of the study. In addition to having nuclear and mitochondrial DNA like humans and animals, plants also have chloroplast DNA (cpDNA). Mitochondrial DNA (mtDNA) is not frequently used in plant aDNA studies because plants have significantly lower mutation rates (1/100) and a lower number of mitochondria (copy number) per cell when compared to human and animal mitochondria (Schlumbaum *et al.* 2008a).

Nuclear DNA (nDNA), which is diploid (*i.e.,* contains two copies of each chromosome) unless the plant is a polyploid, is frequently used in plant aDNA studies, especially those focused on domestication, human selection of traits, or identification of the ploidy level (Allaby *et al.* 1997; Jaenicke-Despres *et al.* 2003; Palmer *et al.* 2009; Schlumbaum *et al.* 1998). The advantage of plant nDNA is that it has a mutation rate approximately four times higher than cpDNA and twelve times higher than plant mtDNA (Zeder *et al.* 2006). This relatively high mutation rate of plant nDNA allows ancient

DNA researchers to investigate domestication events in plants during the last 10,000 years using nDNA markers (Zeder *et al.* 2006). While nDNA is the most variable (polymorphic) DNA marker in plants, the drawback of nDNA is the extremely low copy number, which reduces the likelihood of DNA recovery from highly degraded ancient plant remains (Schlumbaum *et al.* 2008a). In addition, the evolution of plant nDNA can involve crop-weed species hybridization, gene duplications, polyploidy from abnormal cellular division, and gene flow between domesticates and wild progenitors, which can complicate the analysis of nDNA for ancient plant remains (Hancock 2004; Zeder *et al.* 2006). High molecular weight (HMW) glutenin is the nuclear marker commonly selected for PCR amplification in ancient DNA studies of charred wheat samples (Allaby *et al.* 1994; Allaby *et al.* 1999; Brown *et al.* 1998; Schlumbaum *et al.* 1998). In addition, Schlumbaum *et al.* (2008b:8) successfully amplified nDNA from fragments of waterlogged ancient wild apple seeds, but were unable to successfully amplify cpDNA possibly due to "the absence of chloroplast DNA in embryo-free testa fragments".

Despite its lower mutation rate compared to nuclear DNA, chloroplast DNA is also commonly used in ancient plant DNA studies because of its higher copy number per cell (Schlumbaum *et al.* 2008a). One of the most frequently amplified cpDNA markers in plant DNA studies, the *rbc*L marker codes for a subunit of an enzyme, making it a conservative marker that can identify plants to the family or genus level, but not to the species level (Schlumbaum *et al.* 2008a; Taberlet *et al.* 2007). The *trn*L-*trn*F marker is located in a non-coding spacer region between two transfer RNA coding regions, and is more variable than the *rbc*L marker, allowing for identification to the genus or even species level in a limited number of cases (Pollmann *et al.* 2005). More commonly,

species identification with chloroplast markers is difficult because long DNA fragments are generally required due to the low mutation rate. In ancient plant samples, extensive DNA degradation usually prevents amplification of sufficiently long fragments to identify to the species level unless multiple overlapping fragments are amplified by PCR.

To facilitate identification, plant aDNA studies usually involve a comparison of recovered ancient DNA sequences to modern DNA sequences that are either amplified by the researchers themselves or downloaded from an online database called Genbank (http://www.ncbi.nlm.nih.gov/genbank/). In addition to sequence comparisons, reference DNA sequences from Genbank are also required for primer design. A lack of plant DNA reference sequences available in the Genbank database for some species can limit the ability to identify rare ancient plant species and deter researchers from undertaking such projects. Furthermore, plant aDNA studies focusing on domestication require that nuclear DNA markers be reported by modern plant studies before these domestication markers can be applied to ancient plant remains.

Authentication of Results and Contamination Challenges

In response to criticisms related to questionable studies claiming successful recovery and amplification of extremely old DNA (Austin *et al.* 1997; Austin *et al.* 1998), ancient DNA researchers developed a list of ten criteria that they recommended should be met in order to ensure authenticity of ancient DNA results (Cooper and Poinar 2000; Poinar 2003). These criteria included physically isolated work areas, control amplifications, appropriate molecular behaviour, reproducibility, cloning, independent replication by another laboratory, assessment of biochemical preservation, DNA quantification, testing of associated remains, and phylogenetic sense (Cooper and Poinar

2000; Poinar 2003). While initially useful to facilitate a more rigorous approach to data interpretation within the field, the strict adherence to these authentication criteria are no longer considered to be an absolute requirement for all studies that focus on ancient DNA (Gilbert *et al.* 2005; Kemp and Smith 2010). In some research cases, it is not possible to conduct all of the criteria while in other cases some of the authentication criteria may prove prohibitively costly and not provide any additional evidence of authentication (Gilbert *et al.* 2005; Kemp and Smith 2010; Winters *et al.* 2011). Instead, the field of ancient DNA is moving towards a critical evaluation approach to authentication in which researchers provide as much evidence as possible about the authenticity of results, and the scientific community as a whole determines on a case-by-case basis whether the evidence is sufficient (Gilbert *et al.* 2005; Kemp and Smith 2010; Winters *et al.* 2011). In regard to plant aDNA studies, this shift is important because many of the original authenticity criteria, which were designed for ancient human and faunal DNA studies, are often impossible to complete due to the small size of recovered archaeobotanical remains (Schlumbaum *et al.* 2008a). Firstly, plant remains are often physically small and are usually consumed in a single extraction. This prevents re-extraction from the same sample using either the same or a different extraction method, or having the work replicated by and independent laboratory (Schlumbaum *et al.* 2008a). However, some ancient plant DNA studies have tested relatively large plant remains, and have been able to repeat extractions using two different methods in an effort to support the authentication of results (Blatter *et al.* 2002a; Elbaum *et al.* 2006; Pollmann *et al.* 2005; Schlumbaum *et al.* 2008b). Alternatively, several studies (Allaby *et al.* 1997; Blatter *et al.* 2002b; Manen *et al.* 2003) have successfully amplified DNA from different specimens found in close

proximity at the archaeological site, or amplified multiple DNA target sequences or overlapping fragments from the same extract in an effort to address this reproducibility issue. While having an independent laboratory replicate the recovery of DNA from different remains found in close proximity can strengthen the authentication of the results, if the samples were contaminated at any point prior to DNA testing, either by handling or environmental contamination, positive results from the two different laboratories would be expected (Yang and Watt 2005). In addition, due to the low success rate of DNA recovery from ancient plant remains, requiring the results to be independently replicated could result in the dismissal of authentic results if the second laboratory cannot recover DNA from the associated remains being tested.

While biochemical preservation of amino acids in the ancient material was originally argued to provide scientific support for the co-survival of authentic DNA, this has recently been determined to be an unreliable method for the prediction of preserved authentic ancient DNA in bones (Collins *et al.* 2009; Fernandez *et al.* 2009). In addition, while cloning of amplified PCR products was also recommended to help authenticate the results, Winters *et al.* (2011) conducted an experimental study of cloning and found that the consensus DNA sequences generated from the cloning did not differ from the sequence generated from direct sequencing. These results indicated that cloning should not be classified as a criterion for an authentic ancient DNA studies, but rather should be used in situations where mixed DNA samples are expected (Winters *et al.* 2011).

In order to increase the chances of having positive PCR results, many researchers have grouped together identical plant remains into combined bulked samples (Allaby *et al.* 1994, Allaby *et al.* 1997; Banerjee and Brown 2002; Blatter *et al.* 2002b; Brown *et al.*

1998; Manen *et al.* 2003; Schlumbaum *et al.* 1998). However, this approach can be extremely problematic if morphological identification of the species has not been completed or is incorrect (Brown *et al.* 1998). Brown *et al.* (1998) recognized that in order to have accurate species identification and assessment of the genetic diversity of the assemblage, wheat grains should be extracted individually instead of compiled as bulked samples which can result in mixed signals that can complicate DNA sequence analysis. However, while successful extraction and amplification from single grains would yield more reliable information than bulked samples, the individual grain approach is less likely to result in successful recovery of DNA. The single grain extractions conducted by Brown *et al.* (1998) yielded only a 5 % success rate for PCR amplifications and three out of four of these sequences did not correspond to the morphological identification for the seeds, suggesting either that the morphological identification was incorrect or that contamination may have been present. This study highlights the importance of improving the efficiency of the DNA extraction methods to increase the chances of successful extraction of DNA from single archaeological seeds, which would also prevent mixed DNA sequences and add further evidence that the results are indeed authentic.

Even if all the suggested authenticity criteria are followed, the results should not necessarily be considered automatically valid, and researchers should strive to be self-critical (Gilbert *et al.* 2005). When assessing the credibility of ancient DNA studies, one should determine if the data interpretation is logical and if the authors provide enough information to evaluate the authenticity (Gilbert *et al.* 2005). All ancient plant DNA samples should be processed in laboratories that are dedicated solely to ancient DNA research to avoid contamination from modern sources. Fortunately, because many ancient

plant DNA studies specifically target chloroplast DNA, the risk of contamination from human handling is expected to be reduced compared to studies focused on human remains. However, ancient plant DNA studies still carry a significant contamination risk from multiple sources including: modern plant pollen; commercial products (*e.g.,* paper towel, tissue paper); contamination during excavation, sorting or handling of plant remains; and contamination from other ancient plants samples tested in the same laboratory (Gugerli *et al.* 2005; Schlumbaum *et al.* 2008a; Yang and Watt 2005).

DNA Quantification

Polymerase chain reaction (PCR) has revolutionized countless fields of scientific research including ancient DNA since its development in 1985 (Saiki *et al.*). PCR is able to take a limited amount of DNA and generate billions of copies of a specific region within a couple of hours, yielding enough material to be studied in detail. PCR is especially useful for studying limited amounts of degraded DNA in archaeological samples. The specific region of DNA that is amplified depends on the design of a forward and reverse primer, which are short fragments of synthesized DNA that bind to complementary regions of DNA during PCR and direct template amplification. PCR consists of multiple steps called cycles, where one cycle involves a series of temperature changes that promote DNA replication: first the double-stranded DNA separates into single-stranded templates in the denaturation phase; then primers bind to these DNA templates in the annealing phase; and finally, in the extension phase, nucleotides are added to the primers by a polymerase enzyme (Taq) to generate a complementary strand of DNA. When the PCR is efficient, the completion of each PCR cycle results in a doubling in the amount of DNA templates and the same process starts again with the next

cycle. However, a number of variables including primer efficiency, PCR inhibition, and reagent depletion at higher cycle numbers can reduce the overall efficiency of the reaction. It should be noted that it is possible that one single starting DNA template can be amplified enough to be analyzed (*i.e.,* detected and sequenced) if enough PCR cycles are carried out. Due to this sensitivity, it is important to follow strict contamination control procedures to prevent or at least minimize DNA contamination (Cooper and Poinar 2000; Gilbert *et al.* 2005; Knapp *et al.* 2010; Yang and Watt 2005).

Following standard PCR amplification, the presence or absence of amplified bands is visualized by gel electrophoresis and staining. Amplified products are stained with a dye and subjected to gel electrophoresis on an agarose gel, which separates the products into bands based on size (*i.e.,* fragment length). In addition, the strength of the amplified product bands can be compared across samples within a single PCR experiment and to a known DNA ladder as band thickness is proportional to the amount of starting DNA template. While this process is only semi-quantitative, differences in amplification band strength can still be observed and bands can be classified as either strong, medium, weak, or absent. For these reasons, standard PCR was used in the current study as an initial assessment tool rather than as a quantitative tool, and samples were then analysed using quantitative PCR, which is a much more sensitive method for quantifying DNA amounts.

Quantitative PCR (qPCR) uses the exponential phase of PCR, where each cycle theoretically results in a doubling of PCR products, to determine the amount of starting DNA in the sample. Because standard PCR compares samples only at the end of a PCR run and thus includes both the efficient exponential phase and the inefficient plateau

phase wherein reagents are depleted and differences in amounts of starting DNA is at least partially obscured, qPCR is a more sensitive method for quantifying these starting levels of DNA. Quantitative PCR (previously referred to as real-time PCR) consists of the same cycling parameters used in standard PCR but also incorporates a fluorescent dye either in the form of 5'-nuclease hydrolysis probes, molecular beacons, hybridization probes, or intercalating dyes to facilitate template quantification. The current study uses a 5' nuclease hydrolysis probe (also referred to as a Taqman probe) that contains a fluorescent dye (5' 6-FAM) and a quencher (ZEN) attached to the probe. When the probe is intact (either unbound in solution or bound to single-stranded DNA), the quencher prevents the release of the fluorescent signal from the dye. However, during the extension phase of PCR, with the probe bound to the DNA, the 5'-nuclease activity of Taq polymerase separates the dye and quencher both from each other and from the DNA strand, causing them to float apart and resulting in a fluorescent signal that is measured by the qPCR instrument. Thus, the amount of PCR products that is amplified is proportional to the fluorescence that is generated and detected by the qPCR instrument. The incorporation of a second quencher molecule nine basepairs from the fluorescent dye increases the sensitivity of this particular probe and decreases the background fluorescence (http://www.idtdna.com/pages/home/news-events/news/2011/02/17/idt-launches-double-quenched-probes-to-increase-qpcr-sensitivity-and-precision).

During qPCR, the instrument determines the cycle number at which the unknown sample has a fluorescence level that reaches an arbitrary threshold referred to as the cycle threshold (C_t), or more recently called the quantification cycle (C_q) (Bustin *et al.* 2009) (Figure 2). The instrument uses an algorithm to set this threshold value in the exponential

phase of PCR such that it is higher than the amount of background fluorescence in the baseline phase of PCR (Adams 2006). A dilution series of a DNA standard, each with a known DNA quantity, is also run for each qPCR batch. The C_q values of the DNA standards are plotted against DNA concentration to generate a standard curve. The Cq values from the unknown samples are then compared to the standard curve and DNA quantities can be calculated using the qPCR software.

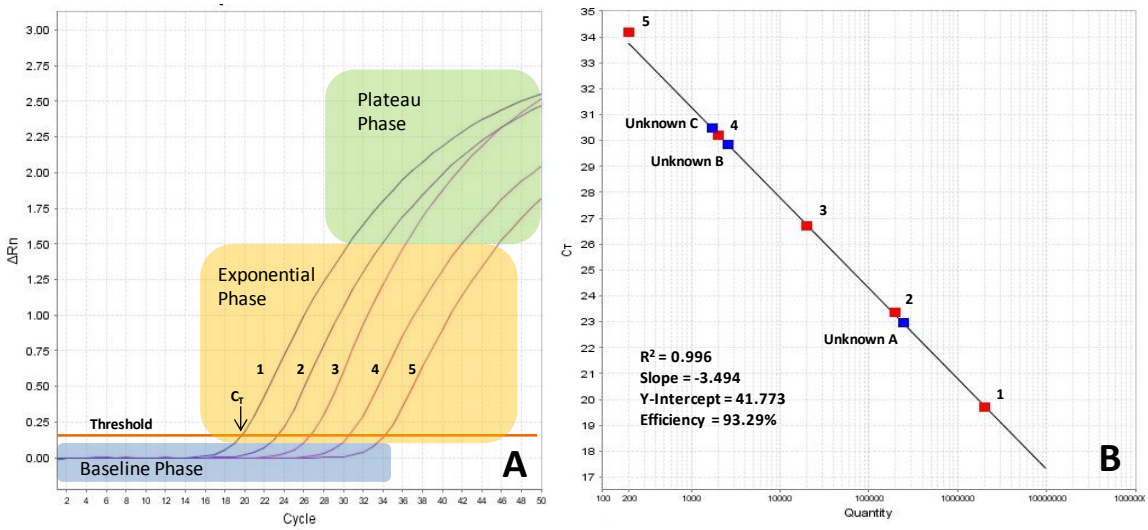

Figure 2: Example of DNA standards used to quantify unknown samples using qPCR. A) amplification curves and B) standard curve.
A) DNA amplification curves using a number of DNA standards (labelled 1-5). The y-axis (ΔRn) is a measure of the amount of fluorescence resulting from PCR amplification. The x-axis indicates the PCR cycle number. Three phases are observed during qPCR. The initial baseline phase is followed by an exponential phase wherein the PCR is most efficient. As the number of PCR cycles increases, the efficiency begins to fall, resulting in the plateau phase of DNA amplification. The qPCR software sets a threshold fluorescence value that falls within the exponential phase of all samples. The program then records the cycle number at which each sample or standard crosses this threshold and assigns each sample/standard with a quantification cycle (C_q). B) The quantification cycle (C_q) data from the DNA standards are then plotted against their known DNA concentrations (labelled 1-5). The amount of DNA in unknown samples can then be quantified by plotting their C_q values on the standard curve. Both figures were generated using Applied Biosystems StepOne™ instrument and software.

Chapter Summary

There are numerous challenges associated with ancient plant DNA studies including: preservation of DNA in macro-botanical remains; difficulty in removing PCR inhibitors (*e.g.,* polyphenols, proteins, and polysaccharides) from samples; presence of inhibitory Maillard products; selection of appropriate DNA markers for each study; appropriate contamination controls and the ability to authenticate results. Any successful DNA extraction method for ancient macro-botanical remains will require the efficient recovery of limited and degraded DNA and the effective removal of PCR inhibitors. In addition, the low success rate of plant aDNA studies and the small size of many preserved macro-botanical remains means that repeating results, a commonly used authentication criteria for ancient DNA studies are difficult or impossible.

Chapter 3: Materials and Methods

Seed Selection and Sample Preparation

In an attempt to account for the diversity of archaeobotantical remains, one species from each of the plant families Cucurbitaceae (cucurbits), Fabaceae (legumes), and Poaceae (grasses/cereals) were selected for analysis in this study: squash (*Cucurbita pepo*); pea (*Pisum sativum*); and corn (*Zea mays*) (Table 1). These plant families were selected in part to reflect global distribution patterns and because of their central roles as both modern and archaeological food sources; in particular, these species represent important domesticates in the New World (squash and corn) and Near East (pea) (Harlan 1971; Zohary and Hopf 2000).

Inclusion of a wide variety of plant specimens is also critical because the amount of recoverable DNA is known to vary depending on both the plant species and tissue type selected for analysis (Rogers and Bendich 1985). Thus, the plant species included in this study encompass a variety of plant tissue types. The cucurbits are characterized as fleshy fruits, which are preserved in the archaeological record as seeds or rinds. Cereals and legumes are both dry, indehiscent fruits with the cereals being monocots (*i.e.,* one cotyledon) and the legumes being dicots (*i.e.,* two cotyledons). In addition, unlike corn, pea has an outer seed coat called a testa, which may provide added protection against DNA degradation. Furthermore, plant seeds and dried fruits included in this study were expected to have variable levels of PCR inhibitors (polysaccharides, polyphenols, and proteins), and the ability of each DNA extraction method to remove these inhibitory

components from the plant samples may also vary. For this study, dried corn and pea fruits and fresh squash were purchased from local food stores. Seeds were harvested from squash and dried prior to testing.

Table 1: Characterization of different plant samples tested.

Common Name	Plant Category	Species	Family	Tissue Type
Corn	Grain	Zea mays	Poaceae	Dried fruit (monocot)
Pea	Legume	Pisum sativum	Fabaceae	Dried fruit (dicot)
Squash	Cucurbit	Cucurbita pepo	Cucurbitaceae	Freshly harvested seed

Note: All plant samples were purchased from local food stores. Corn and pea samples were purchased as pre-dried fruits for planting purposes. Fresh squash was purchased and seeds were harvested and dried over night.

Heat Treatment

Generation of DNA Degradation Patterns

The first phase of this study involved the generation of DNA degradation patterns under varying conditions to identify appropriate experimental conditions for the second phase of this research: a comparison of three extraction methods. Plant samples were subjected to heat treatment in a reduced oxygen environment using either a laboratory oven (Barnstead Thermolyne Type 19200) or a muffle furnace (Thermo Scientific F47925) at 85 ˚C, 140 ˚C, or 200 ˚C for various incubation times (0 - 15 hours). Photographs were taken both before and after heat treatment to document any changes in sample colour or morphology. Seeds and dried fruits were also weighed both before and after heat treatment to determine percentage weight loss. Following heat treatment, plant samples were ground into a fine powder using a cryogenic grinding mill (SPEX Sample Prep, Metuchen, NJ, USA) under the same conditions for all samples. Corn, pea, and squash powders from various heat exposure time points were then weighed using a top loading balance (either the DHaus Scout Pro or the Denver Instruments mxx-123).

For the 85˚ C heat treated plant samples, equal amounts of powder from corn, pea, and squash were combined to yield a mixed plant powder at each of the 0, 5, 10, and 15 hour samples. Approximately 100 mg of the mixed plant powder from each time point was then extracted using a modified silica-spin column method described below (Yang *et al.* 1998, Yang *et al.* 2004). For the 200˚ C heat treated samples, the individual plant powders were not combined, and 25 mg of corn, pea, and squash powder were extracted individually for time points 0, 2.5, 5, 7.5, and 10 hours using the modified silica-spin column method (Yang *et al.* 1998, Yang *et al.* 2004). Next, samples were heat- treated at 140 ˚C, the individual plant powders were again not combined, and 50-60 mg of corn, pea, and squash powder were extracted individually for time points 0, 5, and 10 hours using the modified silica-spin column method (Yang *et al.* 1998, Yang *et al.* 2004). Finally, because the comparison of extraction methods phase of this study was ultimately conducted using 25 mg of plant powder, 140 ˚C heat treated samples (0, 2.5, 5, 7.5, and 10) were also extracted using the silica-spin column with 25 mg of plant powder (Yang *et al.* 1998, Yang *et al.* 2004).

Comparison of Extraction Methods

For the second phase of this study, the three most commonly used DNA extraction methods for archaeobotanical remains were compared to determine whether there were differences in efficiency rates of DNA recovery between the three methods. Corn, pea, and squash samples were prepared and treated at 140 ˚C as described above (0, 2.5, 5, 7.5, and 10 hours), and subjected to DNA extraction using the DTAB/CTAB extraction method (Manen *et al.* 2003) and the DNeasy Plant Mini Kit (Qiagen, Hilden,

Germany). These samples were then compared to those previously extracted under the same conditions with the silica-spin column method.

DNA Extraction Methods

Silica-Spin Column Extraction Method

Plant powder was incubated with 2-3 mL of lysis buffer (0.5 M EDTA pH 8.0; 0.25 % SDS; 0.5 mg/mL proteinase K) at 50 °C in a rotating hybridization oven. After overnight incubation, an additional 50 µL of proteinase K (20 mg/mL) was added to each sample, and samples were then allowed to rotate for another 3 hours at 50 ˚C prior to centrifugation at 4400 *rpm* for 20 minutes. After centrifugation, the supernatant was transferred to an Amicon Ultra-4 centrifugal filter device (30,000 NMWL, Millipore, Billerica, MA) for concentration down to less than 100 µL. The concentrated supernatants were then transferred to QIAquick spin columns - part of the QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) – and the samples were washed according to the manufacturer's protocol (Qiagen QIAquick Spin Handbook 2008). After washing, 100 µL of elution buffer (10 mM Tris-Cl pH 8.5) was added to the QIAquick spin columns, the columns were heated (60-70 ˚C) for 5-10 minutes, and the first elution was centrifuged into a new tube and then transferred into a storage tube. This process was repeated for a second elution, into a separate storage tube. Both the first and second elutions were then stored at -20 °C for further use including PCR amplification. Two extraction blanks were also included for each silica-spin column extraction to test for the presence of systematic contamination from the extraction buffers or the extraction process.

DNeasy Plant Mini Kit Extraction Method

The DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) extraction method was performed as per the manufacturer's protocol with the following modifications (Qiagen DNeasy Plant Handbook 2006): the volume of AP1 buffer was increased to 600 µL (up from 400 µL), and QIAquick spin columns were heated at 60-70 ˚C for 5-10 minutes immediately prior to the DNA elution step to improve DNA recovery from the column.

Prepared plant powder was transferred to either a 2 mL or 15 mL tube with 600 µL of AP1 buffer and 4 µL of RNase A. The powder and buffer were vortexed and then incubated at 65 °C for 10 minutes while inverting the sample tubes two or three times. Next, 130 µL of AP2 buffer was added to the samples, mixed, and incubated on ice for 5 minutes. The AP2 buffer caused the proteins and polysaccharides to form a precipitate. The samples were then centrifuged for 5 minutes at high speed (14,000 *rpm*) to pellet the precipitate. The supernatant was then transferred to a QIAshredder mini spin column (part of the DNeasy Plant Mini Kit) and centrifuged for 2 minutes (14,000 *rpm*) to further remove the precipitate and any remaining cellular components. The flow-through solution was then transferred to a new tube, and approximately 800 µL of AP3/E buffer was added. The resulting mixed solution containing DNA was then added to the kit's spin column approximately 500 µL at a time to bind the DNA to the column. The column was then centrifuged at high speed and the flow-through was discarded. Similar to the silica-spin column extraction method, the DNA binds to the silica membrane of the kits spin column in the presence of high salt concentration. The samples were washed twice with 400-500 µL of AW buffer to remove impurities from the DNA. Finally, 100µL of AE buffer (10mM Tris-Cl, 0.5 mM EDTA, pH 9.0) was added to the DNeasy Plant Mini Kit

spin column, the columns was heated (60-70 ˚C) for 5-10 minutes, and the first elution was centrifuged into a fresh tube and transferred to a storage tube. This process was repeated for a second elution. Both DNA elations were then stored at -20 °C for further use including PCR amplification. As before, two extraction blanks were included for each DNeasy Plant Mini Kit extraction to test for the presence of systematic contamination from the extraction buffers or the extraction process.

DTAB/CTAB Extraction Method

The DTAB/CTAB extraction method was performed as described in the study by Manen *et al.* (2003), which was adopted from a method described by Gustincich *et al.* (1991). Corn, pea, and squash powders from the various time points were transferred to individual 15 mL tubes. The plant powder was then mixed with 700 µL of DTAB buffer (5.5 % DTAB, 1 M NaCl, 70 mM Tris-HCl, 30 mM EDTA), incubated at 65 °C for 30 minutes, and inverted 2-3 times during incubation. Following incubation, the samples were centrifuged for 10 minutes (4400 *rpm*) to pellet the plant powder. The supernatant was transferred to a new 15 mL tube and 700 µL of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (Invitrogen, Carlsbad, CA, USA) was added to each tube. The aqueous and phenol chloroform phases were mixed and then separated by centrifugation for 10 minutes. The upper aqueous phase containing the DNA was transferred to a new 15 mL tube and 1.7 volumes (approximately 1200 µL) of CTAB buffer (0.5 % CTAB, 40 mM NaCl) was added. Samples were then precipitated for 30 minutes at room temperature, prior to centrifugation for 5 minutes (4400 *rpm*) to pellet the precipitated CTAB/DNA complex. A pipette was used to carefully remove and discard the supernatant without disturbing the CTAB/DNA pellet. This pellet was then dissolved in 100 µL of NaCl

solution (1.2 M). Next, 250 µL of 95 % ethanol was added and the samples were vortexed. After incubating the samples at -20 °C for 30 minutes, samples were centrifuged for 10 minutes (4400 *rpm*) to pellet the DNA. The supernatant was then removed by pipette, and the remaining DNA pellet was washed by adding 250 µL of 70 % ethanol (30 % H_2O). The samples were then vortexed again and centrifuged for 10 minutes (4400 *rpm*) to pellet the DNA again. Supernatants were carefully removed as before and the final DNA pellet was allowed to air-dry for at least 1 hour to permit the removal of residual ethanol by evaporation. Finally, 100 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA) was added to the samples to dissolve the DNA pellet. The samples were vortexed to ensure the entire DNA pellet was dissolved, and the resulting DNA in TE buffer was transferred to new tubes for storage at -20 °C. Two extraction blanks were included for each DTAB/CTAB extraction to test for the presence of systematic contamination from the extraction buffers or the extraction process.

Assessment of DNA Recovery

Standard PCR

All primers were designed to bind to chloroplast DNA, more specifically within the ribulose-1,5 bisphosphate carboxylase (*rbc*L) gene. Reference rbcL sequences for *Zea mays* (NC001666.2, Z11973.1), *Pisum sativum* (NC014057.1, X03853.1), and *Cucurbita pepo* (AF206756.1, L219358.1) species were downloaded from Genbank. In addition, *rbc*L sequences from other species of the same plant families - *Poaceae* (common millet and barley), *Fabaceae* (soybean, and kidney bean), and *Cucurbitaceae* (bottle gourd and cucumber) - were also downloaded from Genbank. Primers were designed manually using the downloaded reference sequences, which were aligned using BioEdit software

(http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The quality of primers (*e.g.*, melting temperatures, primer dimerization, and hairpin structures) was determined using the online software Netprimer (PREMIER Biosoft International, Palo Alto, CA, USA). Primers used for standard PCR in this study were manufactured by Integrated DNA Technologies (Coralville, Iowa, USA).

The majority of primers used in this study were designed such that they would amplify DNA from all three species tested in this study (corn, pea, and squash). The benefit of using "universal" primers is that multiple plant species (*e.g.*, corn, pea, and squash) can be amplified using the same primer set. In addition to these universal primers, three reverse primers were also designed to bind specifically to corn, pea, or squash to the exclusion of the other two plant species, but not necessarily other plant species within the particular plant family. To determine the quality of DNA present following heat treatment, primers were designed to amplify DNA fragments of varying lengths. This is an important qualitative assessment tool as the presence of longer fragments can indicate better overall DNA preservation while short fragments are more likely to remain intact following heat treatment and can confirm that DNA is present and amplifiable. In addition, the degradation of larger fragments over time is useful for generating DNA degradation patterns. Following the generation of DNA degradation patterns, some of the primers used for this phase of the study (Table 2) were redesigned for use in the comparison of methods phase of the study (Table 3). Additionally, the F17 (universal) primer was newly designed for this second phase to allow for an amplicon length of 166 bp when used with primer R183 (universal) (Table 3).

Standard PCR amplifications were conducted using either the 25-well or the 96-well Eppendorf Mastercycler Thermocycler (Hamburg, Germany) in a 30 µL reaction volume containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 1.0 mg/mL BSA, 0.3 µM of each primer, 3.0 µL DNA sample (either diluted or undiluted) and 0.75 U of AmpliTaq Gold™ (Applied Biosystems, Carlsbad, CA, USA). PCR amplifications were run for 30-50 cycles at 94 °C for 30 seconds (denaturing step), 55 °C or 58 °C for 30 seconds (annealing step), and 70 °C or 72 °C for 40 or 45 seconds (extension step), with an initial 12 minute denaturing step at 95 °C. All standard PCR amplification experiments included blank extracts and at least one PCR negative amplification control. Following standard PCR amplification, all samples were visualized by gel electrophoresis. PCR products (5 µL) were combined with SYBR Green™ dye (Invitrogen, Carlsbad, CA, USA) and loaded into a 2 % agarose gel, run for 30 minutes at 100 volts, and then visualized under a dark reader (Clare Chemical Research Co., Dolores, CO, USA). Band presence/absence and intensity, as well as the position of each band was noted and compared to a 100 bp ladder (Invitrogen, Carlsbad, CA, USA) to confirm that the expected length of DNA fragment was amplified.

Table 2: Primers used for PCR amplification to establish a degradation pattern

Primer	Position	Sequence (5' to 3')	Region	Amplicon Length
F81 (universal)	56981-57002	TCTTGGCAGCATTCCGAGTAAC	*rbc*L	105, 167 (specific), 255 bp
R186 (universal)	57063-57086	GTCCAMACAGTTGTCCATGTACCA	*rbc*L	105 bp (with F81)
R336 (universal)	57209-57236	ACAATGGARGTAAACATGTTAGTAACAG	*rbc*L	255 bp (with F81)
R248 (corn specific)	57130-57148	CAGGAACGGGCTCGATGTG	*rbc*L	167 bp (with F81)
R248 (pea specific)	57129-57148	CAGGAACAGGCTCGATCTCG	*rbc*L	167 bp (with F81)
R248 (squash specific)	57129-57148	CAGGAACAGGCTCGATTCCA	*rbc*L	167 bp (with F81)
F152 (universal)	57052-57079	GAATCTTCYACTGGTACATGGACAACTG	*rbc*L	472 bp (with R624)
R624 (universal)	57503-57524	CGGTCTCTCCARCGCATAAATG	*rbc*L	472 bp (with F152)

Note: The direction of the primers is indicated by the F and R (forward and reverse primers, respectively) in the primer name. "Universal" in brackets beside the primer name indicates that the primer is common to corn, pea, and squash. "Specific" in brackets indicates that the primer is specific to corn, pea or squash to the exclusion of the other two plant species. The position numbers are based on Zea *mays* NC001666 chloroplast genome, which was downloaded from Genbank.

Table 3: Primers and probe used for standard and quantitative PCR amplification to compare three extraction methods

Primer/ Probe	Position	Sequence (5' to 3')	Region	Amplicon Length
F17 (universal)*	56917-56942	GCTGGTGTTAARGATTATAAATTGAC	*rbc*L	166 bp
R183 (universal)*	57063-57083	CAMACAGTTGTCCATGTACCA	*rbc*L	
*rbc*L_P2 Probe	56973-56999	TACTGATATCTTGGCAGCATTCCGAG	*rbc*L	
F84 (universal)*	56984-57001	TGGCAGCATTCCGAGTAA	*rbc*L	250 bp
R334 (universal)*	57209-57234	AATRGARGTAAACATGTTAGTAACRG	*rbc*L	
F152 (universal)	57052-57079	GAATCTTCYACTGGTACATGGACAACTG	*rbc*L	472 bp
R624 (universal)	57503-57524	CGGTCTCTCCARCGCATAAATG	*rbc*L	

Note: * Indicates that the primers either were redesigned from the primers used to develop degradation curves or were newly designed (F17). The direction of the primers is indicated by the F and R (forward and reverse primers, respectively) in the primer name. "Universal" in brackets beside the primer name indicates that the primer is common to corn, pea, and squash. The position numbers are based on Zea *mays* NC001666 chloroplast genome, which was downloaded from Genbank.

Quantitative PCR

The F17 and R183 universal primers used for standard PCR were also used for quantitative PCR, and yielded a 166 basepair (bp) amplicon (Table 3). Quantitative PCR reactions had a final volume of 20 µL and contained 2 µL DNA sample (diluted or undiluted) and the following reagents: Quanta Bioscience PerfeCta™ qPCR Supermix with ROX (Gaithersburg, MD, USA); UltraPure™ DNase/RNase-free distilled water (Invitrogen, Carlsbad, CA, USA); 0.3 µM F17 primer; 0.3 µM R183 primer; and 0.25 µM fluorescent 5' nuclease hydrolysis probe (5' 6-FAM labelled) with a ZEN quencher from Integrated DNA Technologies (Coralville, IA, USA). The design and selection of the primers and probe for the qPCR (*e.g.,* melting temperatures, primer dimerization, and hairpin structures) were assessed using the online software Beacon Designer™ Free Edition (PREMIER Biosoft International, Palo Alto, CA, USA). Quantitative PCR was

conducted using an Applied Biosystems StepOne™ Real-Time PCR system. Cycling parameters included an initial denaturation at 95 ˚C for 3 minutes followed by 50 cycles (95 ˚C for 30 seconds, 55 ˚C for 30 seconds, and 70 ˚C for 45 seconds) and a final extension at 72 ˚C for 7 minutes.

To obtain a DNA standard for qPCR, artificial DNA (a custom minigene) with a similar sequence to the *rbc*L sequence for corn, pea, and squash was designed and then custom ordered from Integrated DNA Technologies (Coralville, IA, USA). The *rbc*L artificial DNA was designed so that it would amplify the same 166 bp fragment length that is amplified for corn, pea, and squash samples using the same F17 and R183 primers (Figure 3). In addition, the *rbc*L artificial DNA was also designed for the probe (*rbc*L_P2) to bind the same distance from the forward primer as for the corn, pea, and squash samples. All of these conditions were considered during the design of the *rbc*L artificial DNA so that the DNA standards used for the qPCR were as similar to the samples as possible to ensure similar amplification efficiencies for plant samples and standards. The concentration of this artificial DNA was determined by UV absorption using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). In order to prevent supercoiling of the circular DNA standard (Adams 2006; Hou *et al.* 2010), the artificial DNA was then linearized using the restriction enzyme APaI (New England Biolabs, Ipswich, MA, USA) and further dilution series were then prepared fresh for each qPCR. Standards and unknown samples were amplified in duplicate for each qPCR and only runs with standard curves with R^2 values higher than 0.95 were analyzed.

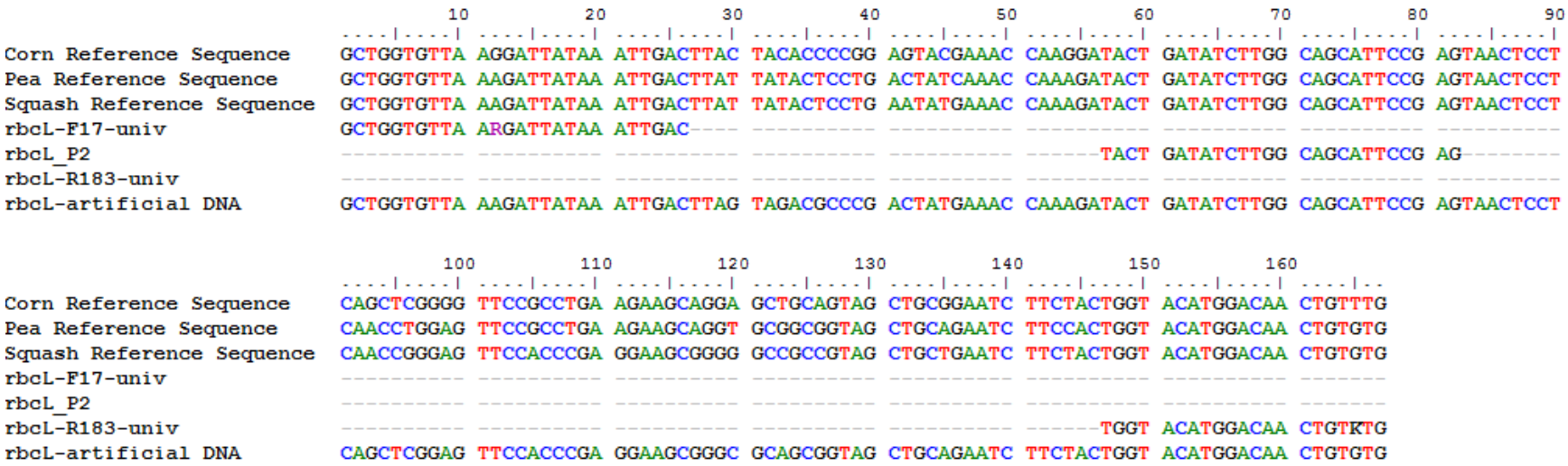

Figure 3: Sequence alignment of reference sequences, primers, probe, and artificial DNA (minigene) used in the design of the 166 basepair *rbc*L qPCR assay.

Two reference sequences each for corn (NC001666.2, Z11973.1), pea (NC014057.1, X03853.1), and squash (AF206756.1, L21938.1) were downloaded from NCBI Genbank. These sequences were used to design the forward primer (*rbc*L-F17-univ), reverse primer (*rbc*L-R183-univ), and probe (*rbc*L_P2). Both primers and the probe were designed so that they would bind corn, pea, and squash DNA. The *rbc*L-artificial DNA (minigene), was designed to be a DNA standard during qPCR. This artificial DNA was designed to have a similar DNA sequence to corn, pea, and squash and also to bind the primers and probe.

Comparison of Efficiency of DNA Extraction Methods

To facilitate the comparison of results across methods, time points, and for each individual plant species, relative-to-best values were determined. At each time point, the highest value of recovered DNA for a particular plant across the three extraction methods was identified. All three DNA quantity values at this time point were then divided by the highest value to obtain a relative-to-best value for each method. This calculation was repeated for all time points and all plant species. The average relative-to-best value for a plant species across all time points for each extraction method was then determined. A paired Student's t-test was performed using the relative-to-best values to determine if observed differences between the extraction methods compared to the best performing method were statistically significant based on the relative-to-best values. The same relative-to best calculations were conducted for the repeatability and reproducibility tests. The only differences were that there was one only time point and the DNA quantities from the extraction replicates for each method were averaged prior to the relative-to-best calculations and the paired Student's t-tests were performed using the replicate DNA quantity values.

Inhibition Testing

In order to determine the extent of PCR inhibition in the undiluted 140 ˚C heat treated sample extracts (0 to 10 hour time points), an Internal Positive Control (IPC) qPCR inhibition assay was designed as follows. Multiple artificial DNA sequences were randomly generated using the Random DNA Sequence option of The Sequence Manipulation Suite (http://www.bioinformatics.org/sms/index.html). These random DNA sequences (400 basepairs in length) were then BLAST searched

(http://www.ncbi.nlm.nih.gov/BLAST/) to ensure that they did not match any corn, pea, squash, or human DNA sequences. One artificial DNA sequence, which had no significant similarity to any of the DNA sequences in Genbank, was selected for primer and probe design using the online software Beacon Designer™ Free Edition (PREMIER Biosoft International, Palo Alto, CA, USA). Multiple forward and reverse primers and two probes were designed within this 400 basepair sequence to allow for the possibility of having Internal Positive Control PCR reactions of different fragment lengths. Primers and probes were custom ordered from Integrated DNA Technologies (Coralville, IA, USA). Ultimately, a 129 basepair fragment within the 400 basepair region was used for the IPC qPCR inhibition assay (Figure 4, Table 4). This 129 basepair fragment was also BLAST searched and no significant similarity to any DNA sequence in Genbank was found. Next, the IPC artificial DNA (a custom minigene including the entire 400 bp IPC sequence) was custom ordered from Integrated DNA Technologies (Coralville, IA, USA) to be the DNA standard for the IPC qPCR inhibition assay. The concentration of this minigene was determined by UV absorption using a NanoDrop 1000 (NanoDrop Technologies, Wilmington, DE, USA). The minigene was then linearized using the restriction enzyme APaI (New England Biolabs, Ipswich, MA, USA), and further dilutions were made.

Table 4: Primers and probe used in the IPC qPCR inhibition assay.

Primer/Probe	Position on Artificial Gene	Sequence (5' to 3')	Amplicon Length
IPC F72 Primer	72-94	GAGCGTAGAGGACTATCTGGAG	129 bp
IPC R200 Primer	180-200	GGTAGTCTGGTAGCAGGAGTG	
IPC_P1 Probe	100-122	ACGAGTGTGGGTAGATTGGCAGC	

Note: The direction of the primers is indicated by the F and R (forward and reverse primers, respectively) in the primer name.

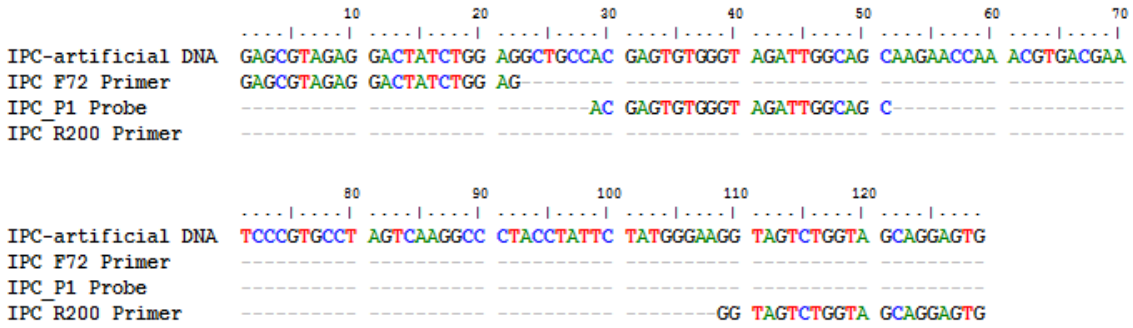

Figure 4: Sequence alignment of Internal Positive Control (IPC) 129 basepair qPCR assay including primers, probe and artificial DNA.
The IPC artificial DNA was randomly generated using The Sequence Manipulation Suite (http://www.bioinformatics.org/sms/index.html). The IPC artificial DNA was used to design the forward IPC F72 primer, reverse IPC R200 primer, and the IPC_P1 probe. Both primers and the probe were designed so that they would not bind corn, pea and squash DNA. The IPC artificial DNA (a custom DNA minigene), was used as the Internal Positive Control during these inhibition assays.

Three different reaction types were included in all IPC qPCR inhibition batches: PCR negative amplification controls (also commonly referred to as a no template control [NTC]); internal positive control standards (10,000 templates/reaction) with 2 µL of distilled water (instead of a sample extract); and 2 µL of undiluted sample extracts with internal positive control standards (10,000 templates/reaction). In addition, each sample extract IPC reaction was tested in duplicate while the IPC reaction (without sample

extract) was run in triplicate. All IPC qPCR inhibition reactions had a final volume of 20 µL and contained the following reagents: Quanta Bioscience (Gaithersburg, MD, USA) PerfeCta™ qPCR Supermix with ROX, UltraPure™ DNase/RNase-free distilled water (Invitrogen, Carlsbad, CA, USA), 0.3 µM IPC F72 primer, 0.3 µM IPC R200 primer, and 0.25 µM fluorescent 5' nuclease hydrolysis probe (5' 6-FAM labelled) with a ZEN quencher. Quantitative PCR was conducted using an Applied Biosystems StepOne™ Real-Time PCR system. Cycling parameters included an initial denaturation step at 95 ˚C for 3 minutes followed by 50 cycles (95 ˚C for 30 seconds, 55 ˚C for 30 seconds, and 70 ˚C for 45 seconds) and a final extension at 72 ˚C for 7 minutes.

The amount of PCR inhibition present in the sample extracts in each batch was determined using two approaches as described by King *et al.* (2009). First, we measured the shift in the quantification cycle (C_q) of the sample extract IPC reaction when compared to the quantification cycle of the IPC standard. This shift is the result of PCR inhibitors in the spiked samples. The C_q values for all samples and standards were automatically calculated by the Applied Biosystems StepOne™ software (version 2.0). All sample extract IPC reactions were run in duplicate and IPC standards were run in triplicate for each batch, and their C_q values were then averaged. Next, the average sample extract C_q value was subtracted from the average IPC standard C_q value, resulting in a calculated ΔC_q value for that sample. To more clearly quantify the effect of PCR inhibition, the "expected recovery" (ER) was calculated as described by King *et al.* (2009). The expected recovery values were calculated from the ΔC_q value for that sample and can be used to predict the effect of PCR inhibition in future PCR reactions using the same sample extract. Expected recovery is calculated using the following equation:

$$ER = 100\ \% \times (\text{dilution factor} \times 2^{\Delta Cq})^{-1}$$

As only undiluted sample extracts were tested in the current study, the dilution factor was always equal to 1. Because the C_q values for the qPCR inhibition reactions are based on a logarithmic scale, a ΔC_q value of 1 (*e.g.*, a shift from C_q of 28 in the IPC alone standard to C_q of 29 for the sample extract IPC inhibition reaction) would result in an expected recovery of 50 %. This means that if one tried to amplify the same sample extract in another qPCR experiment with the same PCR reaction conditions, one would only expect to recovery 50 % of the true amount of DNA present in the sample due to PCR inhibition. As further examples, calculated ΔC_q values of 0, 2, and 4 would result in expected recovery values of 100 %, 25 %, and 6 %, respectively.

The second approach used in this study to assess the extent of PCR inhibition in the extracted samples involved estimating the amplification efficiencies for all sample extract IPC inhibition runs. Again, this was conducted as described by King *et al.* (2009). The raw amplification fluorescence data (ΔRn for all 50 cycles) was plotted using the GraphPad Prism version 5.02 software (GraphPad Software, San Diego, CA, USA). The Hill slopes for each sample were then calculated by fitting the amplification curves to a variable slope sigmoidal dose-response curve using GraphPad Prism software. Steeper amplification curves yielded higher Hill slope values. Next, the average Hill slope values were calculated from the triplicate IPC standard reactions and duplicate sample extract IPC reactions. Finally, estimations of amplification efficiency for each sample were calculated by dividing the average sample extract Hill slope by the average IPC standard Hill slope. King *et al.* (2009:944) explain that any observed deviations from 100 % are the result of PCR inhibitors in the sample extracts, but also "acknowledge that it is in fact

a relative measurement of the shape of an amplification plot." King *et al.* (2009) further emphasize that the two approaches, ΔC_q and amplification efficiency, should be used in combination to assess the extent of PCR inhibition because different inhibitors can affect either or both of these calculated values. For example, some inhibited sample extracts may result in large ΔC_q values, but high amplification efficiencies while other inhibited samples may yield low ΔC_q values, but have very inefficient amplification efficiencies.

DNA Sequencing

A random selection of amplified samples were sequenced to confirm that the obtained DNA sequences corresponded with the expected sequences. Samples were purified using the ExoSAP-IT® kit (USB, Cleveland, Ohio) prior to DNA sequencing. The ExoSAP-IT® kit is designed to eliminate excess primers and nucleotides. The ExoSAP-IT® reagent was added to the amplified sample in a new PCR tube and was run in one of the Eppendorf™ Mastercycler Thermocyclers at 37 ˚C for 15 minutes to degrade the primers and nucleotides, and then at 80 ˚C for 15 minutes to degrade the ExoSAP-IT® enzyme. DNA sequencing was conducted by Eurofins MWG Operon (Huntsville, AL, USA) and the returned sample electropherograms were manually edited using ChromasPro software (www.technelysium.com.au). Sample sequences were initially BLAST searched (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm that the returned sequences were consistent with the expected plant species. In addition, the consensus DNA sequences were prepared using ChromasPro software (www.technelysium.com.au) for the three plant species and further compared to a corresponding reference sequence downloaded previously from Genbank (corn NC_001666.2, pea NC_014057.1, and squash L21938.1).

Repeatability and Reproducibility Tests

To confirm the results obtained in this study, one time point for each plant species was selected, and the DNA extraction methods were repeated – once by myself to test the repeatability of the results, and once by another trained researcher within our laboratory group to test the reproducibility of the results by different researchers. For both experiments, new seeds were obtained, weighed, and artificially degraded at 140 ˚C. The corn and squash samples were heated for 5 hours while the pea samples were heated for 10 hours. These heat treatment time points were selected based on the results of the DNA degradation patterns phase of this research to ensure that at least some degraded DNA would be present following heat treatment and extraction by the three different extraction methods. The plant samples for both the repeatability and reproducibility tests were heated independently (*i.e.,* in different covered weigh boats) but in the same oven at the same time. For the repeatability test, the corn, pea, and squash samples were powdered shortly after heat treatment and each of the plant species was extracted five times using each of the three described extraction methods (silica-spin column, DNeasy Plant Mini Kit, and DTAB/CTAB). Undiluted (1x) DNA from the repeatability test extracts was then quantified in duplicate using qPCR as before. For the reproducibility test, the corn, pea, and squash samples were powdered shortly after heat treatment and the powder was then stored in a freezer (approximately -20 ˚C) for about 6 months prior to extraction. Each of the plant species was extracted three times using each of the three described extraction methods (silica-spin column, DNeasy Plant Mini Kit, and DTAB/CTAB). Diluted (0.1x) DNA from the reproducibility test extracts was then quantified in duplicate using qPCR as before.

Contamination Controls

Strict contamination controls were exercised throughout this study including the use of access-restricted, dedicated SFU Forensic DNA and Post-PCR laboratories, both with separate UV-filtered ventilation. The SFU Forensic DNA laboratory has positive airflow and separate rooms with dedicated laboratory equipment each for sample preparation, DNA extraction, and PCR setup. The SFU Forensic DNA laboratory was designed to deal with samples that fall between ancient and modern samples (*e.g.*, degraded forensic samples and artificially degraded samples) and is physically separated from both the ancient DNA laboratory and the modern DNA laboratory. In addition, strict unidirectional workflow protocols were followed whereby entry into the Forensic DNA laboratory is prohibited after entering the Post-PCR laboratory without showering and changing clothing. This laboratory has been previously used for an artificial DNA degradation study using sheep bones (McGrath 2010). No previous plant samples had been extracted or set up for PCR in the Forensic DNA laboratory prior to the current study. In addition, all researchers wore dedicated lab scrubs and shoes underneath Tyvek body suits, gloves, and masks when in the Forensic DNA laboratory. Laboratory spaces, equipment, and supplies were routinely sterilized with bleach. Filtered pipette tips were used at all steps, extraction blanks were included for each extraction method, and one or multiple PCR negative amplification controls included in each standard and qPCR run.

Chapter Summary

Corn, pea, and squash were used in this study to represent three different plant families and different tissues types (monocot and dicot dried fruits and seeds). Samples were heat treated in an oven or muffle furnace at 85 ˚C, 140 ˚C, or 200 ˚C to artificially

degrade the DNA, generating DNA degradation patterns. DNA recovery efficiency and removal of PCR inhibitors were compared for the silica-spin column, DTAB/CTAB, and DNeasy Plant Mini Kit DNA extraction methods using both standard and quantitative PCR. Repeatability and reproducibility tests were conducted to help determine the strength and authenticity of the results. Strict control measures were utilized throughout the study in order to prevent or minimize contamination.

Chapter 4: Results

DNA Degradation Patterns

Morphological Changes

In order to identify heat treatment conditions that would yield appropriately degraded plant remains for further studies, DNA degradation patterns were generated under a variety of experimental conditions. At 85 ˚C, the dried fruits/seeds showed no significant changes in morphological appearance over 15 hours (Figure 5). Corn and pea samples lost between 2 % and 6 % of their starting weights during heat treatment while the squash samples lost up to 12 % of their starting weight, likely due to the higher water content of the harvested squash seeds compared to the dried corn and pea fruits (Figure 8). Some darkening of the corn and pea samples was observed at 140 ˚C with a more pronounced darkening of all samples at 200 ˚C (Figures 6 and 7). At 140 ˚C, all samples lost between 4 % and 9 % of their starting weights (Figure 8). The most drastic weight losses following heat treatment were observed at 200 ˚C, with corn and pea samples losing 12 -18 % and squash losing up to 35 % of its starting weight (Figure 8). At these three temperatures, none of the samples was completely charred. Even at temperatures as high as 250 ˚C, only the pea samples were completely charred after a one hour heat exposure as interpreted by an SFU expert archaeobotanist (personal communication with Dr. D'Andrea). As the main objectives of this study were to develop DNA degradation patterns for the various plant samples tested and then to conduct a laboratory-controlled comparison of different DNA extraction methods currently used in the field, it was

determined that complete charring of the plant materials was not requirement for this study.

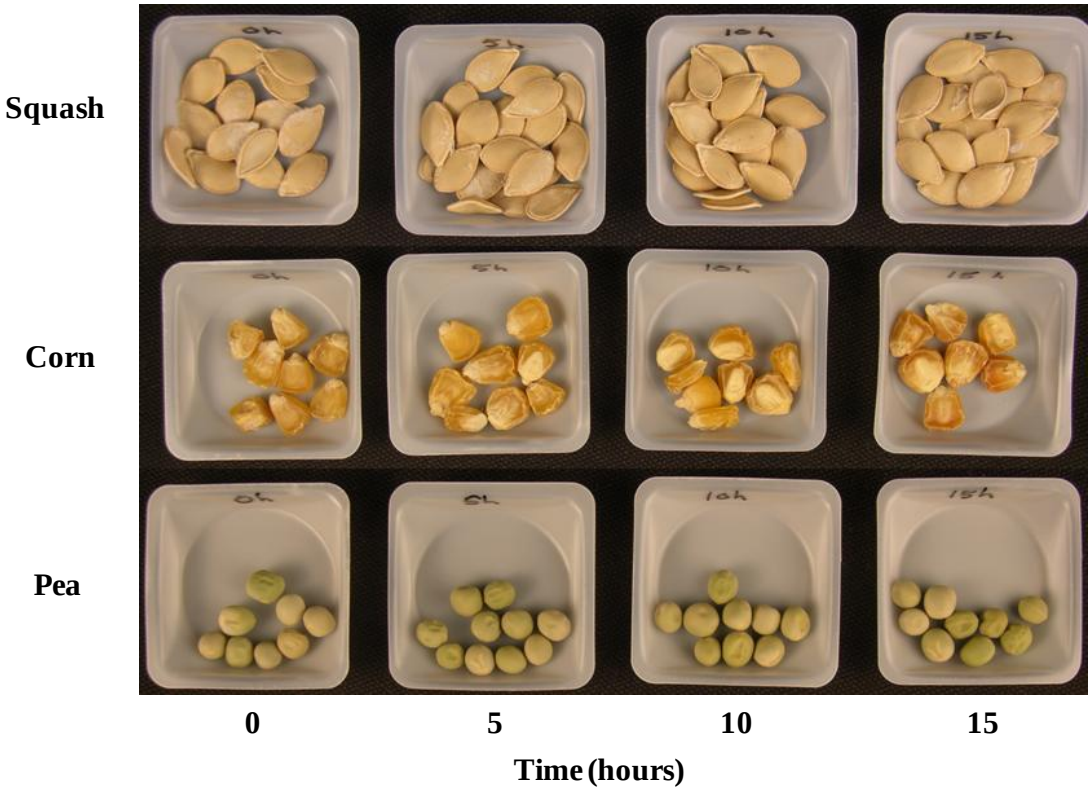

Figure 5: Photographs for seeds exposed to 85 °C for 0, 5, 10 and 15 hours.

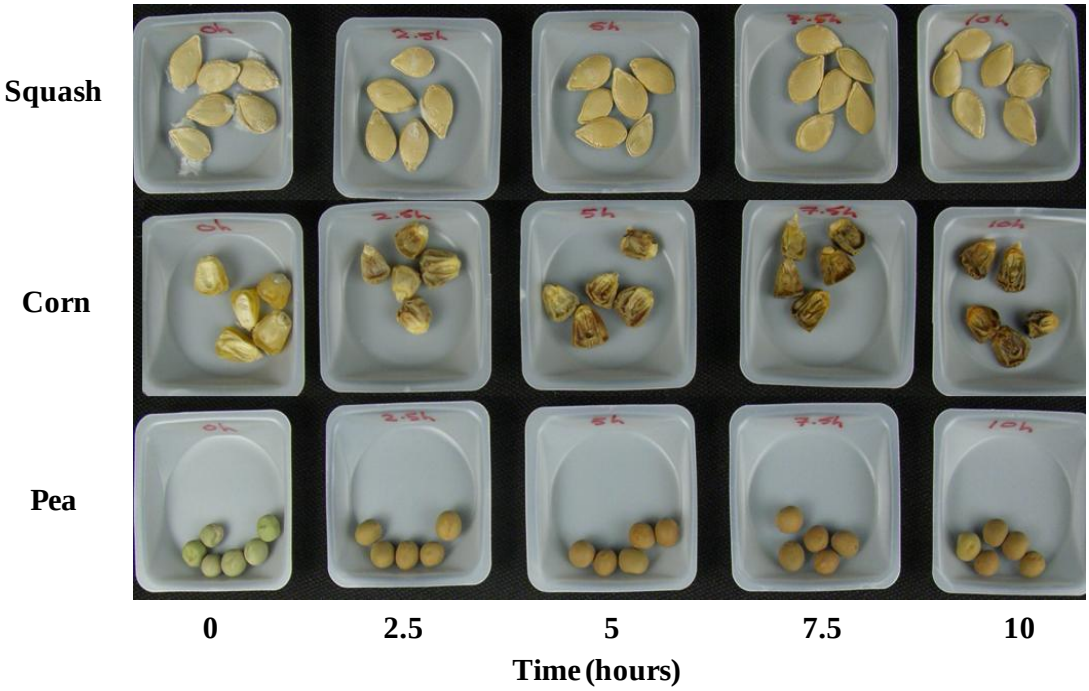

Figure 6: Photographs for seeds exposed to 140 ˚C for 0, 2.5, 5, 7.5, and 10 hours.

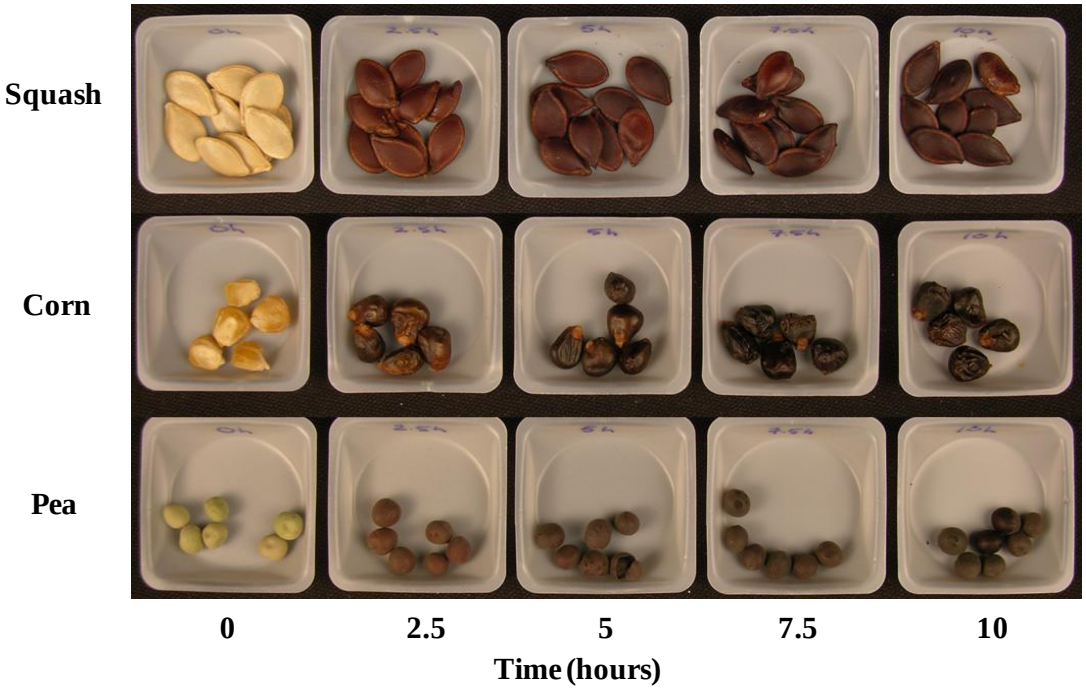

Figure 7: Photographs for seeds exposed to 200 °C for 0, 2.5, 5, 7.5, and 10 hours.

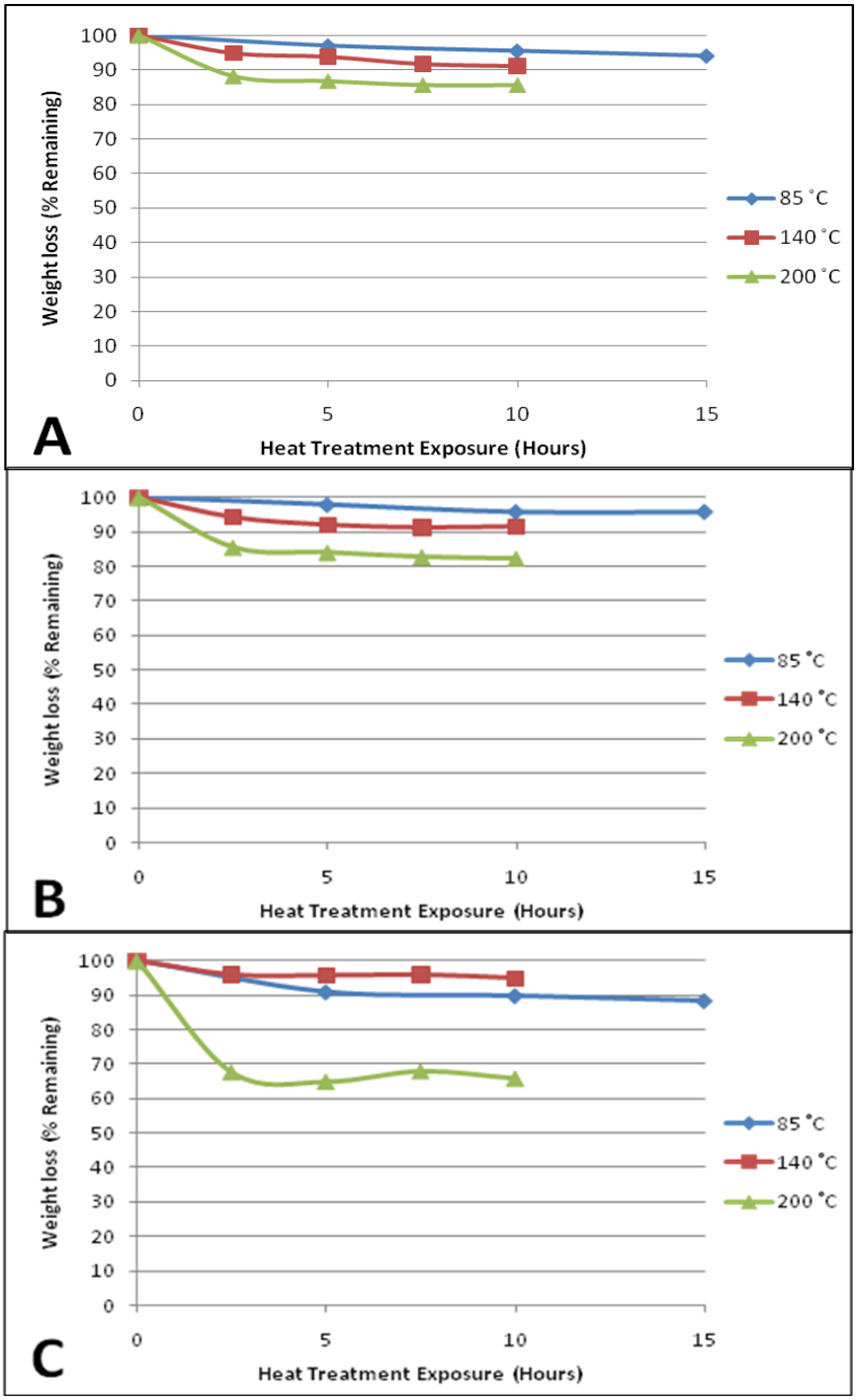

Figure 8: Weight loss in various plant samples as a result of 0-15 hours heat treatment at either 85 ˚C, 140 ˚C, or 200 ˚C.
Prepared plant samples (A: corn; B: pea; C: squash) were subjected to heat treatment for up to 10 hours for the 140 ˚C and 200 ˚C groups and 15 hours for the 85 ˚C group. Weight loss resulting from heat treatment was determined by weighing samples both before and after heating to calculate the percentage weight remaining after treatment.

Standard PCR and Quantitative PCR

To determine how rapidly DNA from each type of plant was degraded under the various heat treatment conditions, DNA from a number of time points throughout the degradation process was extracted using the silica-spin column method, and the quality and quantity of DNA were assessed using standard PCR and qPCR, respectively. All three plant samples (corn, pea, and squash) heated at 85 °C from 0-15 hours showed no significant DNA degradation as standard PCR amplification bands at fragment lengths of 105, 255, and 472 basepairs were present for all four time points (Figure 9). However, because the corn, pea and squash plant powders were mixed together, a further test was conducted to determine if DNA from all three species was the source of the amplified DNA or if rather only one or two species contributed to the amplified DNA while DNA belonging to the other species was too degraded to be amplified. For this reason, species-specific reverse primers were designed to bind to only one of the three species tested in this study to the exclusion of the other two species. When combined in a PCR experiment with the universal forward primer, which binds to DNA of all three plant species, the species-specific reverse primers restrict amplification to only one of the three species per PCR tube.

To validate this approach prior to official sample testing, a specificity test of the universal forward primer and specific reverse primers was conducted using PCR to determine whether unmixed modern DNA (*i.e.,* 0 hour time point) from pea or squash extract would amplify when using the corn specific reverse primer. In addition, similar tests were conducted for the pea and squash specific reverse primers. In all cases, the only DNA amplified was that from the expected species based on the specific primer

used (*e.g.*, the corn specific primer only amplified DNA from the unmixed corn extract) (data not shown). Following specificity testing, DNA extracts from mixed plant samples heat treated at 85 ˚C for 0-15 hours were subjected to PCR using the universal forward primer (F81) and each of the specific reverse primers independently (either R248 corn, pea or squash specific primers) to identify the source of amplifiable DNA in each sample (Figure 10). Species-specific DNA amplification bands (167 bp fragment) were present at all four time points for all plant species, indicating that at 85 ˚C, DNA from all three plant species was amplifiable (Figure 10).

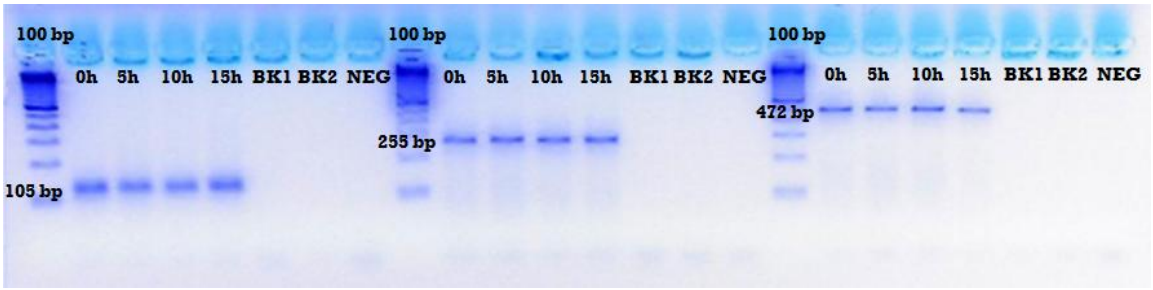

Figure 9: Mixed plant samples heat treated at 85 ˚C for 0 - 15 hours.
Samples (mixed corn, pea, and squash) were diluted ten times (0.1x) and amplified for 30 cycles with amplicon lengths of 105, 255 and 472 basepairs. DNA was extracted using the silica-spin column method from approximately 100 mg of mixed plant powder. Lanes 1, 9, and 17 have 100 bp ladder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

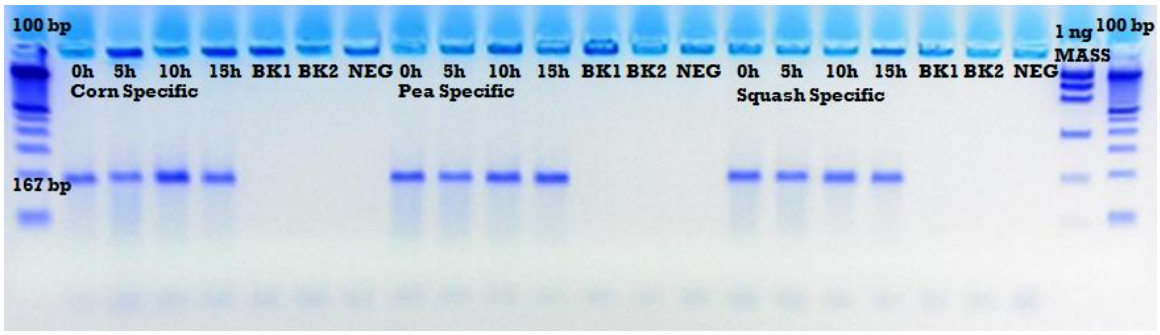

Figure 10: Mixed plant samples heat treated at 85˚C (0 - 15 hours) amplified with corn, pea, and squash specific primers.
Samples (mixed corn, pea, and squash) were diluted ten times (0.1x) and amplified for 30 cycles with amplicon length of 167. Lanes 1 and 24 have 100 bp ladder (Invitrogen). Lane 23 has a 1 ng mass ladder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

While these standard PCR results indicate qualitatively that DNA from corn, pea, and squash is not significantly degraded at 85 ˚C up to 15 hours, standard PCR cannot be used to quantitatively assess the amount of DNA templates present in the sample nor the exact extent of DNA degradation following heat treatment at 85 ˚C. To address these issues, the 85 ˚C heat treated plant samples were also subjected to qPCR (Figure 11). While we did observe a small amount of DNA degradation occurring as a result of heat treatment in this set of experiments, the degradation rate was too slow to permit a comparison of extraction methods. For example, after 15 hours of heat treatment at 85 ˚C, only 13 % of the initial amount of DNA had been degraded (Table 5).

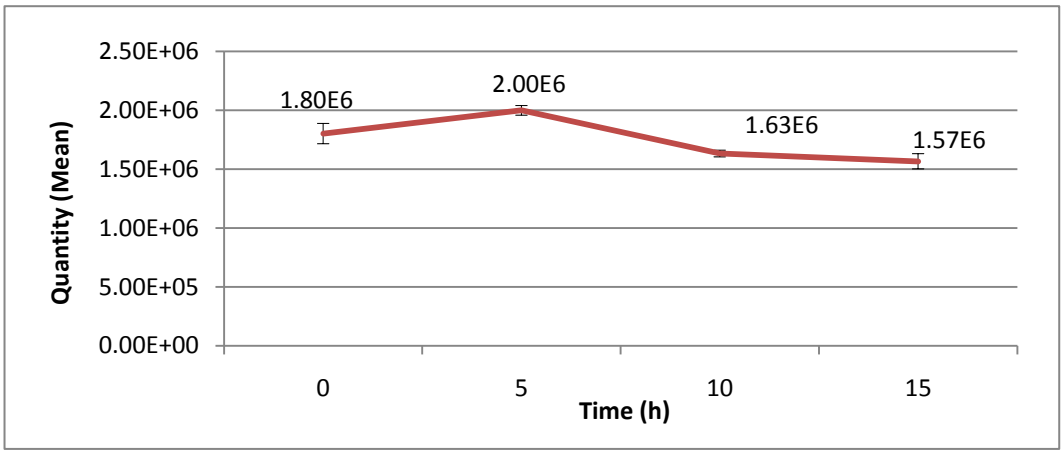

Figure 11: DNA degradation pattern using qPCR for mixed corn, pea, and squash heat treated at 85 ˚C (0 - 15 hours).
Samples (mixed corn, pea, and squash) were diluted ten times (0.1x) and amplified for 50 cycles with an amplicon length of 166 bp. The DNA quantity shown is the mean of duplicate qPCR reactions and error bars indicate the standard deviation in DNA quantity for each of the individual time points.

Table 5: Percent of initial DNA remaining from mixed samples heat treated at 85 ˚C (0.1x dilution).

Heat Treatment (Hours)	**0**	**5**	**10**	**15**
DNA Quantity (Mean)	1801233	1998323	1631777	1565898
Percent of Initial DNA Remaining	**100**	**111**	**91**	**87**

After determining that 85 ˚C was insufficient to produce substantially degraded DNA for further analysis and comparison of methods, a higher temperature was then selected. Corn, pea, and squash samples were heat treated at 200 ˚C (0, 2.5, 5, 7.5, 10 hours), powdered and extracted separately (*i.e.*, corn, pea, and squash were not mixed) using the silica-spin column method. The extent of DNA degradation was again initially determined using standard PCR with amplification fragments of 166 and 250 basepairs (Figure 12). As opposed to the results observed at 85 ˚C, at 200 ˚C only limited amounts of DNA were successfully amplified from the heat treated samples as measured by the appearance of amplification product bands on an agarose gel. Only the pea sample

exposed to 200 ˚C for 7.5 hours yielded DNA amplification bands for both the 166 and 250 bp fragments. However, because the 0 hour time points for each plant species did yield amplifiable DNA (166 and 250 bp), indicating that the overall DNA extraction and amplification procedures worked, it was concluded that this temperature results in complete degradation of DNA prior to 2.5 hours for both corn and squash. Consequently, it was determined that 200 ˚C is too high a temperature to yield degraded but amplifiable DNA and thus, like 85 ˚C, this temperature is unsuitable for further analysis and comparison of extraction methods.

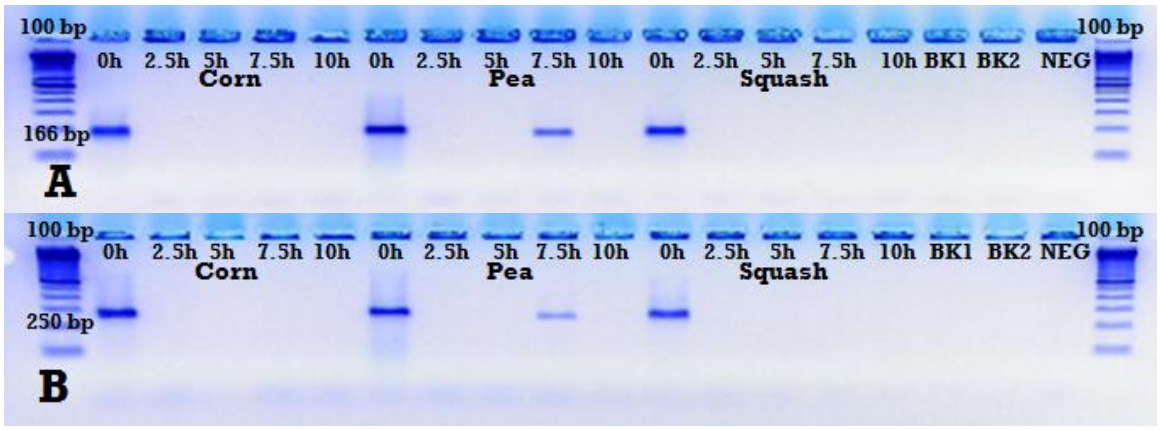

Figure 12: Corn, pea and squash samples heat treated at 200 ˚C (0 - 10 hours). Samples were diluted ten times (0.1x) and amplified for 50 cycles with amplicon lengths of A) 166 basepairs and B) 250 basepairs. DNA was extracted using the silica-spin column method from 25 mg of plant powder. Lanes 1 and 20 have 100 bp 1adder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

After determining that 85 ˚C was too low and 200 ˚C was too high a temperature to obtain appropriate DNA degradation curves for further analysis, an intermediate temperature of 140 ˚C was tested. 50-60 mg of corn, pea, and squash samples were exposed to heat treatment at 140 °C (0, 5, and 10 hours), and DNA was extracted using

the silica-spin column method. Fragments of 166, 250, and 472 basepairs were first individually amplified from the isolated DNA extracts using 50 cycles of standard PCR. Next, amplified products for each extraction sample were subjected to gel electrophoresis by combining 2.5 µL of each amplified sample (166, 250, 472 bp amplicons) with SYBR Green Nucleic Acid Gel stain (Invitrogen, California, USA) and then loading the stained samples onto a 2 % agarose gel (Figure 13). This composite gel demonstrates the variability in DNA degradation for corn, pea, and squash at 140 ˚C with obvious degradation of corn and pea DNA, but no significant degradation of squash DNA under these conditions. We attribute the differences in amplification band patterns between Figure 12 (200 ˚C) and Figure 13 (140 ˚C) to the decrease in temperature with the latter lower temperature resulting in more amplifiable DNA.

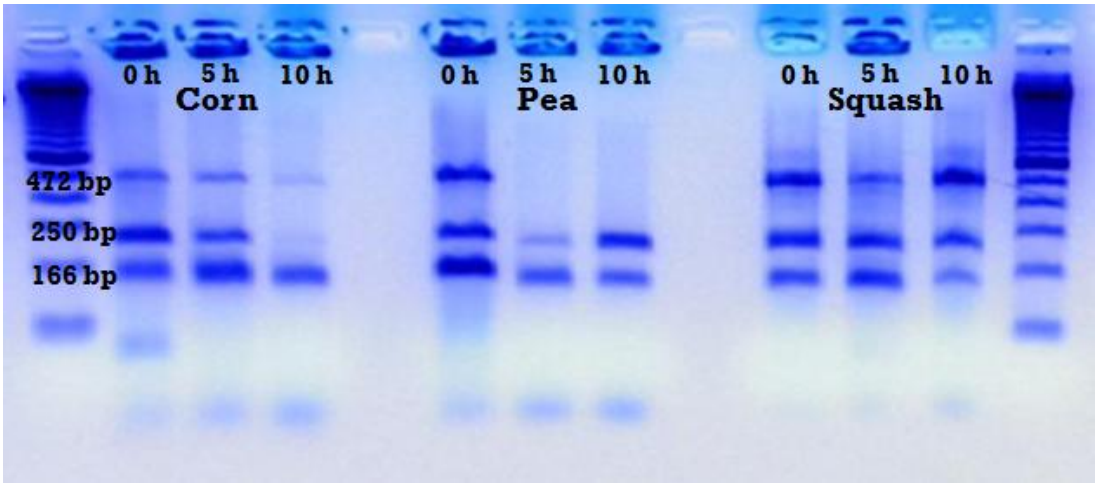

Figure 13: Different DNA degradation patterns for corn, pea, and squash heat treated at 140 ˚C using standard PCR.
DNA was extracted using the silica-spin column method from 50-60 mg of plant powder. Samples were diluted ten times (0.1x) and amplified at 50 cycles. PCR amplicons (166, 250, and 472 bp) from different PCRs were combined in the same electrophoresis gel. Samples were amplified at 50 cycles. Lanes 1 and 13 have 100 bp 1adder (Invitrogen). Row 5 and 9 were left empty.

In order to better adhere to the manufacturer's protocol for the use of the DNeasy Plant Mini Kit in the comparison of extraction methods phase of this research, starting plant powder material was reduced from approximately 50 mg to 25 mg, and samples were re-extracted using the silica-spin column method to confirm that the DNA degradation patterns observed with 50-60 mg starting material could also be obtained with half as much plant powder. This was achieved by extracting DNA from 25 mg of 140 ˚C heat treated corn, pea, and squash powder for the 0, 2.5, 5, 7.5 and 10 hour time points. Isolated DNA was then amplified using standard PCR (255 bp fragment) for 30 cycles (Figure 14). Each plant sample exhibited a variable DNA amplification pattern with corn having amplification bands for only 0, 2.5, and 5 hour time points. Pea samples displayed amplification bands for only the 0 and 10 hour time points while squash had bands at all time points but showed a visible decrease in band intensity as the length of heat treatment increased. The DNA amplification band pattern observed for the 140 ˚C heat treated pea samples was similar to the pattern observed for the 200 ˚C heat treated pea samples. This pattern included a disappearance of amplification bands between the 0-hour sample and the next observed DNA band (*i.e.,* two or more time points after the 0-hour time point failed to yield amplification products visualized on a gel). This may be indicative of PCR inhibition (possibly Maillard products) in the intermediate time points of the pea samples. While this pattern was not observed for the previous 140 ˚C pea samples (Figure 13), this discrepancy could be the result of either differences in the amount of starting plant powder or the number of PCR cycles. It is possible that 30 cycles was insufficient to overcome the presence of PCR inhibitors in these samples.

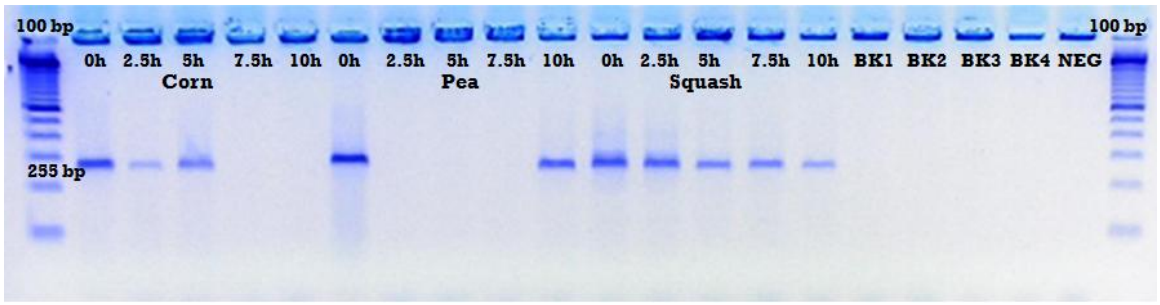

Figure 14: Corn, pea, and squash samples heat treated at 140˚C (0 - 10 hours). DNA was extracted using the silica-spin column method from 25 mg of plant powder. Extracts were diluted ten times (0.1x) and amplified for 30 cycles with an amplicon length of 255 basepairs. Lanes 1 and 22 have 100 bp 1adder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

Based on our results obtained at 140 ˚C, which demonstrated variable DNA degradation patterns of the three plant species tested, the 25 mg samples extracts were subsequently subjected to qPCR to obtain finer resolution DNA degradation patterns (Figure 15). The 0-hour time points for corn and pea samples were found to contain similar DNA template levels with rapid degradation observed prior to 2.5 hours. The squash degradation curve originated at a lower DNA template level and showed a more gradual decline in template levels over time. The qPCR results for pea yielded little DNA between 2.5 and 7.5 hours, but demonstrated an unexpected increase in DNA quantity at the 10 hour time point, suggesting that the presence of inhibitors in pea DNA extracts for the 2.5, 5 and 7.5 hour time points that interfered with PCR amplification.

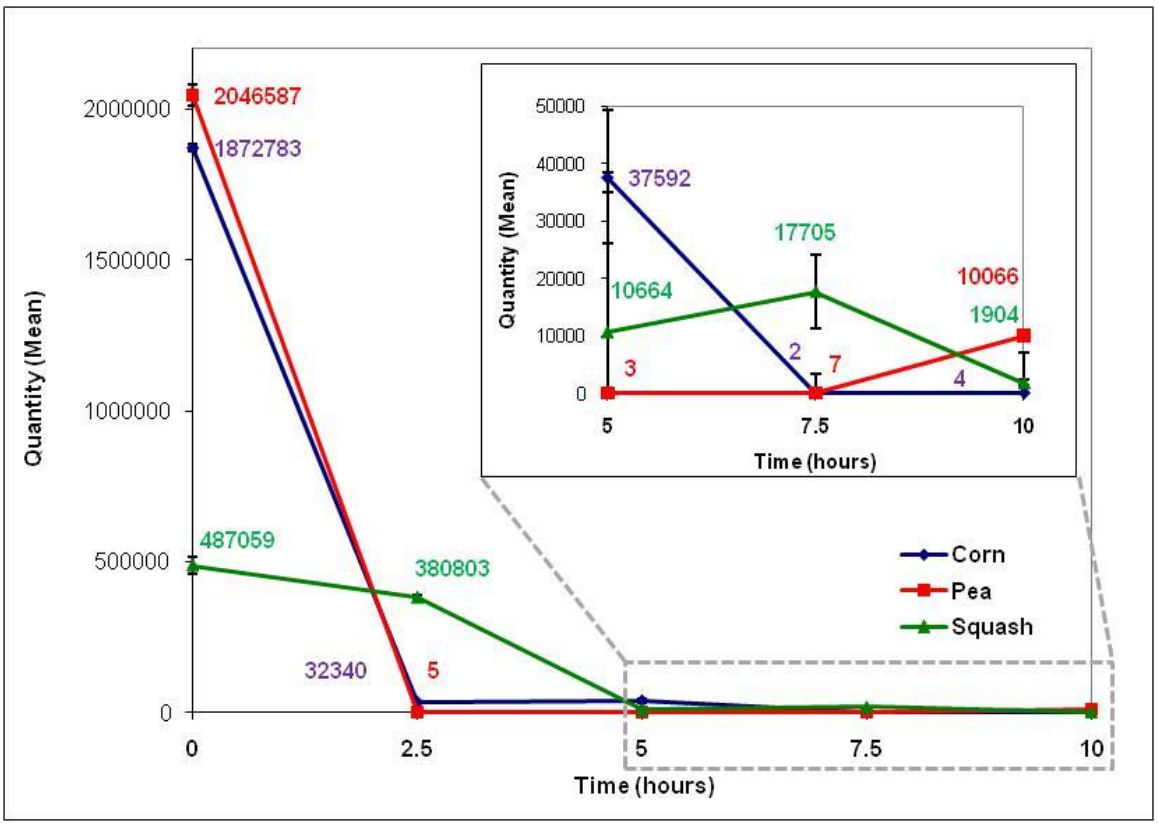

Figure 15: DNA degradation patterns of corn, pea, and squash from qPCR following heat treatment at 140 ˚C (0 - 10 hours).
Extracted samples were diluted ten times (0.1x) and DNA quantity was determined by qPCR amplification of a 166 bp fragment. Insert shows a zoomed in image of the 5, 7.5, and 10 hour time points. The DNA quantity showed is the mean of duplicate qPCR reactions and error bars indicate the standard deviation in DNA quantity for each of the individual time points.

To further characterize the degradation of DNA in the plant samples, the percentage of initial DNA remaining at each time point was calculated for corn, pea, and squash samples that were heat treated at 140 ˚C (Table 6). Squash had the slowest DNA degradation pattern of all three plant samples while both corn and pea samples were approximately 98% degraded from the initial starting DNA quantity. While the observed increase in DNA quantity in the pea sample at the 10 hour time point may seem surprising upon first glance, this increase represents only 0.5 % of the starting DNA

template. Ultimately, as a result of the successful generation of DNA degradation patterns at 140 °C, this temperature was selected for the second phase: the comparison of the different extraction methods.

Table 6: Percent of initial DNA remaining from samples heat treated at 140˚C (0.1x dilution).

Heat Treatment (Hours)	0	2.5	5	7.5	10
Corn					
DNA Quantity (Mean)	1872783	32340	37592	2	4
Percent of Initial DNA Remaining	**100**	**1.7268**	**2.0073**	**0.0001**	**0.0002**
Pea					
DNA Quantity (Mean)	2046587	5	3	7	10066
Percent of Initial DNA Remaining	**100**	**0.0002**	**0.0001**	**0.0003**	**0.4918**
Squash					
DNA Quantity (Mean)	487059	380803	10664	17705	1904
Percent of Initial DNA Remaining	**100**	**78.1843**	**2.1894**	**3.6351**	**0.3910**

DNA Recovery Efficiency of the Three DNA Extraction Methods

Comparison of Extraction Methods using Standard PCR

DNA was extracted from 25 mg of corn, pea, and squash powder previously exposed to heat treatment at 140 °C (0, 2.5, 5, 7.5, and 10 hours) using either the DNeasy Plant Mini Kit or the DTAB/CTAB method. All samples were then amplified at two different fragment lengths (166 and 250 bp) for 50 cycles. The samples previously extracted using the silica-spin column method were also re-amplified so that they could be compared directly to the samples extracted with the other two methods. In all experiments, the extraction blanks for the three DNA extraction methods and PCR

negative amplification controls yielded no bands. While all three extraction methods yielded 166 bp amplification bands for the 0, 2.5, and 5 hour time points of the corn samples, only the silica-spin column method yielded a 166 bp amplification band at the 10 hour time point (Figure 16). In addition, the silica-spin column method and the DNeasy Plant Mini Kit yielded 250 bp amplification bands at the 0, 2.5, and 5 hour time points while no such band was observed at the 2.5 hour time point for the DTAB/CTAB method.

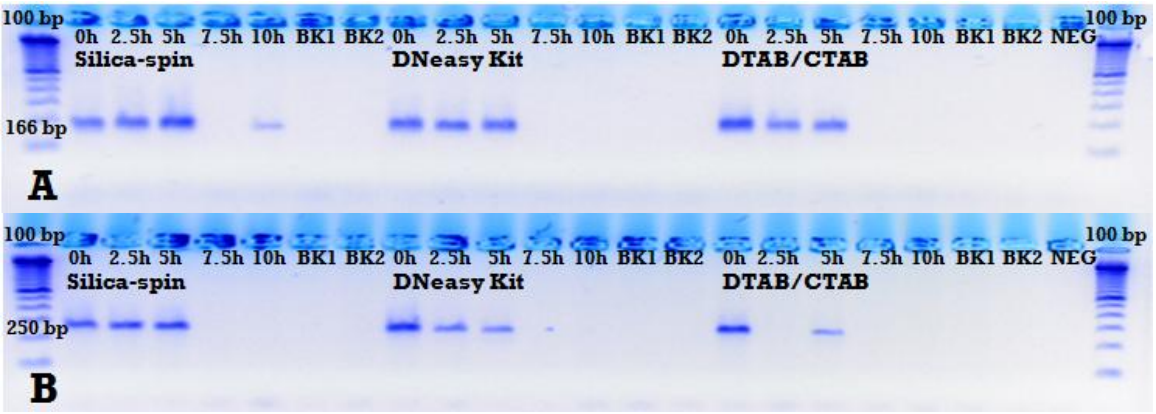

Figure 16: Comparison of DNA extraction methods with corn samples heat treated at 140 ˚C (0 - 10 hours).
Extracted Samples were diluted ten times (0.1x) and amplified for 50 cycles with amplicon lengths of A) 166 basepairs and B) 250 basepairs. Lanes 1 and 24 have 100 bp 1adder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

For the pea samples, the silica-spin column method yielded 166 bp DNA amplification bands at all time points (Figure 17). Conversely, the DNeasy Plant Mini Kit was missing a 166 bp amplification band at the 7.5 hour time point while the DTAB/CTAB method was missing 166 bp amplification bands at 5, 7.5, and 10 hour time points. At the longer 250 bp fragment of the pea samples, amplification bands were

absent at the 5 hour time point for the silica-spin column method, and the 5 and 7.5 hour time points for the DNeasy Plant Mini Kit with only a weak band present at the 2.5 hour time point for the latter extraction method. Furthermore, the 250 bp amplification bands were also absent for the DTAB/CTAB method at the 5, 7.5, and 10 hour time points.

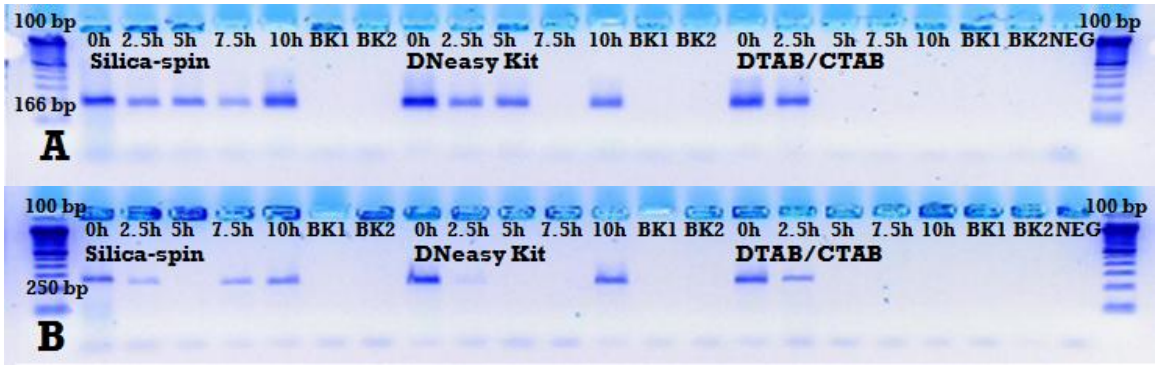

Figure 17: Comparison of DNA extraction methods with pea samples heat treated at 140 ˚C (0 - 10 hours).
Extracted samples were diluted ten times (0.1x) and amplified for 50 cycles with amplicon lengths of A) 166 basepairs and B) 250 basepairs. Lanes 1 and 24 have 100 bp 1adder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

Initial results for the squash samples demonstrated that 166 and 250 bp amplification bands were present for all three extraction methods at all time points providing no indication of any variability in the extraction efficiency of the three different methods (data not shown). Therefore, the standard PCR reactions were repeated at a lower cycle number (35) in an attempt to provide a qualitative comparison of the variability between extraction methods (Figure 18). Under these revised conditions, sample extracts from all three DNA extraction methods still yielded 166 bp amplification bands at all time points, but the bands for the silica-spin column method were on average

thicker than the bands present for the other two extraction methods (Figure 18). In addition, the 250 bp amplification bands for the silica-spin column method yielded strong DNA bands for all time points while the DNeasy Plant Mini Kit and DTAB/CTAB methods each had amplification clear bands present at the 0 and 2.5-hour time points, extremely weak bands for the 5 and 7.5 hour time points and no bands present for the 10 hour time points.

Figure 18: Comparison of DNA extraction methods with squash samples heat treated at 140 ˚C (0 – 10 hours).
Extracted samples were diluted ten times (0.1x) and amplified for 35 cycles with amplicon lengths of A) 166 basepairs and B) 250 basepairs. The second extraction blank for the DNeasy Plant Mini Kit was not amplified at 166 bp but was amplified at 166 bp previously for the corn and pea samples and did not produce any amplification bands. Lanes 1 and either 23 (A) or 24 (B) have 100 bp 1adder (Invitrogen). BK: extraction blank; NEG: PCR negative amplification control.

Combined, the results from these standard PCR runs provide a qualitative assessment and comparison of the three different DNA extractions tested in this study. These results indicate that the silica-spin column is the most efficient DNA extraction method for the plant samples tested. However, because of the qualitative nature of these measurements, further testing using qPCR on the same DNA extracts was conducted to

obtain a more accurate and quantitative measurement of how much DNA template was recovered using each extraction method.

Comparison of Extraction Methods using Quantitative PCR

To determine more conclusively whether one extraction method is optimal for corn, pea, and squash or if they require different extraction methods, qPCR was conducted on samples heat treated at 140 ˚C and extracted using the three previously described methods (Figures 19-21). All sample extracts were quantified as both undiluted (1x) and ten times diluted (0.1x) in an effort to identify any effects of PCR inhibition on the recovery of DNA (Kemp *et al.* 2006). DNA template quantity was measured by qPCR amplification of a 166 bp fragment from each extract in duplicate. To facilitate the comparison of results across extraction methods, time points, and individual plant species, relative-to-best values were determined, and then these relative-to-best values were averaged over the five time points (Table 7).

Corn samples yielded DNA from all three extraction methods for the first three time points (0 – 5 hours) with a significant reduction in DNA recovery from 7.5 – 10 hours (Figure 19). For pea samples, the same pattern observed using standard PCR was also observed using qPCR: an overall reduction in DNA recovery at intermediate time points (2.5 – 7.5 hours) from all extraction methods followed by an increase in DNA recovery at 10 hours with the silica-spin column and DNeasy Plant Mini Kit extraction methods (Figure 20). Squash was the only plant species tested that yielded recoverable DNA at all time points using all extraction methods (Figure 21), which may be explained by its more gradual DNA degradation, as noted by our earlier findings.

On average, the silica-spin column method outperformed both the DNeasy Plant Mini Kit and the DTAB/CTAB method, particularly at all time points for the corn and squash samples (Figures 19-21). The DNeasy Plant Mini Kit was the second most efficient DNA extraction method for corn and squash samples based on the average relative-to-best values (Table 7). The DTAB/CTAB method was the least efficient DNA extraction method for corn and squash samples based on the average relative-to-best values (Table 7). The results of a paired Student's *t*-test ($p < 0.05$) indicated that, for the corn and squash samples, the DNeasy Plant Mini Kit and the DTAB/CTAB extraction methods performed significantly worse than the silica-spin column method (Table 7).

The superiority of the silica-spin column method was less pronounced with the pea samples as each extraction method outperformed the other two at least one time point (Figure 20, Table 7). When the average relative-to-best values for the 1x and 0.1x pea samples were combined, the silica spin column method still had a small advantage (0.85) over the next best method, the DNeasy Mini Kit (0.71), followed by the least efficient method, the DTAB/CTAB method (0.21). Unlike the corn and squash relative-to-best values, the pea relative-to-best values did not reach statistical significance.

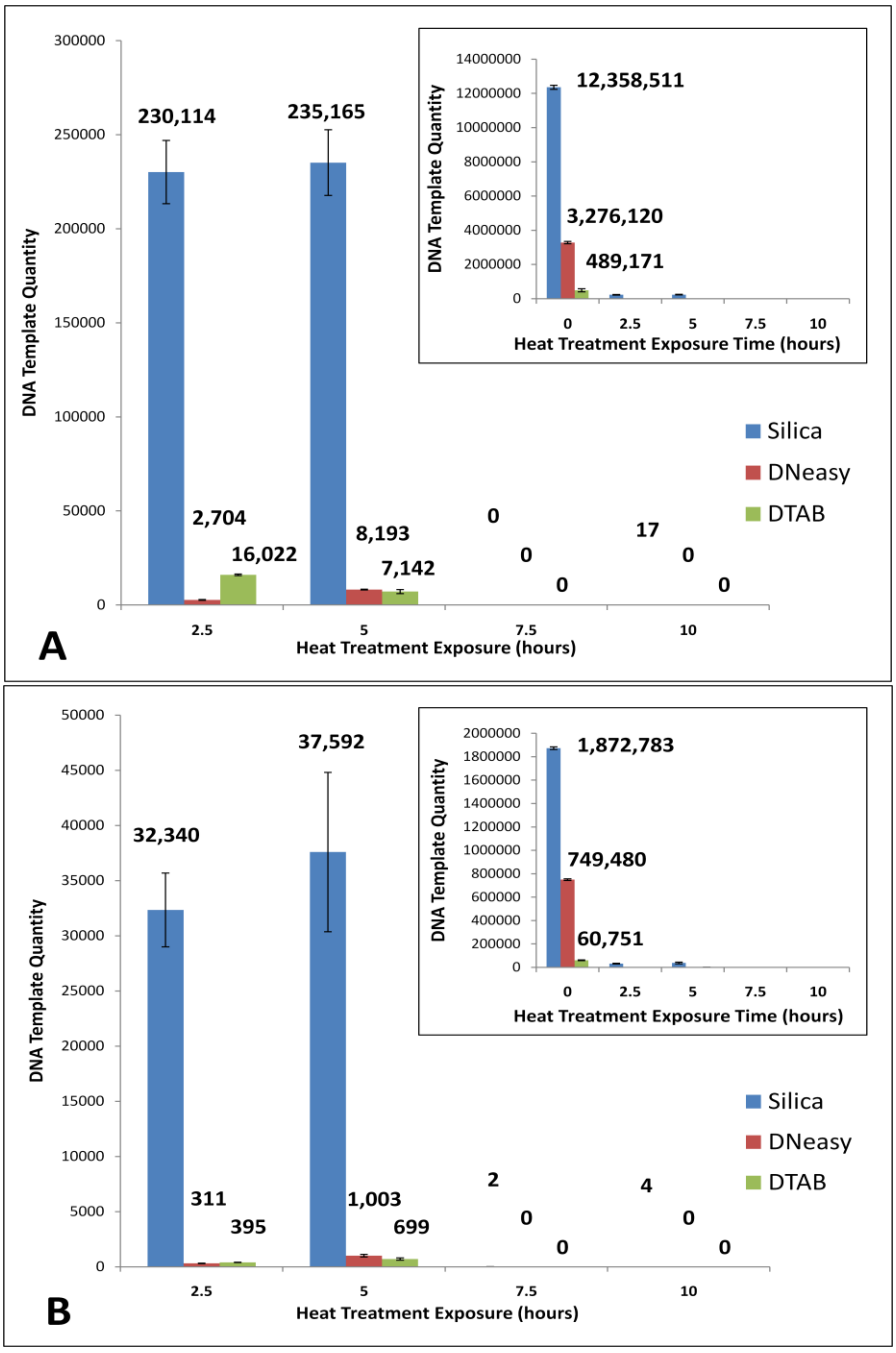

Figure 19: Comparison of efficiency of DNA extraction methods for 140 ˚C heat treated corn samples using qPCR.

DNA was extracted using the silica-spin column method (silica), the DNeasy Plant Mini Kit (DNeasy) and the DTAB/CTAB method (DTAB). The 2.5 to 10 hour time points are highlighted because these samples are no longer at modern DNA levels. Inserts show all data points collected (0-10 hour time points). Error bars indicate standard deviation. A) DNA quantities for undiluted sample extracts. B) DNA quantities for ten times (0.1x) diluted sample extracts.

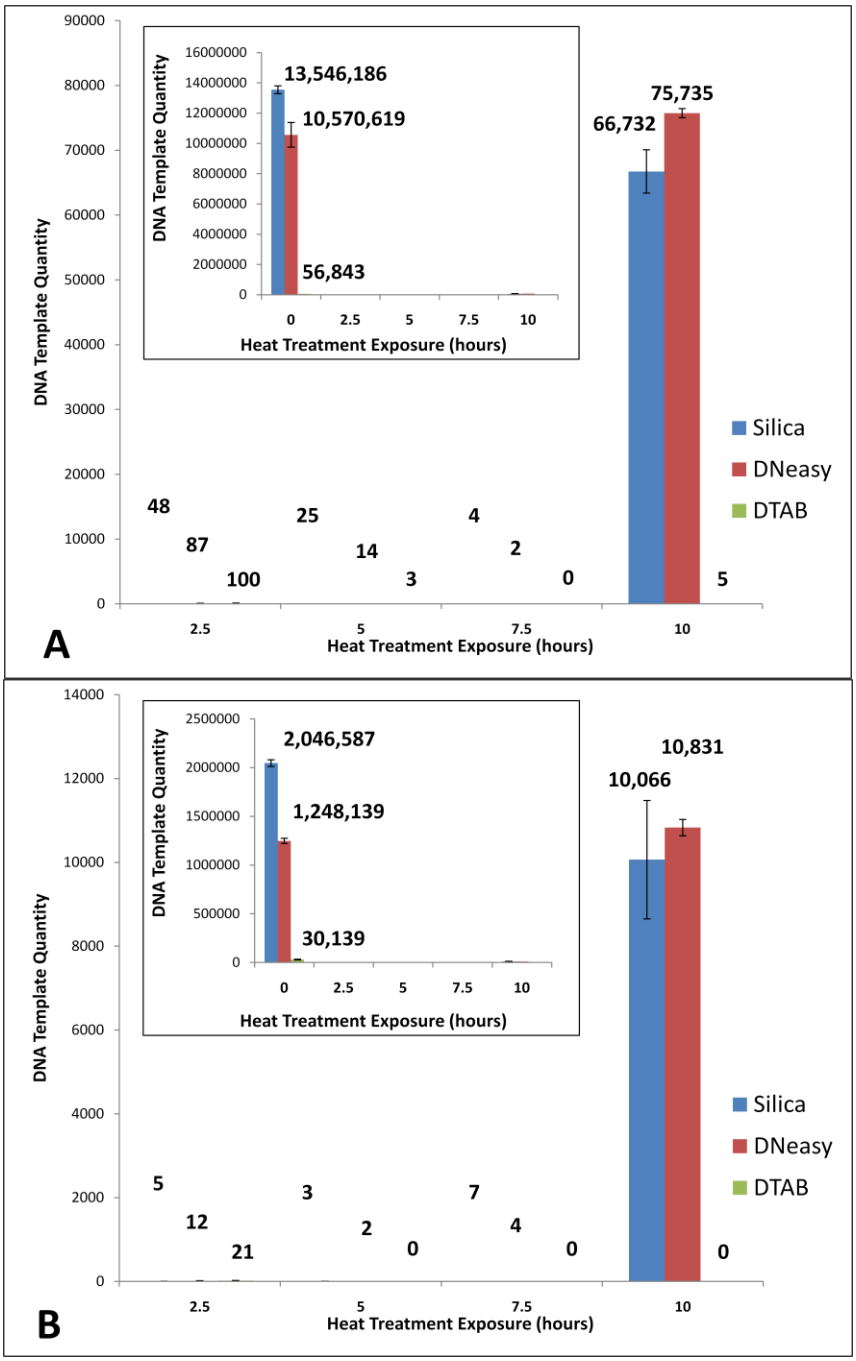

Figure 20: Comparison of efficiency of DNA extraction methods for 140 ˚C heat treated pea samples using qPCR.

DNA was extracted using the silica-spin column method (silica), the DNeasy Plant Mini Kit (DNeasy) and the DTAB/CTAB method (DTAB). The 2.5 to 10 hour time points are highlighted because these samples are no longer at modern DNA levels. Inserts show all data points collected (0-10 hour time points). Error bars indicate standard deviation. A) DNA quantities for undiluted sample extracts. B) DNA quantities for ten times (0.1x) diluted sample extracts.

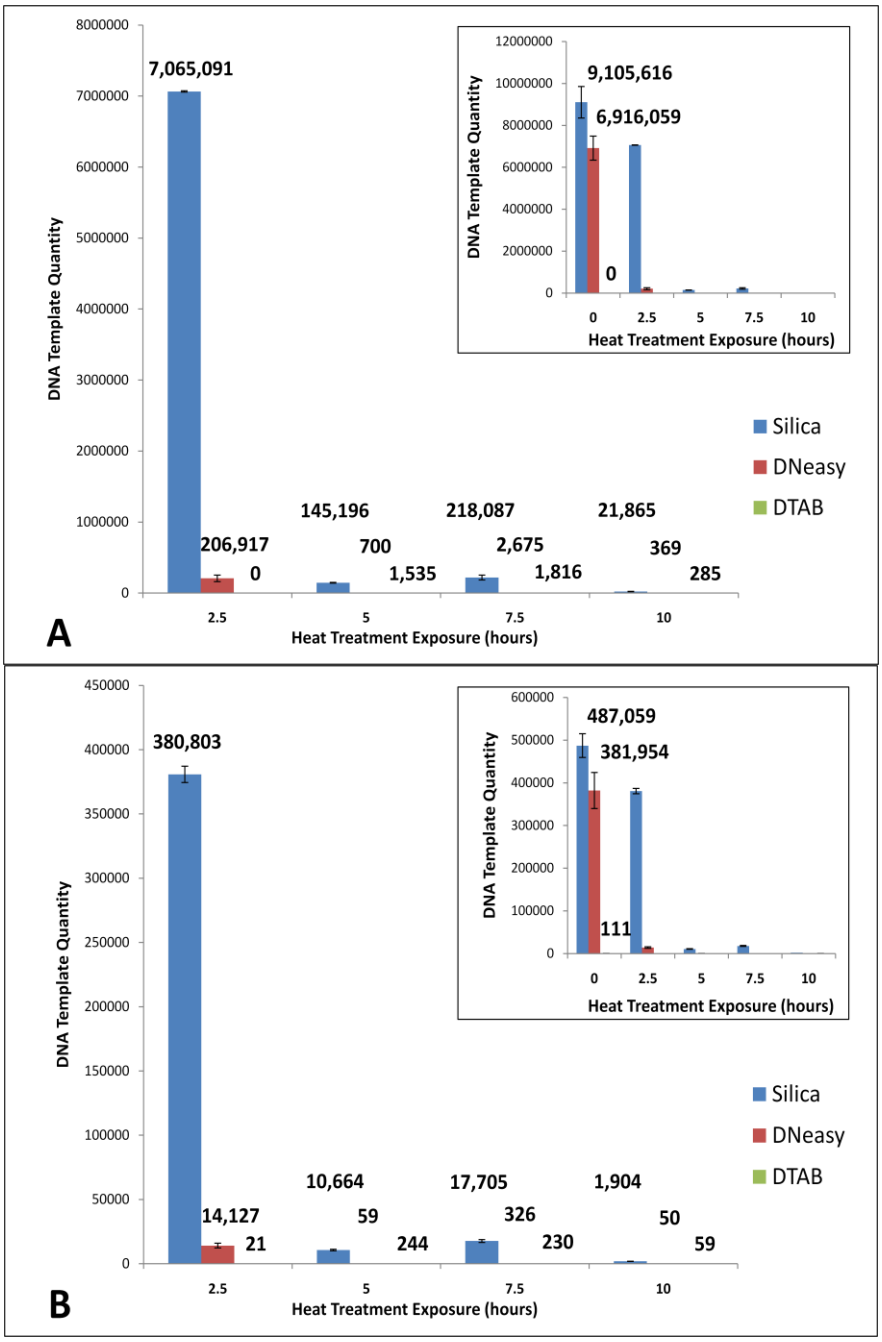

Figure 21: Comparison of efficiency of DNA extraction methods for 140 ˚C heat treated squash samples using qPCR.

DNA was extracted using the silica-spin column method (silica), the DNeasy Plant Mini Kit (DNeasy) and the DTAB/CTAB method (DTAB). The 2.5 to 10 hour time points are highlighted because these samples are no longer at modern DNA levels. Inserts show all data points collected (0-10 hour time points). Error bars indicate standard deviation. A) DNA quantities for undiluted sample extracts. B) DNA quantities for ten times (0.1x) diluted sample extracts.

Table 7: Relative-to-best method values for the 140 ˚C heat treated corn, pea, and squash samples as calculated from the DNA quantities recovered from the undiluted (1x) and ten times diluted (0.1x) sample extracts.

Extraction Method	Dilution	Relative to Best Method					*Average*
		Hours					
		0	*2.5*	*5*	*7.5*	*10*	
Corn							
Silica-spin column	**1x**	1.00	1.00	1.00	N/A	1.00	***1.00***
	0.1x	1.00	1.00	1.00	1.00	1.00	***1.00***
DNeasy Mini Kit	**1x**	0.27	0.01	0.03	N/A	0	***0.08****
	0.1x	0.40	0.01	0.03	0	0	***0.09****
DTAB/ CTAB	**1x**	0.04	0.07	0.03	N/A	0	***0.03****
	0.1x	0.03	0.01	0.02	0	0	***0.01****
Pea							
Silica-spin column	**1x**	1.00	0.48	1.00	1.00	0.88	***0.87***
	0.1x	1.00	0.24	1.00	1.00	0.93	***0.83***
DNeasy Mini Kit	**1x**	0.78	0.87	0.56	0.50	1.00	***0.74***
	0.1x	0.61	0.57	0.67	0.57	1.00	***0.68***
DTAB/ CTAB	**1x**	0.00	1.00	0.12	0	0	***0.22***
	0.1x	0.01	1.00	0	0	0	***0.20***
Squash							
Silica-spin column	**1x**	1.00	1.00	1.00	1.00	1.00	***1.00***
	0.1x	1.00	1.00	1.00	1.00	1.00	***1.00***
DNeasy Mini Kit	**1x**	0.76	0.03	0	0.01	0.02	***0.16****
	0.1x	0.78	0.04	0.01	0.02	0.03	***0.17****
DTAB/ CTAB	**1x**	0	0	0.01	0.01	0.01	***0.01****
	0.1x	0	0	0.02	0.01	0.01	***0.01****

Note: Samples were heat treated at 140 ˚C for various time points, powdered, and then extracted with either the silica-spin column method, the DNeasy Plant Mini Kit, or the DTAB/CTAB method. DNA quantity was determined from both the undiluted (1x) and diluted (0.1x) sample extracts using qPCR amplification of a 166 bp fragment. The DNA quantity from which the relative-to-best values were calculated are provided in figures 19-21 above. Relative-to-best values were calculated based on the DNA template quantity divided by the highest DNA template quantity for each time point. These values were averaged over the five time points. DNA extraction methods that performed significantly worse than the best performing DNA extraction method (using the relative-to-best values) according to a paired Student's *t*-test ($p < 0.05$) are indicated by an asterisk (*). N/A indicates that no extraction method gave any DNA quantity for that time point and therefore no relative-to-best values could be calculated.

To examine whether PCR inhibition had any effect on the different DNA recoveries for the three extraction methods, samples were quantified in both undiluted (1x) and diluted (0.1x) form (Figures 19-21). This was done to investigate the possibility that different extraction methods would perform better when diluted due to the dilution of PCR inhibitors. Similar relative-to-best values were obtained for both the undiluted and ten times diluted samples, indicating that PCR inhibition was not a significant factor in the determination of which extraction method was the most efficient at recovering DNA from artificially degraded corn, pea, and squash samples (Table 7). However, this does not conclusively eliminate the possibility of PCR inhibition, but rather it is possible that PCR inhibitors could be having similar effects across all extraction methods. This suggests that the sample dilution approach of detecting PCR inhibition may not be the most effective method for these particular samples, and a different approach is needed.

Inhibition Testing

To assess the extent of PCR inhibition in the samples extracted using the three different DNA extraction methods, two approaches were used as described above: 1) the shift in the quantification cycle (ΔC_q value) compared to the IPC standard, and corresponding expected recovery values; and 2) the estimated PCR amplification efficiencies, which were determined from the Hill slope values calculated from the raw fluorescence data. First, the expected recovery (ER) values for the sample extracts of corn, pea, and squash (0 - 10 hour time points) were compared for the three different DNA extraction methods (Figure 22). For the corn sample extracts, both the silica-spin column method and the DNeasy Plant Mini Kit had very high ER values at all time points

(ER range = 86 – 100). The corn sample extracts using the DTAB/CTAB method, however, had a much wider range of expected recovery values, and the ER value increased progressively from the 0 hour time point (ER = 17) to the 10 hour time point (ER = 96). For the pea sample extracts, the overall pattern observed for the expected recovery values was much more variable than was corn. Similar to the variability noted in the relative-to-best values in the original pea samples, in the IPC inhibition assay, each extraction method had the highest ER value at one time point and also had the lowest ER value at another time point, highlighting the importance of investigating the effects of PCR inhibition over time rather than at a single time point. For the squash sample extracts, the silica-spin column had the highest ER values for the 0 - 7.5 hour time points. The DTAB/CTAB method had very low or uncalculatable ER values for the squash 0, 2.5 and 7.5 hour time points. It should be noted that none of the PCR negative amplification controls used in the IPC inhibition assay yielded detectable levels of DNA.

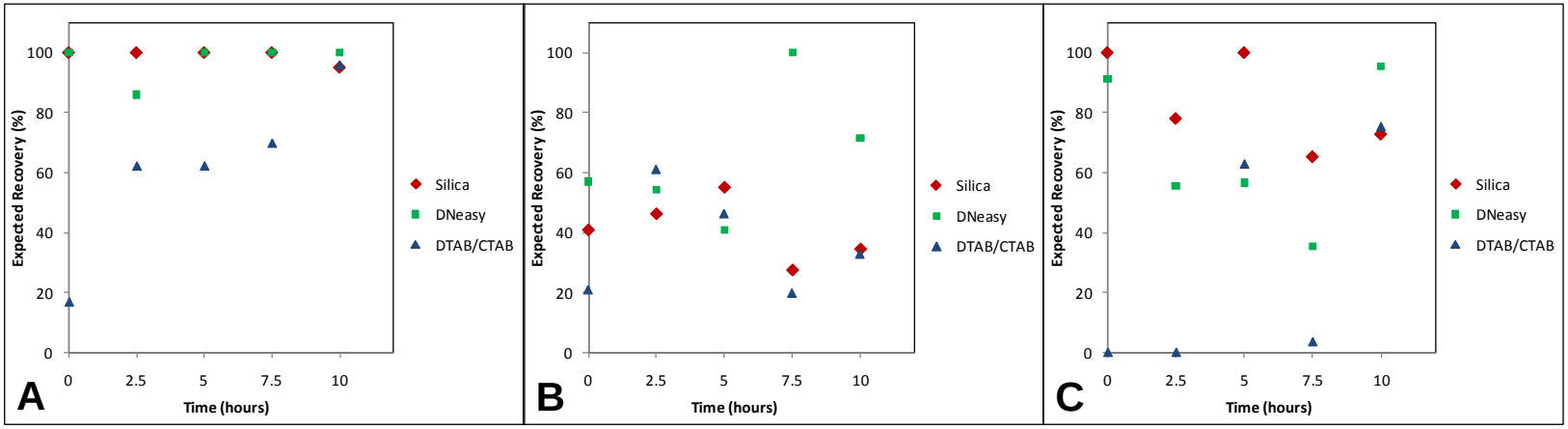

Figure 22: Assessment of PCR inhibition in 140 ˚C heat treated undiluted sample extracts (0 to 10 hour time points) using an Internal Positive Control standard and qPCR.
DNA was extracted using the silica-spin column method (silica), the DNeasy Plant Mini Kit (DNeasy), and the DTAB/CTAB method (DTAB). The extent of PCR inhibition was assessed by the expected recovery (%), which was calculated from the ΔC_q values for the A) corn, B) pea, and C) squash samples. Note that while the squash 0 hour time point extracted by the DTAB/CTAB method resulted in an undetectable amount of IPC standard DNA, it is plotted as an ER value of 0.

To help further compare the ability of the three different extraction methods to remove PCR inhibitors during sample extraction, the average ΔC_q values and amplification efficiencies were calculated from all five time points (Table 8). To better understand what the differences in the average ΔC_q values mean, expected recovery values were calculated from these average values. In addition, the variation in the IPC standard triplicate runs was measured to determine which calculated values for the samples could be attributed to PCR inhibition as opposed to normal variation in the IPC standard itself. The IPC standards had a mean intra-assay variation in C_q (SD_{Cq}) of 0.63 cycles while the mean intra-assay variation in the Hill slope of the standards (CV_{HS}) was 2.6 %. For the ΔC_q values, six of the average values exceeded the variation found in the IPC standards (SD_{Cq} = 0.63 cycles): the corn samples extracted using the DTAB/CTAB method; pea samples extracted using all three of the extraction methods; and squash samples extracted using both the DNeasy Plant Mini Kit and the DTAB/CTAB method (Table 8). For the PCR amplification efficiency, only two average values exceeded the variation found in the IPC standards: the corn samples extracted using the DNeasy Plant Mini Kit (97 %) and the corn samples extracted using the silica-spin column method (92 %).

Table 8: PCR inhibition assessment based on the average values of the Δ C_q and amplification efficiency (%) for the 140 ˚C heat treated 0 to 10 hour time points for corn, pea and squash.

	Average Δ C_q (Expected Recovery %)		
	Average Amplification Efficiency (%)		
Plant Species	Silica	DNeasy	DTAB/CTAB
Corn	0.02 **(99)**	0.04 **(97)**	0.90 **(54)**
	92	97	99
Pea	1.33 **(40)**	0.69 **(62)**	1.60 **(33)**
	98	98	100
Squash	0.28 **(82)**	0.67 **(63)**	5.77 **(2)**
	99	100	100

Note: The squash 0 hour time point for the DTAB/CTAB extracted sample is excluded from these averages as the IPC standard was completely undetected and therefore no expected recovery or amplification efficiency could be calculated. The Expected Recovery (%) value was calculated from the average ΔC_q values. The mean intra-assay variation in C_q and Hill slope values for the triplicate IPC standard reactions was measured to be $SD_{Cq} = 0.63$ and $CV_{HS} = 2.6$ %, respectively.

Despite the large ΔC_q shifts in the DTAB/CTAB extracts, the average amplification efficiency of these samples was very high (99 %, 100 %, and 100 % for corn, pea and squash, respectively). This type of inhibition pattern was also observed by King *et al.* (2009) and they hypothesized that it could be due to the presence of thermolabile PCR inhibitors. These types of inhibitors prevent efficient PCR amplification during the initial cycles and subsequently degrade over the life of the PCR reaction, resulting in an efficient end to the PCR reaction. The average amplification efficiencies for the silica-spin column method were high for the pea (98 %) and squash (99 %) sample extracts and lower for the corn sample extracts (92 %). The average amplification efficiency for the corn silica-spin column sample extracts steadily decreased as the time exposed to 140 ˚C increased, going from 96 % for the 0 hour time point to 84 % for the 10 hour time point. This indicates that there may be a form of PCR inhibitor present in the later time points that is decreasing the PCR amplification efficiency while not increasing the ΔC_q values.

Collectively, the results from the IPC inhibition assay indicates that the silica-spin column method and the DNeasy Plant Mini Kit are effective at removing PCR inhibitors from corn samples, the DNeasy Plant Mini Kit is most effective at removing PCR inhibitors from pea samples, and the silica-spin column method is the most effective extraction method for removing PCR inhibitors from squash samples. Ultimately, while both the ΔC_q values and the amplification efficiencies should be taken into consideration when selecting which method is best suited for extraction of a particular sample, we place greater emphasis on the ΔC_q values in the current study because most samples demonstrated very high amplification efficiencies.

DNA Sequencing

A random selection of amplified samples was sequenced to confirm that the obtained DNA sequences correspond with the expected sequences. DNA sequences that were of sufficient quality (*i.e.,* signal not too low) were manually edited in Chromas Pro software (www.technelysium.com.au), and then DNA sequences BLAST searched (http://www.ncbi.nlm.nih.gov/BLAST/). Nine out of the ten BLAST searched sequences yielded DNA sequences consistent with the expected sequences. The one sample which yielded an unexpected sequence (squash 140 ˚C 5 hour time point) is likely the result of non-specific PCR amplification.

Repeatability and Reproducibility Tests

To confirm the validity of the results obtained in this study, one time point for each plant species was selected for additional testing. For the repeatability test, new seeds were artificially degraded at 140 ˚C as before, and the samples were then extracted five

times with each of the three described methods. Additionally, for the reproducibility test, the three plant species were also extracted three times each with the three described extraction methods by another qualified laboratory researcher within our laboratory. Then, either the undiluted (1x) DNA or diluted (0.1x) sample extracts were quantified in duplicate using qPCR as described above.

The results from the repeatability tests showed similar DNA recovery trends to our original findings and confirm that the silica-spin column method is the most efficient of the three extraction methods investigated in this study, as demonstrated by the highest relative-to-best values for all three species (Figure 23, Table 9). In addition, the average relative-to-best values obtained for corn and squash replicates were virtually identical to those obtained in the original experiment at the 5 hour time point (Tables 9 and 11). However, we were not able to reproduce the slight advantage of the DNeasy Plant Mini Kit over the silica-spin column method that was observed for the original 10-hour time point pea samples. In the five pea replicates in the repeatability test, the silica-spin column method outperformed the DNeasy Plant Mini Kit with average relative-to-best values of 1.00 and 0.20, respectively.

In addition to determining which extraction methods would recover the most DNA, replicating the extraction methods five times allowed for a measurement of the variation in DNA template recovery between replicates in the three different extraction methods and for the three different plant species. The amount of variation in each dataset, the coefficient of variance (COV), was calculated by dividing the standard deviation from the mean DNA template number for each extraction method and each plant species (Table 9). The silica-spin column method had the lowest variation between replicates for

the corn and squash samples with COV values of 14 and 6, respectively. The DTAB/CTAB method was the most variable for these samples with COV values of 120 and 157 for corn and squash, respectively. This pattern was reversed, however, for the pea samples as the silica-spin column was the most variable with a COV value of 45 while the DTAB/CTAB method and the DNeasy Plant Mini Kit had COV values of 25 and 15, respectively.

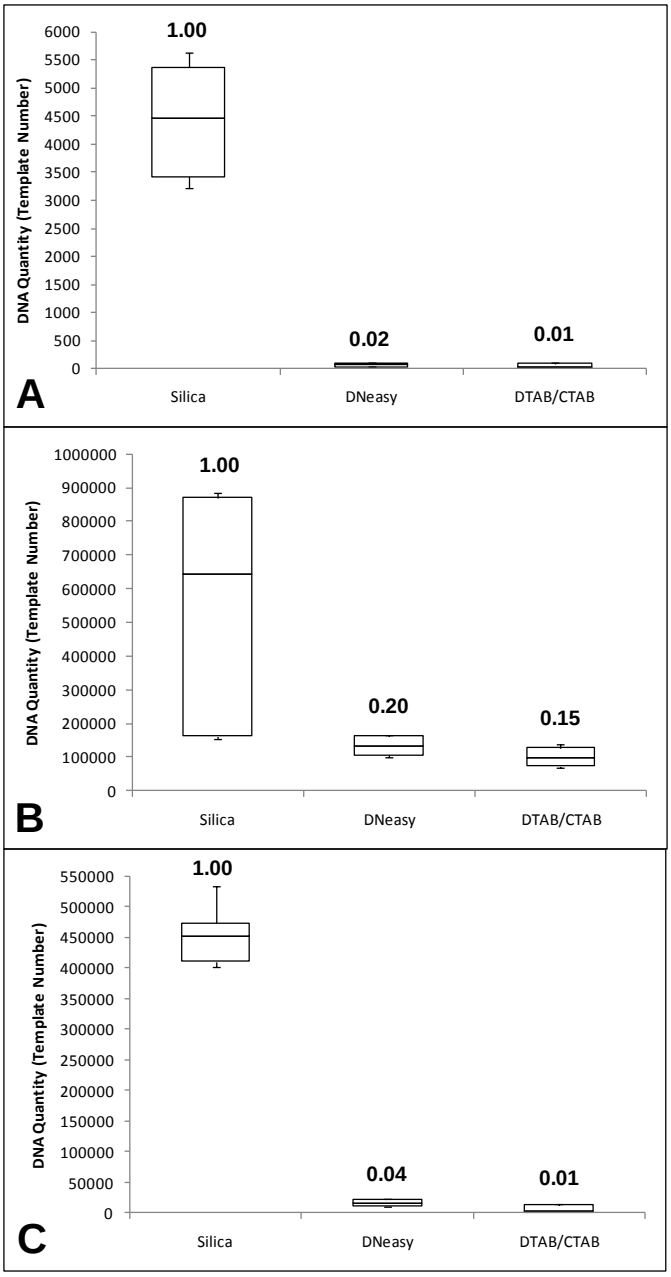

Figure 23: Repeatability test - comparison of DNA extraction method efficiency for 140 ˚C heat treated corn, pea, and squash samples.

Heat treated (140 ˚C) A) corn (5 h), B) pea (10 h), and C) squash (5 h) samples were extracted five times with each DNA extraction method (Silica, DNeasy, and DTAB/CTAB). The DNA quantity of each undiluted (1x) sample extract was quantified in duplicate using qPCR (166 bp fragment), and DNA quantities from the replicates were averaged. Box plots indicate the range of DNA quantities obtained for the five replicates with the middle line indicating the averaged value. Error bars indicate the maximum and minimum possible DNA quantities given the standard deviation of the highest and lowest values, respectively. In addition, the relative-to-best values for each extraction method are indicated above each box.

Table 9: Repeatability test for the comparison of DNA extraction methods.

Extraction Method	Silica-spin Column	DNeasy Plant Mini Kit	DTAB-CTAB
Corn 5h (140 ˚C)			
Avg. DNA Quantity of Replicates	***4456***	***68****	***29****
Standard Deviation	620	16	35
Coefficient of Variance	*14*	*23*	*120*
Relative to Best Method	**1.00**	**0.02**	**0.01**
Pea 10h (140 ˚C)			
Avg. DNA Quantity of Replicates	***643209***	***131809***	***97066***
Standard Deviation	286696	20113	23904
Coefficient of Variance	*45*	*15*	*25*
Relative to Best Method	**1.00**	**0.20**	**0.15**
Squash 5h (140 ˚C)			
Avg. DNA Quantity of Replicates	***451559***	***15865****	***3569****
Standard Deviation	25236	4496	5622
Coefficient of Variance	*6*	*28*	*157*
Relative to Best Method	**1.00**	**0.04**	**0.01**

Notes: DNA was extracted from each plant species five times using each extraction method. Undiluted (1x) extracts were quantified using qPCR in duplicate. Average DNA quantity values were determined from duplicate reactions for each of the five undiluted extract replicates. These DNA quantity values for all five replicates were then averaged to obtain the reported average value for all replicates. The standard deviation was calculated from the five replicate DNA quantities. The coefficient of variance was calculated by dividing the standard deviation by the average DNA quantity. Relative-to-best values were calculated from the mean DNA template number. DNA extraction methods that performed significantly worse than the best performing DNA extraction method (using the DNA template quantities), according to a paired Student's *t*-test ($p < 0.05$) are indicated by an asterisk (*).

The results from the reproducibility test using the DNA quantities from the 0.1x diluted samples provide further confirmation that the silica-spin column method is the most efficient of the three extraction methods investigated in this study (Figure 24, Table 10). The average relative-to-best values obtained for corn and squash replicates were very similar to those obtained both in the original extractions and in the repeatability tests

(Table 11). However, the relative-to-best values for the pea reproducibility tests were more similar to the results of the repeatability test than the results from the original pea DNA extractions at this time point.

For the pea and squash samples, the DNeasy Plant Mini Kit yielded the least amount of variation between replicates during the reproducibility test, as indicated by the lowest COV values. The DTAB/CTAB method, on the other hand, showed the most variability in DNA recovery from these samples. The corn COV values indicated that the silica-spin column method had the least variability, followed by the DNeasy Plant Mini Kit. Because no DNA was recovered from the corn samples using the DTAB/CTAB method, no COV values could be calculated. However, it should also be noted that all of the methods yielded very little DNA recovery for the corn samples, which would affect those particular COV values. Based on the general pattern obtained from the COV values, the DTAB/CTAB method is likely more variable in the amount of DNA recovered from sample to sample than either of the silica-spin column method or the DNeasy Plant Mini Kit.

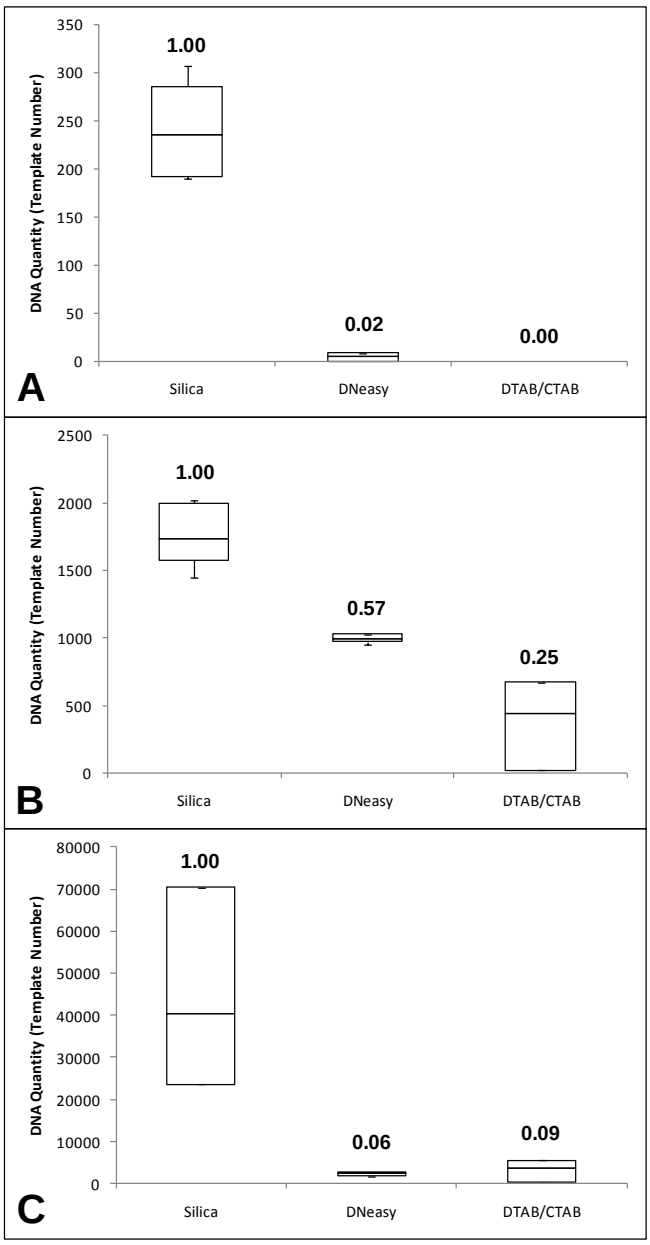

Figure 24: Reproducibility test - comparison of DNA extraction method efficiency for 140 ˚C heat treated corn, pea, and squash samples.
Heat treated (140 ˚C) A) corn (5 h), B) pea (10 h), and C) squash (5 h) samples were extracted three times with each DNA extraction method (Silica, DNeasy, and DTAB/CTAB). The DNA quantity of each ten times diluted (0.1x) sample extract was quantified in duplicate using qPCR (166 bp fragment), and DNA quantities from the replicates were averaged. Box plots indicate the range of DNA quantities obtained for the three replicates with the middle line indicating the averaged value. Error bars indicate the maximum and minimum possible DNA quantities given the standard deviation of the highest and lowest values, respectively. In addition, the relative-to-best values for each extraction method are indicated above each box.

Table 10: Reproducibility test for the comparison of DNA extraction methods.

Extraction Method	Silica-spin Column	DNeasy Plant Mini Kit	DTAB-CTAB
Corn 5h (140 ˚C) (0.1x)			
Avg. DNA Quantity of Replicates	***236***	***6***	***0***
Standard Deviation	47	5	N/A
Coefficient of Variance	*20*	*89*	*N/A*
Relative to Best Method	**1.00**	**0.02**	**0**
Pea 10h (140 ˚C) (0.1x)			
Avg. DNA Quantity of Replicates	***1735***	***995***	***440***
Standard Deviation	232	29	363
Coefficient of Variance	*13*	*3*	*82*
Relative to Best Method	**1.00**	**0.57**	**0.25**
Squash 5h (140 ˚C) (0.1x)			
Avg. DNA Quantity of Replicates	***40395***	***2321***	***3620***
Standard Deviation	26012	404	2868
Coefficient of Variance	*64*	*17*	*79*
Relative to Best Method	**1.00**	**0.06**	**0.09**

Notes: DNA was extracted from each plant species three times using each extraction method. Diluted extracts (0.1x) were quantified using qPCR in duplicate. Average DNA quantity values were determined from duplicate reactions for each of the three undiluted extract replicates. These DNA quantity values for all three replicates were then averaged to obtain the reported average value for all replicates. The standard deviation was calculated from the three replicate DNA quantities. The coefficient of variance was calculated by dividing the standard deviation by the average DNA quantity. Relative-to-best values were calculated from the mean DNA template number. None of the DNA extraction methods performed significantly worse than the best performing DNA extraction method (using the DNA template quantities), according to a paired Student's *t*-test ($p < 0.05$).

Collectively, the relative-to-best values for the original extractions, the repeatability test extractions, and the reproducibility test extractions demonstrate a pattern wherein the silica-spin column method is the most efficient method at recovering DNA from the 140 ˚C heat treated corn (5 hour time point), pea (10 hour time point), and squash (5 hour time point) samples (Table 11). The only sample and time point where the silica-spin column did not have a relative-to-best value of 1.00 was the original pea

10-hour time point (undiluted and 0.1x dilution) in which the DNeasy Plant Kit slightly outperformed the silica-spin column.

Table 11: Comparison of the relative to best values for all DNA extractions of the corn 5 hour, pea 10 hour, and squash 5 hour time points.

Relative to Best Values (Mean DNA Template Quantities)				
Extraction Method	Original (1x)	Original (0.1x)	Repeatability (1x) 5 Replicates	Reproducibility (0.1x) 3 Replicates
Corn 5h (140 C)				
Silica-spin column	**1.00** (235165)	**1.00** (37592)	**1.00** (4456)	**1.00** (236)
DNeasy Plant Mini Kit	**0.03** (8193)	**0.03** (1003)	**0.02** (68)	**0.02** (6)
DTAB-CTAB	**0.03** (7142)	**0.02** (699)	**0.01** (36)	**0** (0)
Pea 10h (140 C)				
Silica-spin column	**0.88** (66732)	**0.93** (10066)	**1.00** (643209)	**1.00** (1735)
DNeasy Plant Mini Kit	**1.00** (75735)	**1.00** (10831)	**0.20** (131809)	**0.57** (995)
DTAB-CTAB	**0** (5)	**0** (699)	**0.15** (97006)	**0.25** (440)
Squash 5h (140 C)				
Silica-spin column	**1.00** (145196)	**1.00** (10664)	**1.00** (451559)	**1.00** (40395)
DNeasy Plant Mini Kit	**0** (700)	**0.01** (59)	**0.04** (15865)	**0.06** (2321)
DTAB-CTAB	**0.01** (1535)	**0.02** (244)	**0.01** (4462)	**0.09** (3620)

Notes: All relative-to-best method values were calculated from the average DNA template quantities (in brackets) which were determined by using real-time PCR. The average DNA template numbers for the repeatability and reproducibility were averaged from five and three extraction replicates, respectively.

Contamination Controls

A number of controls were included in this study to identify possible instances and sources of contamination. Extraction blanks were included for each extraction method and one or more PCR negative amplification controls were set up for each

standard and qPCR run. All of the 140 ˚C extraction blanks for all three extraction methods including the original, repeatability, and reproducibility experiments showed no contamination when tested in duplicate with qPCR. The two 85 ˚C extraction blanks at 0.1x dilution yield very low contamination levels of 2.20 and 3.44 templates, respectively. In addition, 30 of the 32 PCR negative amplification controls tested using qPCR in this study yielded no detectable DNA. Of the two PCR negative amplification controls that did yield low levels of contamination, the amount of DNA present was estimated to be approximately 5-10 templates per reaction (with an average C_q value of 38.43). For both of these two minimally contaminated controls, which were detected in different amplification batches, there was at least one other negative amplification control in each batch that did not yield any detectable DNA, suggesting the presence of low levels of sporadic rather than systematic contamination. Of note, the Applied Biosystems StepOne™ real-time PCR instrument has a demonstrated sensitivity of 10 DNA copies, indicating values below 10 DNA templates may not be reliable.

Chapter Summary

DNA degradation patterns were generated by heat treating corn, pea, and squash samples at 85 ˚C, 140 ˚C, or 200 ˚C in an attempt to simulate the limited amounts of degraded DNA found in ancient macro-botanical remains. DNA degradation patterns at 85 ˚C and 200 ˚C were determined to be too slow and too rapid, respectively to be of use for further comparison of extraction methods. Because DNA degradation patterns suitable for further experimentation were obtained at 140 ˚C from 0-10 hours for corn, pea, and squash samples, comparison of extraction methods using each of the three plant species was conducted under these conditions. The silica-spin column method was found

to be more efficient at recovering DNA for corn, pea, and squash based on both standard and qPCR results. Calculated relative-to-best values indicate that for the corn and squash samples, the silica-spin column method performed significantly better than the other two methods based on paired Student's *t*-tests. The superiority of DNA recovery in the silica-spin column method was confirmed using both repeatability and reproducibility tests.

The extent to which the three extraction methods removed PCR inhibitors was assessed using an IPC qPCR assay. These results indicate that the DTAB/CTAB extraction method was the worst at removing PCR inhibitors from all three plant species and that the most effective method was either the silica-spin column method or the DNeasy Plant Mini Kit depending on the species.

Chapter 5: Discussion

While the study of ancient plant DNA can and has been used to investigate migration, trade, plant domestication, regional vegetation, and diet, extensive application of this field of inquiry to address these and other important archaeological questions is limited largely by technical challenges. Unlike the study of ancient human and faunal remains, which has benefited from both the homogeneous nature of samples and comprehensive comparisons of extraction methodology, plant aDNA studies samples are more heterogeneous in terms of internal structures and no comprehensive comparison of methods on multiple plant species has ever been conducted. To address these issues, the current study used heat treatment to artificially degrade modern corn, pea, and squash samples to simulate the limited amounts of degraded DNA associated with archaeological macro-botanical remains. We then compared DNA recovery efficiencies and abilities to remove PCR inhibitors of three commonly used DNA extraction methods: the silica-spin column method; a commercially available kit, DNeasy Plant Mini Kit; and the DTAB/CTAB method.

Plant DNA Degradation Patterns

In order to identify the experimental conditions that would generate artificially degraded DNA suitable for comparison of the three extraction methods, macro-botanical samples were exposed to three different heat treatment temperatures. Because artificial DNA degradation is a function of both temperature and time, there may be multiple

experimental conditions that can facilitate the generation of a useful DNA degradation pattern. Our results indicated that 85 ˚C and 200 ˚C were not suitable temperatures for artificial degradation of DNA for corn, pea and squash. A temperature of 85 ˚C was found to be unsuitable because the resulting DNA was not significantly degraded at this lower temperature while 200 ˚C was eliminated because of rapid and complete DNA degradation at this higher temperature. In this study, suitable degradation patterns were successfully generated at 140 ˚C from 0 to 10 hours. While corn and pea had significantly higher starting DNA template quantities than did squash samples, both degraded more rapidly during the first 2.5 hours compared to squash. By 5 hours, all samples were greater than 96 % degraded from the initial starting DNA template quantities. Our findings are in general agreement with other artificial degradation studies that have reported rapid initial DNA degradation followed by a plateau phase (Dobberstein *et al.* 2008; McGrath 2010; Threadgold and Brown 2003). Furthermore, our observation that corn and pea had more starting DNA than squash seeds was expected because dried fruits, which have cotyledons (corn has one and pea has two), have more DNA than seeds, which have reduced cotyledons (Kasem *et al.* 2008). However, the different number of cotyledons between corn and pea had no limited effect on the starting quantities of DNA templates in our study. Finally, our finding that corn, pea, and squash samples yielded different DNA degradation patterns suggests that the plant structure itself may be providing some level of protection to the DNA. This observation could be further confirmed and expanded by investigating an even wider range of plant species with varying plant structures.

It should be noted that the DNA template quantities reported here, as in all such studies, do not necessarily reflect the true levels of DNA in the artificially degraded samples, but rather the ability of the respective extraction methods to recover DNA from said samples. In a sense, the DNA degradation curve for any controlled study will depend on the efficiency of the extraction method at recovering limited amounts of DNA, and its ability to remove PCR inhibitors. It is possible that little or no DNA would be recovered from a particular sample using one inefficient extraction method, but a second more efficient method may be able to recover DNA from the same sample. This underscores the importance of using the most efficient extraction method to develop the most realistic DNA degradation patterns possible and provides the rationale for this study.

Different DNA Recovery Efficiency from DNA Extraction Methods

As discussed above, the successful recovery of ancient DNA from archaeological plant remains can be hampered by the use of inefficient DNA extraction methods and the presence of PCR inhibitors. Thus, identification of the most efficient extraction method and modifications to improve individual methods may lead to increased recovery of ancient DNA from plant remains. In the current study, the abilities of three different extraction methods to recover DNA from artificially degraded plant samples are compared. One possible outcome was that degraded DNA from different plant species might require different extraction methods for optimal recovery, possibly due to variations in plant internal structures. However, our results indicate that the silica-spin column method was the most efficient extraction method in terms of DNA recovery for all three plant species tested while the DNeasy Plant Mini Kit and the DTAB/CTAB method ranked a distant second and third place, respectively. This suggests that, in

addition to being the universally best method for ancient faunal remains (Rohland and Hofreiter 2007), a silica-based extraction method (silica-spin column or silica slurry) may also be most suited for all degraded plant remains. While this study examined corn, pea, and squash samples, as representatives of the cereal, legume, and cucurbit plant families, expansion of this study to include other plant types as well as true ancient plant remains is recommended before we can unequivocally determine if the silica-spin column method is the universally best method for ancient plant remains.

In an effort to provide an explanation for the observed differences in efficiency of DNA recovery, technical differences between the three extraction methods were identified (Table 12). The amount of force used during the sample grinding process with a mortar and pestle can result in differences in recovery of DNA (Kasem *et al.* 2008). In an effort to minimize this source of variability, all plant samples were ground into fine powder using a cryogenic mill for the same length of time and at the same rate. Thus, variability in DNA recovery rates between extraction methods was expected to be due primarily to differences in the cell lysis and DNA recovery phases of the three methods. The differences in the three lysis protocols include 1) the length of lysis time; 2) the reagent and sample mixing method (inversion *vs.* continual rotation); 3) the ratio of lysis buffer to plant powder; 4) the DNA isolation method; and 5) the method of protein removal (Table 12). In particular, the dissimilarities between the extraction methods during the concentration and purification of the DNA released from the lysed plant cells may play an important role in determining the overall effectiveness of the extraction methods. While this step involves a silica-spin column for both the DNeasy Plant Mini

Kit and the silica-spin column method (although with different buffers and steps), the DTAB/CTAB method involves alcohol precipitation for DNA recovery.

Table 12: Summary of differences during the extraction and DNA isolation phases of the three tested extraction methods.

	Extraction Method		
Protocol Variable	**Silica-Spin Column**	**DNeasy Plant Mini Kit**	**DTAB/CTAB**
Lysis Time	Overnight	10 min	30 min
Lysis Temperature	50 ˚C	65 ˚C	65 ˚C
Lysis Mixing	Constantly rotating	2-3 inversions	2-3 inversions
Lysis Volume	2000 or 3000 µL	600 µL	700 µL
Lysis/Powder Ratio	80 or 120 µL/mg	24 µL/mg	28 µL/mg
Isolation Method	Silica-spin column	Silica-spin column	Alcohol precipitation
Protein Removal	Proteinase K, filtration	Buffer precipitation	Phenol-chloroform

Note: The lysis volume tested for the DNeasy Plant Mini Kit was increased to 600 µL for this study from the manufacturer's recommend 400 µL (which would have resulted in a lysis/powder ratio of 16 µL/mg). The lysis volume used in the silica-spin column extractions for the original comparison of methods was 2000 µL while the lysis buffer volume for the repeatability and reproducibility tests was 3000 µL of lysis buffer.

The DTAB/CTAB method, the most inefficient method identified in our study, is one of a number of published modifications of the CTAB method, the most commonly used DNA extraction method for modern plant samples (Kasem et al. 2008; Weising et al. 2005). Due to the relative abundance of DNA in modern plant samples, the CTAB method usually recovers sufficient quantities of DNA for most researchers' applications regardless of the method's DNA recovery efficiency. However, the results of the current study clearly indicate that there are more efficient extraction methods available, which may be critical for the successful recovery of limited amounts of degraded DNA from ancient plant remains. We attribute the overall poor performance of the DTAB/CTAB method compared to the most efficient method, the silica-spin column method, to a

combination of sub-optimal DNA isolation method (alcohol precipitation) and inefficient cell lysis conditions. We further speculate that any differences observed between the DNA recovery efficiencies between the DTAB/CTAB method and the DNeasy Plant Mini Kit, although small, were likely the result of differences in the DNA isolation methods - alcohol precipitation and silica-spin column, respectively - as the cell lysis conditions of both extraction methods were relatively similar.

In the current study, the second most efficient extraction method was the DNeasy Plant Mini Kit. This commercially available kit has a number of advantages over the other two methods: it can be completed in less time than the other two methods; it has an additional step to prevent plant material from clogging the silica-spin column; and the lysis buffer contains RNase to prevent RNA contamination, which can inhibit PCR (Kasem *et al.* 2008; Qiagen DNeasy Plant Handbook 2006). While this method consistently ranked higher than the DTAB/CTAB method, with the exception of the single time point used for the squash reproducibility test, the differences in DNA recovery efficiencies between these two methods were not statistically significant. As discussed above, we speculate that the trend toward superior performance of the DNeasy Plant Mini Kit over the DTAB/CTAB method is likely due to differences in the DNA isolation method. On the other hand, the DNeasy Plant Mini Kit was less efficient at recovering DNA than was the silica-spin column method. We attribute this inferior performance primarily to differences in the cell lysis conditions of the two methods because both methods use a silica-spin column for DNA isolation from lysed cells. An additional disadvantage of the DNeasy Plant Mini Kit is the relatively limited amount of starting material recommended by the manufacturer (20 mg for dry plant material). The

DNeasy Plant Mini Kit is designed to handle small quantities of modern plant powder and thus, according to the manufacturer, increases in starting plant material may not yield improved DNA recovery rates (Qiagen DNeasy Plant Handbook 2006). Furthermore, while the recommended 20 mg of starting dried plant powder may be enough powder to result in the successful recovery of DNA from modern plants, it is likely that ancient plant remains with limited amounts of fragmented DNA may require more starting plant powder.

Ultimately, we found that the silica-spin column method, which is already considered the universally best method for DNA extraction from faunal remains, was also the most efficient at extracting artificially degraded plant DNA from all species tested in our study. This finding agrees with a previous study, which also found that a modified silica-spin column method outperformed the DTAB/CTAB extraction method for artificially degraded wheat seeds (Giles and Brown 2008). While the DNeasy Plant Mini Kit also uses a silica-spin column for isolation of DNA from lysed cells, the modified silica-spin column method used in this study (Yang *et al.* 1998; Yang *et al.* 2004) has the added advantage of being readily adaptable to a variety of experimental conditions: lysis buffer volume can be increased; more plant material can be used; and additional reagents including PTB can potentially be included in the lysis to improve DNA recovery rates. In addition, this method has the highest lysis buffer to plant powder ratio of the three methods examined in this study, has an extended lysis time, and is the only method that contains proteinase K, which facilitates protein degradation. However, this extraction method is the lengthiest of the three tested here, requiring at least two days for completion. However, while it may not be necessary for the majority of modern plant

samples, our findings suggest that the extra investment of time in this method may increase the chances of successful DNA recovery from ancient plant remains.

A study by Schlumbaum *et al.* (2008b) used both the CTAB method and the DNeasy Plant Mini Kit to extract DNA from ancient apple seeds. The results from this study, which is the only study on ancient plant remains to report directly on the efficiency of the extraction methods used, indicated that the DNeasy Plant Mini Kit outperformed the CTAB method. Schlumbaum *et al.* (2008b) found that no DNA was recovered from six apple seed fragments samples using a CTAB extraction, but they were able to recover amplifiable nuclear DNA from two out of four of the same samples when using the DNeasy Plant Mini Kit. Of note, the authors admittedly had a small sample number and did not include the silica-spin column method in their comparison. They also did not quantify recovered DNA using qPCR. Despite these limitations, this study highlights the importance of selecting an optimal extraction method for plant aDNA studies. In general, the testing of ancient plant material with different extraction methods and explicit reporting of the results from all extraction methods tested can facilitate our understanding of which extraction methods are most appropriate for future ancient plant DNA studies, and can help corroborate findings from studies using artificially degraded plant remains. While extraction of ancient plant remains with two different extraction methods has been conducted as a part of other studies (Blatter *et al.* 2002a; Elbaum *et al.* 2006; Pollmann *et al.* 2005), this approach has been strictly for authentication purposes, and only the overall success rates of PCR amplification have been reported. In addition, no study to date has compared the efficiency of DNA recovery for the silica-spin column, DNeasy Plant Mini Kit or the DTAB/CTAB extraction methods using ancient plant remains.

Our study, in conjunction with the studies of Giles and Brown (2008) and Schlumbaum *et al.* (2008b), confirms that silica-spin column-based methods - either the silica-spin column method itself or the DNeasy Plant Mini Kit - are more efficient at recovering DNA from degraded plant samples than are CTAB-based methods. It should be noted that, collectively, these three studies examined a diverse range of plant species including corn, pea, squash, wheat, and apple, from either ancient or artificially degraded plant materials. In addition, while the heat treatment studies conducted by our group and Giles and Brown (2008) simulated the charring process, the ancient apple seeds tested by Schlumbaum *et al.* (2008b) were preserved waterlogged. This suggests that the type of plant preservation (*e.g.,* waterlogged *vs.* charred) may not play a significant role in determining the most efficient extraction methods. Ultimately, the identification of the silica-spin column-based methods as the most efficient extraction method is all the more relevant in light of the varied sample types and experimental conditions of these studies. Our study adds to the previous body of literature by indicating that, of the two described silica-based methods, the silica-spin column method is superior to the DNeasy Plant Mini Kit.

Removal of PCR Inhibitors by the Three DNA Extraction Methods

Because optimizing DNA recovery during the extraction process is futile if subsequent PCR is unsuccessful due to excessive amounts of PCR inhibitors, inhibition testing is a critical component of comparing the overall efficiency of different DNA extraction methods. In the current study, an Internal Positive Control (IPC) qPCR assay was designed to quantitatively assess the amount of PCR inhibitors present in the sample extracts for the three DNA extraction methods. This analysis allowed for the comparison

of the effectiveness of the silica-spin column method, the DNeasy Plant Mini Kit, and the DTAB/CTAB method in removing PCR inhibitors during the extraction process. In addition, as the corn, pea, and squash samples could contain different types and levels of PCR inhibitors, conducting IPC inhibition assays on all three species was important. While there are other methods that can be used to assess PCR inhibition (*e.g.,* dilution of samples), there are a number of advantages to using an IPC qPCR assay including: assessment of PCR inhibition regardless of the amount of DNA templates present in the sample extract (which is not possible for sample dilution with limited DNA template amounts); the measurement of PCR inhibition by two approaches (PCR efficiency as calculated by the Hill slope and the shift of ΔC_q values); and the detection of different forms of PCR inhibition as a result of these two measurements (*e.g.,* thermolabile or non-thermolabile inhibitors). There are also, however, some disadvantages to this method that must be considered: it is assumed that the PCR inhibitors are equally affecting the IPC reaction and the other qPCR or standard PCR reactions; and inhibitory compounds may prevent the IPC probe from binding to the control DNA without negatively affecting the standard PCR reaction.

A study by King *et al.* (2009) effectively used an IPC qPCR inhibition assay to detect and quantify PCR inhibitors in different sample types (*i.e.,* bone, feces, hair, and soil). This allowed them to determine the effectiveness of adding PCR facilitators (*i.e.,* BSA and additional Taq enzyme) and compare the effectiveness of different extraction methods to remove PCR inhibitors from soil samples (King *et al.* 2009). While the current study did not investigate the effects of PCR facilitators on the amplification of DNA from artificially degraded plant samples, we did test the ability of the various DNA

extraction methods to remove PCR inhibitors from these samples. The inhibition results for the corn samples indicate that both the silica-spin column method and DNeasy Plant Mini Kit were more efficient at removing PCR inhibitors than the DTAB/CTAB method. When combined with the limited amounts of recoverable DNA (0 – 17 templates) at the 7.5 and 10 hour time points, these inhibition results indicate that the loss of recoverable DNA compared to the earlier time points was the result of DNA degradation by heat treatment rather than the presence of PCR inhibition. Interestingly, as the heat treatment of corn samples progressed over time, the expected recovery values for the DTAB/CTAB steadily increased, yet the PCR amplification for the silica-spin column method decreased. This may indicate that these two extraction methods were affected by different forms of PCR inhibitors with opposing effects – thermolabile inhibitors in the DTAB/CTAB extracted samples may be degrading as the heat treatment progresses while a different form of inhibitor present in the silica-spin column extracted samples may be increasing as the heat treatment progresses.

The inhibition results for the squash samples indicate that, on average, the silica-spin column method was the most efficient at removing PCR inhibitors from these samples. In addition, while the DTAB/CTAB squash sample extracts had very large ΔC_q shifts, indicating the presence of extreme PCR inhibition, the amplification efficiency of these samples was 100%. This may indicate the presence of thermolabile PCR inhibitors that degrade over the span of the PCR run, resulting in a delayed but efficient amplification curve. This type of inhibitor prevents efficient PCR amplification during the initial cycles, which delays the sample from reaching the threshold, resulting in a shift

in the quantification cycle (C_q) value. These inhibitors are subsequently degraded over the life of the PCR reaction, ultimately resulting in an efficient end to the PCR reaction.

As was done with the corn and squash samples, the extent of PCR inhibition in the pea samples was also assessed using the IPC qPCR inhibition assay. We found that the DNeasy Plant Mini Kit was the most efficient at inhibitor removal as seen by the ΔC_q values compared to the silica-spin column and DTAB/CTAB method. All three extraction methods, however, had high PCR amplification efficiencies for these samples. Using this information, we then attempted to explain the unexpected results obtained in the original pea sample experiment in which a decrease in recovered DNA was observed at intermediate time points (2.5 – 7.5 hours) followed by an increase in DNA template numbers at 10 hours using the silica-spin column method and the DNeasy Plant Mini Kit. Prior to conducting the inhibition assays, we hypothesized that this pattern could be due to formation of Maillard products during the heat treatment process. These inhibitory compounds could have been subsequently broken down between 7.5 and 10 hours, permitting successful amplification of the 10 hour time point sample. This hypothesis was based on observations made in another publication on artificially degraded plant remains (Threadgold and Brown 2003). In an artificial charring study using 200 ˚C heat treated wheat samples, Threadgold and Brown (2003) determined that, while the onset of Maillard products was dependent on the temperature of the experiment, these inhibitors were generally present between 30 minutes and three hours and then ceased to interfere with PCR amplification of DNA. While the experimental conditions for the current study are clearly different from those used by Threadgold and Brown (2003), we did observe a

similar pattern, suggesting that Maillard products may be negatively affecting our ability to recover or amplify DNA from the pea samples.

Based on the original degradation patterns we observed and our hypothesis that Maillard products were involved, we expected that the 2.5 – 7.5 hour time point samples would show increased levels of inhibition compared to the 0 and 10 hour samples. While the IPC qPCR assay indicated that the pea sample extracts did have PCR inhibitors that were affecting the ΔC_q values, these values did not significantly change over the five time points tested (0 – 10 hours). While unexpected, this observation raises questions about exactly how Maillard products interfere with PCR amplification of DNA and how well the IPC qPCR inhibition assay is able to detect these inhibitory compounds. Maillard products are cross-linked compounds resulting from the chemical reaction between an amino group in proteins or DNA and a sugar molecule, thereby preventing amplification by PCR. This cross-linking reaction is promoted by heat and thus is particularly problematic for the recovery of DNA from heat treated samples (*e.g.,* artificially degraded DNA or charred ancient plant remains). Given that Maillard products bind directly to the sample DNA during heat treatment, the failure of PCR experiments in the presence of these inhibitory compounds is due to the unavailability of the cross-linked DNA to act as a template in PCR. However, in the case of the IPC qPCR inhibition assays, because these Maillard product-DNA complexes are formed during heat treatment prior to PCR amplification, they would not be expected to interfere with amplification of the IPC standards. Thus, it is questionable if either the IPC qPCR inhibition assay or the sample dilution approach are suitable for assessing the extent of PCR inhibition resulting from Maillard products in DNA extracts.

In addition to negatively affecting DNA amplification during PCR, another possible mechanism of inhibition is the interference of Maillard products during the DNA extraction process itself. Because the silica-based extraction methods remove proteins and carbohydrates from the sample extract using a silica-spin column, it is possible that DNA bound to the Maillard products is being lost during the extraction process. Thus, disruption of Maillard products during the cell lysis stage, prior to the DNA isolation phase, may drastically improve both the DNA recovery and PCR amplification rates. To address the issue of Maillard products, many researchers in the field have recommended the addition of N-phenalcylthiazolium bromide (PTB) during cell lysis to facilitate cleavage of the protein-DNA cross-links, thereby releasing ancient DNA from Maillard products and making it available for PCR amplification (Giles and Brown 2008; Hofreiter *et al.* 2000; Poinar *et al.* 1998). While others have added PTB to either the silica-spin column method (Giles and Brown 2008), the silica slurry extraction method (Hofreiter *et al.* 2000; Poinar *et al.* 1998), or the DNeasy Plant Mini Kit (Erickson *et al.* 2005), to our knowledge there are no reports in the literature regarding effects of PTB on the DTAB/CTAB extraction method. In addition, some researchers have experimentally tested PTB and have observed no improvement in PCR success rates, indicating that there is a lack of overall consensus about the benefits of this reagent for removal of PCR inhibitors (Kemp *et al.* 2006; Rohland and Hofreiter 2007). As a result, for the sake of consistency, PTB was not used in the current study although this is certainly an area that needs to be studied further in the future. In general, it is important to remember that PCR inhibitor removal is only one component of an effective DNA extraction method and that

these results should be combined with the DNA recovery efficiency results to determine which DNA extraction methods are the most optimal for ancient plant remains.

Authentication of Results

Contamination Controls and Authenticity

Contamination control steps are a critical component of all ancient DNA research, including the current study, because of the low levels of degraded starting DNA preserved in ancient remains or artificially degraded samples and the sensitivity of the PCR method. As mentioned previously, efforts were made throughout this study to prevent or at least minimize DNA contamination to ensure that the DNA degradation patterns and the subsequent comparison of extraction methods were both reliable and authentic. As outlined in the methods section, this study incorporated the following steps as part of our contamination control measures: 1) restriction of laboratory access to only laboratory personnel; 2) the physical separation of pre- and post-PCR laboratory work; 3) the separation and restriction of all laboratory work into the appropriate laboratory based on the sample type (*e.g.*, ancient, forensic, or modern DNA laboratories); 4) the separation and dedication of individual rooms and equipment within the forensic laboratory for specific purposes (*e.g.*, sample preparation room, DNA extraction room, PCR setup room); 5) strict protocols regarding unidirectional movement of laboratory personnel were followed; 6) regular decontamination of laboratory spaces and equipment with bleach; 7) appropriate use of clean personal protective equipment including fresh gloves, Tyvek suits, and masks; and 8) use of lab-dedicated clothing and shoes under Tyvek suits. In addition, the 0 hour time point samples were used as positive controls for the DNA extractions, standard PCR, and quantitative PCR. Two extraction blanks were

included for each extraction batch to monitor the levels of systematic contamination. For both standard PCR and qPCR, negative amplification controls, which consisted of the PCR master mix in the absence of any sample extract, were also used also to monitor contamination levels. All extraction blanks for samples heat treated at 140 ˚C using the three different extraction methods showed no contamination for either standard PCR or qPCR. Furthermore, no contamination was observed for the 200 ˚C heat treated samples using standard PCR. Low levels of contamination were detected, however, in the two 85 ˚C extraction blanks using qPCR. However, it was determined that this low level contamination was insignificant when compared to the DNA quantities recovered in the 0 to 15 hour time points, which ranged from 1.8 million templates to 1.5 million templates, respectively. None of the PCR negative amplification controls gave bands by standard PCR, and 30 of 32 PCR negative amplification controls for the quantitative PCR yielded no detectable DNA. We attribute the two positive PCR negative amplification controls to sporadic contamination as other PCR negative controls in the same batch were negative.

In addition to rigorous contamination controls, a number of other factors can be used to confirm the authenticity of aDNA studies. In the current study, we used the following factors to confirm the authenticity of the DNA degradation patterns and the results of our extraction method comparison study: 1) appropriate molecular behaviour was observed using multiple PCR fragment lengths with standard PCR; 2) both standard PCR and qPCR yielded similar results with respect to the most and least efficient DNA extraction methods; 3) repeatability tests were conducted in which one time point sample was extracted five times with each extraction method by the primary investigator; 4) reproducibility tests were conducted in which one time point sample was extracted three

times with each extraction method by an independent laboratory member; 5) quantification of DNA templates was conducted in duplicate; 6) testing for PCR inhibition was conducted; and 7) selected results were sequenced to confirm that recovered DNA was consistent with the expected species. Collectively, confidence in our findings is possible due to the use of dedicated DNA laboratories at SFU, stringent contamination controls, and the general design of the study to include multiple experimental approaches and repetition of testing, all of which contribute to the authenticity of our results.

Repeatability and Reproducibility Tests

As described above, both repeatability and reproducibility tests were significant components of our study. A selection of the initial time points was repeated from the beginning for authentication purposes. New corn, squash, and pea samples heated at 140 ˚C for 5, 5, and 10 hours, respectively, were each extracted five times using the three different extraction methods and subjected to qPCR. These results confirmed our initial findings that the silica-spin column was the most efficient at recovering DNA from the artificially degraded corn, pea, and squash samples. While the DNA template numbers obtained from the replicates were not identical in the original samples, the calculated relative-to-best values were very similar for the selected time points. The corn samples yielded fewer DNA templates on average for the repeats than were obtained from the original corn sample. Conversely, both the pea and squash repeats averaged more DNA templates than were obtained from the original samples. There are a number of possible reasons why the replicate DNA template numbers differ from the original extraction: 1) the position of the samples in the oven and the resulting differences in heat exposure may

have affected DNA degradation; 2) the use of new seeds for the replicates means that the starting levels of DNA were not expected to be identical; and 3) general extraction variability was expected as the original extractions were only done once for each time point while the replicates were done five times.

Further extractions were conducted to test the reproducibility of the results by an independent researcher. These experiments were carried out as described for the repeatability tests, but in triplicate by another laboratory member. The results from this set of experiments further confirmed that the silica-spin column method was the most efficient at recovering DNA from the degraded corn, pea, and squash samples. In addition to the variables that were described for the repeatability tests, two additional factors could have affected the amount of DNA templates recovered during the reproducibility tests: 1) while the same protocol was followed to ensure consistent methodology amongst laboratory personnel, we cannot rule out that there are no differences in technical ability or experience between researchers; 2) these samples were heated and powdered independently from previous samples, and were stored in the freezer for approximately six months prior to DNA extraction. Despite these variables, the results from these reproducibility tests indicate that our initial findings are real, and are not dependent on the experience of a single laboratory member. In general, our repeatability and reproducibility tests demonstrated that there is some degree of variability in the extraction processes for all three methods even when the same starting material is used. This highlights the importance of conducting extraction replicates whenever possible when working with either artificially degraded samples or ancient samples to ensure the results are valid.

Applications of Artificial DNA Degradation Patterns

In order to overcome low PCR amplification success rates that can be a challenge in the field of plant aDNA, thereby encouraging more research focusing on this area, significant methodological improvements are required. Efforts to advance the field should focus on systematic comparison and/or optimization of DNA extraction techniques. The comprehensive controlled study conducted by Rohland and Hofreiter (2007) using ancient faunal remains can be used as a model for future studies on ancient plant remains. However, because macro-botanical remains are generally small in size compared to most ancient faunal remains, artificially degraded plant samples, which can be prepared in larger volumes, may have to be used to simulate ancient remains in these comparison and optimization studies. Use of artificially degraded plant samples for such methodology improvement studies will also prevent the consumption of valuable ancient plant remains for these purposes. Thus, ancient samples can be conserved and tested only once an optimal method has been identified, thereby limiting sample destruction and maximising the chances of successful DNA amplification.

The results from the current study indicate that the artificial DNA degradation model can be used to compare the efficiency of DNA recovery using various extraction methods. We further demonstrate that this model is applicable to multiple plant species, and that appropriately heat treated modern plant remains can simulate the limited quantities of degraded DNA expected to be found in charred macro-botanical remains. While heat treatment is currently the most common method used to artificially degrade modern DNA, other DNA degradation methods (*e.g.,* heating with water, UV irradiation, and submersion in bogs) could simulate other forms of plant preservation (Lindahl 1993;

Campos *et al.* 2010). This would allow researchers to gain more valuable insight into the process of DNA degradation and preservation in plants, and could also be used to further test the ability of different extraction methods to recover aDNA from plants preserved waterlogged or desiccated.

Another important potential application of this work relates to the degradation of DNA at the lower temperature examined in this study. While the level of DNA degradation observed at 85 ˚C (13 % at 15 hours) was small in terms of simulating ancient remains, the observed degradation levels do raise potential concerns for a number of other applications. In particular, there is currently no uniform protocol for storage of macro-botanical remains. Some researchers may store samples in non-climate controlled areas, which may subject the limited amounts of DNA in these ancient samples to even further degradation over extended storage. Thus, further studies assessing the extent of DNA degradation in artificially degraded plant samples at lower temperatures (25-50 ˚C) may provide invaluable information both about DNA preservation in desiccated ancient plant remains from hot and dry climates, and about appropriate storage conditions for all ancient plant remains to maximize future DNA recovery.

Ultimately, the main goal of the current study was to compare the efficiencies of three currently used DNA extraction methods to determine which method gives the best chance at recovering of limited amounts of degraded plant DNA. The purpose of this study was not to conclusively identify the exact conditions (temperature and exposure time) under which artificially degraded DNA could and could not be recovered. Currently, there are no morphological markers that can accurately indicate the temperature at which a plant seed was charred or which samples are likely to contain

amplifiable DNA (Schlumbaum *et al.* 2008a). Thus, despite our observation that the 200 ˚C plant samples exhibited morphological darkening yet were not completely charred and yielded little or no DNA amplification, no negative conclusions should be drawn about the likelihood of recovering ancient DNA from charred plant remains in general. This is because not all charring is the result of exposure to high temperatures as charring can also occur at lower temperatures over long periods of time (Schlumbaum *et al.* 2008a). Furthermore, technology in the field of aDNA is continually and rapidly changing and therefore, the current study should be interpreted as a building block rather than a finish line in methodology improvement. In particular, the use of new technology called Next-Generation-Sequencing (NGS), which to date has been used to analyze extremely short DNA fragments of ancient human and animal remains, may be used to study previously unsuccessfully amplified degraded plant remains in the near future (reviewed in Knapp and Hofreiter 2010). The importance of this ever-evolving and improving technology in the field of aDNA is evident when comparing the attempts of two different research groups to amplify degraded barley seeds. While Chalfoun and Touross (1999) conducted the first type of artificial degradation study by heating modern barley seeds at various temperatures and time points, they were unable to recover DNA after 1 hour at 150 ˚C. These unsuccessful findings may have deterred some researchers from attempting to recover DNA from ancient barley seeds. However, ten years after the Chalfoun and Touross (1999) study, Palmer *et al.* (2009) successfully recovered ancient DNA from desiccated barley remains from the site Qasr Ibrim in Egypt dated to as old as 3000 years. This type of positive result is not a comment on the scientific abilities of the different research teams, but rather was likely not possible ten years ago due to limitations in both

extraction and PCR amplification technologies. This example illustrates that the findings of our current study should be interpreted only as a comparison of extraction methods and should not discourage any attempts at recovering ancient DNA from charred corn, pea, and squash remains.

Future Directions for Improving Extraction Efficiency

While the silica-spin column method is commonly used for human and faunal aDNA studies, relatively few studies on ancient plant remains have used this method. The DNeasy Plant Mini Kit, with a number of published modifications, has been more frequently used for plant aDNA studies. Because this kit limits the amount of recommended starting plant material, these modifications are aimed at improving the overall extraction efficiency. However, many of these published modifications have never been explicitly compared to the unmodified protocol, and thus it is unclear if the modification actually makes the DNeasy Plant Mini Kit more efficient. The current study, which is the first laboratory controlled study to directly compare the silica-spin column and the DNeasy Plant Mini Kit, suggests that it may be more beneficial to plant aDNA researchers to adopt the silica-spin column method rather than modifying the DNeasy Plant Mini Kit method. Further optimization of the silica-spin column method could be achieved by assessing some of the published modifications of the DNeasy Plant Mini Kit either to improve the DNA recovery efficiency or to help remove or neutralize PCR inhibitors. A number of the published modifications appear to make the DNeasy Plant Mini Kit more like silica-spin column: addition of proteinase K and ethylenediaminetetraacetic acid (EDTA) to the lysis buffer (Erickson *et al.* 2005; Speirs *et al.* 2009); and increasing the amount of lysis buffer (Elbaum *et al.* 2006). In addition,

in order to increase the amount of starting plant material, one study divided up 80-100 mg of sample powder into five separate (approximately 25 mg) samples and then combined all five into one silica-spin column at the final stage (Speirs *et al.* 2009). The silica-spin column method has the capacity to extract 100 mg of sample powder without the need for sample division, saving time and reagents. A number of other modifications, however, may be applicable to the silica-spin column method: increasing the amount of starting material (Dumolin-Lapegue *et al.* 1999; Elbaum *et al.* 2006; Erickson *et al.* 2005); and addition of dithiothreitol (DTT), N-phenalcylthiazolium bromide (PTB), and polyvinyl pyrrolidone (PVP) to address PCR inhibition issues (Erickson *et al.* 2005; Speirs *et al.* 2009). We recommend further testing of these modifications to determine if they improve the overall effectiveness of the silica-spin column method.

In particular, a number of both modern and ancient DNA studies, not just those using the DNeasy Plant Mini Kit, have used a variety reagents in an effort to minimize the negative impact of PCR inhibition during DNA extraction. As described above, PTB has been added to lysis buffer in an effort to cleave Maillard products in coprolite samples (Hofreiter *et al.* 2000; Poinar *et al.* 1998). Another type of PCR inhibitor, polyphenol compounds are released from plant cell vacuoles during the grinding phase and are more prevalent in aged plant samples (Kasem *et al.* 2008; Weising *et al.* 2005). Some modern plant DNA studies have used reagents such as bovine serum albumin (BSA) or polyvinyl pyrrolidone (PVP) in the lysis buffer in an attempt to bind polyphenols or antioxidants such as β-mercaptoethanol to prevent oxidation of polyphenols (Couch *et al.* 1990; Jobes *et al.* 1995; Kim *et al.* 1997; Ouenzar *et al.* 1998).

The lysis buffer of the silica-spin column method could easily be modified to add these reagents and a controlled laboratory comparison similar to the one conducted here would determine the effectiveness of these reagents for different plant samples.

Another potentially useful modification was outlined by Kemp *et al.* (2006) who tested ancient bone and coprolite samples and observed a relationship between the darkness of DNA extracts and high concentrations of PCR inhibitors. They found that repeating the last step of the silica-spin column extraction (potentially multiple times) resulted in successful removal of PCR inhibitors from samples that they were previously unable to amplify using sample dilution or addition of BSA or PTB to the lysis buffer (Kemp *et al.* 2006). This "repeat silica extraction" method has not yet been test using either artificially degraded plants or ancient plant remains, and could potentially remove the dark colour and PCR inhibitors present in many ancient plant DNA extracts. Currently, these samples are often diluted as much as one hundred times for successful PCR amplification, and the "repeat silica extraction" method may prove more effective at removing PCR inhibitors (Kemp *et al.* 2006; Schlumbaum *et al.* 2008b).

In addition to the removal of PCR inhibitors, the other common challenge associated with efficient recovery of DNA from plant material is the effective breakdown of the plant tissue and individual cells. In order to recover more DNA from plant powder, the differences between animal and plant cells must be considered. Animal cells lack the rigid cell wall structures that are present in plant cells. These plant cell walls are comprised of three main components: cellulose microfibrils, hemicelluloses, and pectin (Black *et al.* 2006). The cell wall acts as a barrier to the release of DNA from plant cells and represents an additional obstacle for plant aDNA extraction compared to animal

aDNA studies. There are various ways of disrupting plant cell walls to release DNA for extraction including physical disruption in liquid nitrogen using a mortar and pestle or a cryogenic grinder (Kasem *et al.* 2008). While the amount of DNA released depends on the force used during the physical grinding of the plant sample, too much force has the potential to degrade the DNA into shorter fragments (Kasem *et al.* 2008). A number of non-grinding disruption methods also exist. Potassium or sodium ethyl xanthogenate (PEX or SEX) has been used as a non-grinding chemical treatment method for disruption of plant cell walls in modern plant DNA studies (Jhigan 1992; Williams and Ronald 1994). In addition, various fungi naturally produce enzymes to break down cell walls including cellulase, pectinase, xylanase, mannanase, xyloglucanase, and arabinofuranosidase (Rether *et al.* 1993; Rogstad *et al.* 2001; Woodhead *et al.* 1998). Currently, these fungal enzymes are not widely used among researchers who extract DNA (ancient or modern) from plants. It is possible that non-grinding methods could be combined with physical disruption in an effort to release more of the preserved DNA present in ancient plant remains during extraction. This would increase the overall efficiency of DNA recovery, especially from ancient plant remains, allowing these techniques to be more successfully applied to important archaeological questions.

Chapter Summary

Our findings are in general agreement with other artificial degradation studies that have reported rapid initial DNA degradation followed by a plateau phase (Dobberstein *et al.* 2008; McGrath 2010; Threadgold and Brown 2003). In addition, our results indicate that an artificial DNA degradation model can be used to compare the efficiency of DNA recovery and the removal of PCR inhibitors using various extraction methods. Because

one of the possible outcomes of this study was that degraded DNA from different plant species may require different extraction methods for optimal recovery, it was vital to include a variety of plant species in our study. Our results indicate that the silica-spin column method was the most efficient extraction method in terms of DNA recovery for all three plant species. These findings suggests that a silica-based extraction method could potentially be used as a universal extraction method for ancient plant remains. The observed variability in DNA recovery rates between extraction methods could be due to differences in the cell lysis and DNA recovery phases of each method. Findings of this study suggest avenues for further investigation to potentially improve extraction methods for ancient plant remains.

Chapter 6: Conclusion

A systematic comparison of the three most commonly used DNA extraction techniques in the field of plant aDNA was conducted in this study to determine whether there is the potential for one universal method that is ideal for a variety of ancient plants remains or if the optimal extraction method depends on internal structures and composition of each plant. Our results indicate that the silica-spin column method was the most efficient method for recovery of DNA from artificially degraded corn, pea, and squash samples. These findings are consistent with a previous study on artificially degraded wheat (Giles and Brown 2008) as well as with numerous studies on both human and faunal remains (Bouwman and Brown 2002; MacHugh *et al.* 2000; Yang *et al.* 1998). Furthermore, our results suggest that there may be a single optimal extraction method for all ancient plant remains, despite differences in plant internal composition. Expansion of this study to include other plant types as well as ancient remains is recommended to unequivocally confirm the existence of such a universal extraction method. Future testing of technical modifications of the silica-spin column method including lysis buffer modification using a controlled laboratory experiment is also recommended to potentially improve the recovery of DNA and limit the extent of PCR inhibition.

In addition to the comparison of different extraction methods, this study was also able to develop detailed DNA degradation patterns for corn, pea, and squash samples at 140 ˚C using standard and qPCR. Generally, at 140 ˚C significant DNA degradation

occurred within the first 2.5 hours followed by a slower rate of DNA degradation. Furthermore, we demonstrated that corn, pea, and squash had very limited DNA degradation at 85 ˚C and extreme degradation at 200 ˚C. These findings suggest that DNA degradation in plants is significantly affected by exposure to temperature over time. Finally, our results also indicate that artificial heat treatment can be used as an experimentally controllable model in order to conduct systematic comparisons of different extraction methods or modifications of extraction methods.

This study underscores the fact that researchers studying ancient plant remains may not currently be using the most efficient DNA extraction method available and should consider changing their laboratory protocols to improve DNA recovery rates. Furthermore, ancient DNA researchers already using the silica-spin column method for DNA extraction from ancient human or faunal remains could easily expand their focus to include ancient plant remains with little or no modifications to their current methods. This would facilitate rapid advancements in the field of ancient plant DNA, ultimately allowing for broader applications of the field to address more archaeological questions.

Chapter 7: Appendices

Appendix A – DNA degradation pattern sequence alignment

Figure A1: Sequence alignment of corn, pea, and squash reference sequences and universal and specific primers used for the generation of DNA degradation patterns.
Reference sequences for corn (NC001666.2), pea (NC014057.1), and squash (AF206756.1) were downloaded from NCBI Genbank.

Appendix B – qPCR data for the repeatability and reproducibility tests

Table B1: Repeatability test for the comparison of DNA extraction methods.

Extraction Replicates	**Mean DNA Template Number (Standard Deviation)**		
Extraction Number	Silica-spin Column	DNeasy Plant Mini Kit	DTAB-CTAB
Corn 5h (140 ˚C)			
1	5376 (256)	67 (13)	97 (25)
2	4441 (24)	40 (1)	20 (4)
3	3428 (219)	87 (21)	0 (N/A)
4	4449 (150)	71 (11)	11 (5)
5	4583 (266)	77 (8)	16 (4)
Avg. DNA Quantity of Replicates	***4456***	***68****	***29****
Standard Deviation	620	16	35
Pea 10h (140 ˚C)			
1	855898 (31036)	162373 (3271)	114890 (2463)
2	639049 (33783)	107322 (15620)	93364 (4447)
3	869855 (13575)	134293 (17986)	74124 (22319)
4	687794 (33709)	132085 (5926)	127830 (9887)
5	163450 (9123)	122971 (10139)	75123 (62081)
Avg. DNA Quantity of Replicates	***643209***	***131809***	***97066***
Standard Deviation	286696	20113	23904
Squash 5h (140 ˚C)			
1	472897 (59649)	18519 (165)	4359 (6164)
2	448086 (42638)	22209 (1491)	0 (N/A)
3	470525 (34453)	12867 (186)	13074 (479)
4	455972 (34560)	11053 (468)	412 (408)
5	410316 (9295)	14677 (442)	1 (0)
Avg. DNA Quantity of Replicates	***451559***	***15865****	***3569****
Standard Deviation	25236	4496	5622

Notes: Comparison of DNA extraction methods replicated five times for one time point for each plant species. DNA template numbers determined from quantitative PCR. DNA quantity was determined from duplicate reactions using undiluted extracts using duplicate quantitative PCR amplifications. The DNA quantity is provided as template number and the standard deviation is provided in brackets. An average DNA template number were determined from the five extractions for each method and each species. DNA extraction methods that performed significantly worse than the best performing DNA extraction method (using the DNA template quantities), according to a paired Student's *t*-test ($P < 0.05$) are indicated by an asterisk (*). The standard deviation was calculated from the five replicate DNA quantities.

Table B2: Reproducibility test for the comparison of DNA extraction methods.

Extraction Replicates	**Mean DNA Template Number (Standard Deviation)**		
Extraction Number	Silica-spin Column	DNeasy Plant Mini Kit	DTAB-CTAB
Corn 5h (140 ˚C) (0.1x)			
1	229 (36)	9 (N/A)	0 (N/A)
2	193 (2)	0 (N/A)	0 (N/A)
3	286 (21)	7 (N/A)	0 (N/A)
Avg. DNA Quantity of Replicates	***236***	***6***	***0***
Standard Deviation	47	5	N/A
Pea 10h (140 ˚C) (0.1x)			
1	2001 (150)	972 (18)	675 (75)
2	1627 (27)	1028 (76)	623 (198)
3	1578 (131)	987 (117)	22 (N/A)
Avg. DNA Quantity of Replicates	***1735***	***995***	***440***
Standard Deviation	232	29	363
Squash 5h (140 ˚C) (0.1x)			
1	70361 (3483)	2563 (273)	5049 (1408)
2	23637 (444)	1855 (45)	318 (183)
3	27186 (767)	2547 (86)	5494 (1400)
Avg. DNA Quantity of Replicates	***40395***	***2321***	***3620***
Standard Deviation	26012	404	2868

Notes: Comparison of DNA extraction methods replicated five times for one time point for each plant species. DNA template numbers determined from quantitative PCR. DNA quantity was determined from duplicate reactions using undiluted extracts using duplicate quantitative PCR amplifications. The DNA quantity is provided as template number and the standard deviation is provided in brackets. An average DNA template number were determined from the five extractions for each method and each species. None of the DNA extraction methods performed significantly worse than the best performing DNA extraction method (using the DNA template quantities), according to a paired Student's *t*-test ($P < 0.05$). The standard deviation was calculated from the five replicate DNA quantities.

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