Ausmer, Alea, A Comparative Study of Three Methods to Enhance the Collection of DNA from Plant Material. Master of Science (Forensic Genetics), May, 2013, 36 Pages, 2 tables, 6 figures, references, appendix.

The Botanical Research Institute of Texas is using two methods of DNA extraction from plants, an automated method called Bullet Blender® and a manual method of grinding. A third method, using an instrument called the Fast Prep-24™, was evaluated and the DNA yield obtained was compared to the other methods. Eight plant species were chosen and two sample preparation methods, wet and dry, were evaluated. DNA yield gels were run in order to compare DNA quality and UV spectroscopy was used to evaluate quantity. Independent Student *t*-tests were performed to compare means variation between the DNA yield on the wet and dry samples and one-way ANOVA was used to compare variation between the three extraction methods. No significant difference was found between the wet and dry samples for DNA concentrations, but a significant difference was observed between the Fast Prep-24™ instrument and the other two methods.

A COMPARATIVE STUDY OF THREE METHODS TO ENHANCE THE COLLECTION OF DNA FROM PLANT MATERIAL

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center of Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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Fort Worth, Texas

May 2013

ACKNOWLEDGEMENTS

I would like to acknowledge my family and friends back in Mississippi for their prayers, support, encouragement, and faith in me during my academic pursuit. I would like to thank my committee at the University of North Texas Health Science Center for their guidance throughout this project and Dr. William McClatchy and the staff at BRIT for all of their help. I also would like to recognize each of my classmates. I appreciate all the guidance and support. I couldn't have asked for a better group of friends to have the grad school experience with.

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CHAPTER I

INTRODUCTION

Forensic botany is the application of using plant evidence to aid in criminal and civil investigations. This can incorporate many aspects of plant biology such as the study of pollen, taxonomy and growth patterns. When a crime occurs, according to Edmond Locard theory, information in the form of evidence is transferred between two objects like the perpetrator and the crime scene (1). An unlikely item of evidence is plant material. It can be found on clothes, in hair, in vehicles and even inside the body. Once this crucial evidence is discovered, it can further aid in the investigation (2). An optimized DNA extraction protocol for plant cells is crucial for genetic analysis of botanical evidence. Therefore, establishing an optimal method to lyse a plant's cell wall and extract the DNA is critical.

All plant cells have a rigid cell wall made of cellulose that must be ruptured before DNA can be removed from the nucleus (2). Plants also contain high levels of compounds that can interfere with DNA purification such as polysaccharides, ribonucleases and polyphenolics. These compounds bind to the DNA (3). Thus extracting DNA from plants and plant material can be challenging. A DNA extraction method for plants must be able to provide both adequate amounts of DNA, as well as, DNA free from contaminants such as proteins and inhibitors, for further downstream applications (3). This research project evaluated the usefulness of the Fast Prep-24™ (MP Biomedicals, Solon, OH) instrument on plant material. Current methods used by the

Botanical Research Institute of Texas (BRIT) are the Bullet Blender® (Next Advance, Averill Park, NY) and a mortar and pestle (Creative Home, Manalapan, NJ) to grind and lyse plant cells.

Plants from four categories were chosen for this project to give a total of eight different plants. The categories were landscape/horticulture plants, house plants, poisonous plants, and wild plants. The plants chosen were: Heavenly bamboo (*Nandia domestica),* Live Oak (*Quercus fusiformis),* Christmas mistletoe (*Phoradendron sp*), Chinese elm *(Ulmus parvifolia*), African violet (*Saintpaulia sp*.), Poinsettia (*Euphorbia pulcherrima*), Oleander *(Nerium oleander*) and Pine (*Pinus*).

The samples were lysed using the Fast Prep-24™ instrument, Bullet Blender® and a mortar and pestle. The extracted DNA was purified using a protocol created by a BRIT staff member. Purified DNA samples were then quantified by yield gel analysis and UV spectroscopy. The results from all three lysing methods were then compared using statistical analysis. Information obtained from this study can assist BRIT in deciding which method to implement for future genetic studies.

Statement of Problem

This project aimed to determine which of the three lysing methods evaluated is the most efficient process to release DNA from difficult plant cells and obtain a high quantity and quality of DNA for further studies.

The Null Hypothesis: There will be no statistically significant differences in DNA recovery between the three methods.

The Alternate Hypothesis: One of the three methods will demonstrate a statistically significant higher rate of DNA recovery than the other two methods.

Research significance

The purpose of this research was to compare the effectiveness of lysing plant cells with the Fast Prep-24™ instrument, the Bullet Blender® instrument and the mortar and pestle method process. All cells have a plasma membrane and protein-lipid bilayer that separate the intracellular and the extracellular environment. In addition, peripheral proteins are imbedded in the inner and outer surface of the bilayers. The arrangement of these proteins and lipids differs with the cell type and species of the plant (4). Plants can also contain high levels of polysaccharides and many types of secondary metabolites affecting DNA purification. These components interfere with cell lysis which decreases optimal DNA from extractions (3). Therefore, a plant's cell wall must be disrupted carefully so the DNA can be extracted from the nucleus without being degraded by the harmful cellular components. In addition to plant biological components, plant material collected in the field is seldom stored in a manner that preserves DNA quality. Improper collection or storage techniques could explain why retrieving sufficient DNA can be difficult. For many studies, DNA of high quality is essential, especially in the case of sequencing (3).

Cell lysis is the first step in isolation and purification of DNA. Cell lysis is the process of releasing the biological components of a cell such as proteins, DNA, RNA and lipids (4). Many techniques have been developed for the disruption of cells, including physical and chemical-based methods. These methods include: mechanical agitation, manual grinding, pressure, sonication, freeze/thaw and chemical methods such as detergents and enzymatic hydrolysis (4).

The main purpose of these methods is to physically break the cell envelope (cell wall, cell membrane and outer membrane) to release the intracellular components. A common method

used is manual grinding with a mortar and pestle. Plant tissue is frozen in liquid nitrogen and then crushed with the mortar and pestle. Due to the strength of cellulose and polysaccharides comprising the cell wall, mortar and pestle is the fastest, most efficient and cheapest way to release plant DNA (4). Unfortunately, this process can be time-consuming and manually demanding when handling a large number of samples (5).

In this research, the Fast Prep-24™ was compared to currently used techniques. The Fast Prep-24™ was predicted to save time during the sample preparation stage and provide higher yields of intact DNA. Fast Prep-24™ uses a unique motion to disrupt cells through multi-directional simultaneous beating of specialized matrix beads on the sample material. The Fast Prep-24™ can thoroughly and quickly disrupt the cell structure releasing DNA. The instrument is self-contained which helps reduce the risk of cross-contamination. This method reduces the cleanup time compared to previous methods (6).

The Fast Prep-24™ method works by adding a plant sample and buffer to the Lysing Matrix D tube (MP Biomedicals). Matrix D consists of an impact resistant 2 mL tube containing 1.4 mm ceramic spheres. Up to 200 mg of a plant sample can be processed. Twenty-four samples can be homogenized at once. The homogenization speed and duration time are digitally controlled for consistency. Samples are processed for 60 seconds (6).

The unique, vertical angular motion of the Fast Prep-24™ causes the lysing matrix particles to impact the sample from all directions simultaneously, releasing DNA into the protective buffer faster than with any other method (Figure 1). High performance Fast Prep-24™ Purification Kits (MP Biomedicals), when used in conjunction with the Fast Prep-24™, can provide an easy method for the release and purification of DNA (6). Once the DNA is extracted, the next step is to determine how much DNA is present within the sample.

Figure1. Fast Prep-24™ Procedure (6). Diagram describing the Fast Prep-24™ procedure. Step one illustrates tube preparation, step two the mechanical processing with the instrument, step three centrifugation to pellet and remove debris and step four transfer of purified lysate.

Background

Visual identification is often used for distinguishing and categorizing plant material. However, in order to gain genetic information, the DNA from the plants must be extracted. Prior to the introduction of DNA analysis in forensic science, observational evidence was used to compare plant evidence with a known exemplar. This is only successful if a good physical description is given of the original evidence (2). For example, in the Lindberg baby kidnapping

case the analysis of a wooden ladder was used to help determine its origin. The kidnapper gained access to the child's second floor bedroom by use of a homemade ladder. The wood used to construct the ladder was demonstrated to have originated from the same wood that made up the floor of the perpetrator's attic (2). In another forensic botany-related case, a grave discovered in Germany contained 32 male skeletons. Pollen spores found in the nasal cavities of the skulls were analyzed. The pollen was identified and helped to determine that the murders were committed in the summer season due to the fact that the particular pollen is only abundant in the months of June and July (7).

A more recent case involved the DNA analysis from Palo Verde trees. A woman's body was discovered in the Arizona Sonoran Desert. Her body was found in close proximity to a Palo Verde tree which is native to that desert. Physical evidence at the scene included a recent scrape on a branch of the Palo Verde and the Palo Verde seed pods. Similar seed pods were found in the back of a suspect's truck (8). These seed pods were analyzed by a geneticist using multiple primer RAPD (randomly amplified polymorphic DNA) analysis and it was determined that the seed pods from the truck produced a DNA profile that matched the tree from the crime scene (9). The suspect was convicted and this case became the first of its kind to use plants genetics in a court of law (8).

The use of forensic botany has become increasingly important in determining the origin of marijuana evidence. Marijuana may be identified using microscopic examination and the Duquenois-Levine chemical test (10). Alternative, identification may use gas chromatography mass spectrometry (GC-MS), thin layer chromatography, or high performance liquid chromatography (HPLC) with known reference samples. In addition, genetic tests using chloroplast DNA sequencing analysis of spacer region have been employed (11). Once

marijuana has been positively identified it is possible to determine populations of origin through comparison with reference samples using STRs (short tandem repeats) and AFLPs (amplified fragment length polymorphisms) (12).

To generate plant STRs, DNA must first be extracted from the cells. Extraction consists of separating and removing the genomic DNA from the other components and purifying it to yield a genetic profile. In forensic sciences, the most common and well-documented method for extraction is an organic extraction. This procedure uses organic chemicals to denature and remove proteins from the cell to purify the DNA. To prevent the DNA from being degraded by nucleases EDTA (ethylenediaminetetraacetic acid) a chelating agent is added to a lysis extraction buffer that contains, NaCl (sodium chloride), SDS (sodium dodecyl sulfate), and Tris-HCL (Tris-hydrochloric acid) (13). The combination of these reagents are used to break down the cell, release its contents and protect the DNA from degradation. Following incubation the proteins need to be further denatured and removed from the solution. This is accomplished by using a phenol, chloroform and isoamyl alcohol (PCIA) combination. Following the PCIA addition, the DNA is then cleaned, purified and concentrated with an ethanol precipitation (13).

Another type of extraction used is a Chelex® 100 (Bio-Rad, Hercules, CA) extraction. This method consists of a chelating resin that is used to extract DNA from many types of forensic sample. This method is favored because it saves time, money, and has less chance for contamination. The Chelex resin works well because it acts as a chelator to bind metal ions in boiling temperatures. This process denatures damaging proteins and denatures the DNA from being double stranded to single stranded. The resin is then removed by centrifugation to help clean the remaining DNA (14).

In addition, solid phase extraction can be used to isolate genomic DNA. The process involves silica beads that are in an aqueous medium and combined with the sample. Proteins are then removed while the DNA binds to silica in a high salt concentration. The DNA is then washed and released with an ethanol solution for purification. MO BIO offers an UltraClean™ kit (MO BIO Laboratories, Inc, Carlsbad, CA) for this extraction process (15).

In regards to this research project there are few extraction kits available that can easily extract and isolate plant DNA. For instance, Qiagen produces a DNeasy Plant Mini Kit (Qiagen, Germantown, MD). This kit can use fresh, frozen or dried samples. The procedure uses a filtering and homogenization spin column that removes cellular debris to yield total cellular DNA by first mechanically disrupting the cells then chemically lysing them. The DNA then binds to a silica membrane while the other cellular components are washed away. The isolated DNA is then eluted and immediately ready for downstream applications (16).

For the Fast Prep-24™, Bullet Blender® and mortar and pestle, the extraction process was the same throughout. However the lysing step was different between the three methods. For the Fast Prep-24™ and Bullet Blender®, the process of breaking open the cell consists of small ceramic beads inside a tube that contains an extraction buffer. This mechanical method uses beads that are beat against the plant material to breakdown the cell components and release the genomic DNA. The tubes are shaken at a very high speed to allow the beads to puncture the plant material and break it down into its cellular components. After the lysing is complete, the extraction procedure can be followed.

For the mortar and pestle, manual grinding is needed to disrupt the cell wall and grind the plant products until they are broken down. Most times, plant tissue is made fragile with the

addition of liquid nitrogen, which makes it easier for the plant cells to be broken down by the pestle. This process requires more time and can be dependent on the strength of the individual processing the samples. The plant material is then placed in a tube along with extraction buffer and the extraction procedure can be completed.

CHAPTER II

Methods and Materials

Specimens of eight different species of plants were provided by the Botanical Research Institute of Texas. The species of plants used in this research project were chosen because they often appear in botanically relevant forensic cases. Fresh and dry sample of each the plants were provided. Fresh samples were placed in labeled plastic bags that were kept frozen. Dry samples were in labeled brown paper bags kept in a dry storage area.

Plant ID	Scientific Name	Common Name	Category
N/A	*Euphorbia pulcherrima*	Poinsettia	Fresh-poison plants
N/A	*Euphorbia pulcherrima*	Poinsettia	Dry-poison plants
M.B. Byerley 331	*Quercus fusiformis*	Live Oak	Fresh-landscape
M.B. Byerley 331	*Quercus fusiformis*	Live Oak	Dry-landscape
M.B. Byerley 333	*Nerium oleander*	Oleander	Fresh-poison plants
M.B. Byerley 333	*Nerium oleander*	Oleander	Dry-poison plants
Tiana F. Rehman 477	*Ulmus parvifolia*	Chinese Elm	Fresh–landscape/horticulture
Tiana F. Rehman 477	*Ulmus parvifolia*	Chinese Elm	Dry–landscape/horticulture
Tiana F. Rehman 476	*Saintpaulia* sp.	African Violet	Fresh-houseplant
Tiana F. Rehman 476	*Saintpaulia* sp.	African Violet	Dry-houseplant
M.B. Byerley 332	*Nandina domestica*	Heavenly bamboo	Fresh–landscape/horticulture
M.B. Byerley 332	*Nandina domestica*	Heavenly bamboo	Dry–landscape/horticulture
M.B. Byerley 335	*Phoradendron tomentosum*	Christmas mistletoe	Fresh–landscape
M.B. Byerley 335	*Phoradendron tomentosum*	Christmas mistletoe	Dry–landscape
M.B. Byerley 334	*Pinus* sp.	Pine	Fresh–wild plant
M.B. Byerley 334	*Pinus* sp.	Pine	Dry–wild plant

Table 1. Table containing samples used in the project. Samples were collected and provided by the Botanical Research Institute of Texas (BRIT).

Extraction

Each sample was manually cut into squares ranging from 0.5cm to 1.5cm. The samples were then subdivided between the three test methods, Fast Prep-24™, Bullet Blender® and mortar and pestle and subject to treatment. Upon completion, each of the samples was treated with the same DNA extraction buffer.

Extraction buffer was prepared for each of the methods. A stock solution of 250mM NaCl (Sigma Aldrich, St. Louis, MO); 200mM Tris-HCL pH 8.0 (Life Technologies); 25mM EDTA (Life Technologies) and 0.5% SDS (Life Technologies) was combined.

Fast Prep-24™ method

Thirty-two green top 1.5mL Fast Prep-24™ Matrix D tubes were labeled with the plant name. Two tubes contained a wet cutting of a plant and two tubes contained a dry cutting of the same plant for a total of four tubes with the same plant for each of the eight different species. A cutting of approximately 0.5 to 1cm of plant material was placed in the tube that contained 2mm ceramic beads. The tubes were placed on the Fast Prep-24™ and set at 6 m/s for 40 seconds. For dried plant samples a total of 500µL of extraction buffer was added to the tubes and shaken for 40 seconds. Then the plant samples were then subsequently added to the tubes and shaken for another 40 seconds. For the fresh samples the extraction buffer was added the first time and shaken twice with the samples.

Samples were placed in the centrifuge for 2 minutes at 13,300 rpm. The liquid was removed with a large pipette and placed into a tube labeled the same as the previous tube. 250µL of 7.5mM ammonium acetate (SigmaAldrich, St. Louis, MO) was added and the tubes were vortexed for 10 seconds. Samples were then put in the freezer for 10 minutes. Samples were then centrifuged for 10 minutes to pellet the leaf material. After the centrifugation, the supernatant was poured into a clean tube. 750µL of isopropanol (Target, Minneapolis, MN) was added and the tubes were inverted for 15 seconds. The tubes were then centrifuged at 13,300 rpm for 15 minutes to pellet DNA.

Isopropanol is decanted and the tubes were inverted onto paper towels for 2 minutes. The DNA pellet was then washed with 400µL of 70% ethanol. They were then placed in the centrifuge for 5 minutes. The alcohol was poured off and the tubes were placed in a vacuum for 10 minutes with the tops open. Once dry, 100µL of 10mM Tris pH 8.0 was added to the tube and placed in the refrigerator for storage.

Bullet Blender® method

Thirty-two labeled 1.5mL tubes were filled with 1.0mm Next Advance Zirconium Oxide beads (Next Advance) to the 100µL level. A cutting of approximately 0.5 to 1cm of plant material was placed in the tubes that contained beads. The tubes were placed in the Bullet Blender® instrument and set for 1 minute at speed of 7. For dried plant samples a total of 500µL of extraction buffer was added to the tubes and shaken for 40 seconds. Then the plant samples were then subsequently added to the tubes and shaken for another 40 seconds. For the fresh samples the extraction buffer was added the first time and shaken twice with the samples.

Samples were removed and placed in the centrifuge for 2 minutes at 13,300 rpm. The liquid is removed with a large pipette and placed into a tube labeled the same as the previous tube. 250µL of 7.5mM ammonium acetate (Sigma Aldrich, St. Louis, MO) was added and the tube was vortexed for 10 seconds. Samples were then put in the freezer at -20 °C for 10 minutes. Tubes were centrifuged for 10 minutes to pellet the leaf material. After the centrifugation was complete, the supernatant was poured into a clean tube. 750µL of isopropanol was added and the tubes were inverted for 15 seconds. The tubes were then centrifuged at full speed at 13,300 rpm for 15 minutes to pellet DNA.

The isopropanol was poured off and the tubes were inverted onto paper towels for 2 minutes. The DNA pellet was then washed with 400µL of 70% ethanol, placed in the centrifuge for 5 minutes. The alcohol was poured off and the tubes were placed in a heating block for 10 minutes with the tops open. Once dry, 100µL of 10 mM Tris pH 8.0 was added to the tubes and placed in the refrigerator for storage.

Mortar and pestle method

Plant samples were grinded one at a time in a ceramic mortar using a pestle for about 2 minutes. The plant material was scraped into a labeled 1.5 tube and 500µL extraction buffer is added. The samples were then vortexed for 10 seconds. In between the grinding of samples the mortar and pestle was washed with a 10 % bleach solution, distilled water and dried with paper towels. Once all the samples were vortexed they were placed in the centrifuge for 2 minutes at 13,300 rpm. The liquid was removed from the tube with a large pipette and placed into a tube labeled the same as the previous tube. 250µL of 7.5mM ammonium acetate was added and the tube was vortexed for 10 seconds. Samples were then put in the freezer at -20 °C for 10 minutes. Tubes were centrifuged at 13,300 for 10 minutes to pelletize the leaf material. After the centrifugation, the supernatant was poured in a clean tube. 750µL of isopropanol was added and the tubes were inverted for 15 seconds. The tubes were then centrifuged at 13,300 rpm for 15 minutes to pelletize the cleaned DNA.

The isopropanol is poured off and the tubes were inverted onto paper towels for a few minutes. The DNA pellet was then washed with 400µL of 70% ethanol, placed in the centrifuge for 5 minutes. The alcohol was poured off and the tubes placed in a heating block for 10 minutes

with the tops open. Once dry, 100µL of 10 mM Tris PH 8.0 was added to the tube and placed in the refrigerator for storage.

Quantification

Gel preparation

1.5% agarose gel was created by combining 4.5g of Agarose LE powder (Light Labs USA, Dallas, TX) and 300mL of 1X TAE buffer (Thermo Scientific, Pittsburgh, PA) in a beaker. The beaker was microwaved for 5 minutes. The beaker was taken out after every minute and swirled until the powder was dissolved. The beaker was allowed to cool slightly. As the solution was cooling, the gel apparatus was assembled with the correct comb to get the preferred number of wells. Once the gel was cooled enough it was poured into the gel box and the comb was placed in the appropriate spot. The gel was allowed to firm. This took approximately 30 minutes. Once the gel had set, the comb was pulled out, to reveal the desired wells.

Sample preparation

A portion of wax paper was cut and labeled L for ladder, 1-18 for the number of samples in the gel and C for control. For the first gel, 1µL of 6x bromophenol blue dye was placed onto the wax paper in the same spots for the samples and control. 2µL of the plant sample was added to each of the drops of the blue dye and mixed well. A control of 9947A was prepared with 5µL and 1µL of the dye. 2µL of a 100bp ladder was added. Plant samples and control were vortexted and spun down prior to pipetting.

The total amount of DNA sample and blue dye mixture was pipetted into the desired wells. The electrical leads from the power supply were connected to the gel apparatus. The power source was turned on and the gel ran at 130V for approximately 30 minutes. The power was then disconnected.

For the second gel the amounts of DNA was increased to 10 µL. As previously stated a portion of wax paper was cut and labeled L for ladder, 1-18 for the number of samples in the gel and C for control. 2µL of 6x bromophenol loading blue dye was placed onto the wax paper in the same spots for the samples and control. 10µL of the plant sample was added to each of the drops of the blue dye and mixed well. A control of 9947A was prepared with 10µL and 2µL of the dye. 2µL of a 100bp ladder was added. Plant samples and control were vortexted and spun down prior to pipetting.

The total amount of DNA sample and blue dye mixture was pipetted into the desired wells. The electrical leads from the power supply were connected to the gel apparatus. The power source was turned on and the gel ran at 130V for approximately 30 minutes. The power was then disconnected.

For the last gel the amounts of DNA was maintained at 10µL. As previously stated a portion of wax paper was cut and labeled L for ladder, 1-18 for the number of samples in the gel and C for control. 2µL of 6x bromophenol loading blue dye was placed onto the wax paper in the same spots for the samples and control. 10µL of the plant sample was added to each of the drops of the blue dye and mixed well. A control of 9947A was prepared with 10µL and 2µL of the dye. 7µL of a 1kb ladder was added. Plant samples and control were vortexted and spun down prior to pipetting.

The total amount of DNA sample and blue dye mixture was pipetted into the desired wells. The electrical leads from the power supply were connected to the gel apparatus. The power source was turned on and the gel ran at 130V for approximately 30 minutes. The power was then disconnected.

All three gels were carefully cut and added to a plastic container. A staining dye solution of Gel Red (Phenix Research Products, Candler, NC) was poured to cover the gels. The gels were placed in a dark cabinet for one hour. The gels were then removed from cabinet and placed on the bio imaging system (UVP, Upland, CA). The DNA bands were captured with the imaging system and sent to the computer.

UV spectroscopy

To determine the amount and purity of nucleic acid in the samples NanoDrop 2000 spectrometer (Thermo Scientific, Pittsburgh, PA) was used. To calibrate the instrument a blank sample of Tris buffer, the same Tris buffer used to preserve the samples, was placed on the instrument and allowed to obtain a reading of 0.0ng of DNA. Next, 1µL of the first sample was placed on the instrument and the nucleic acid was measured. The liquid is then wiped away with a Kim™ wipe and the next sample was placed on the instrument in the same manner. These two steps are repeated until all the samples have been measured. A260/A280 ratio was used to determine DNA purity and optical density (OD) readings were used to determine DNA concentration.

Statistics

IBM SPSS Statistics (IMB, Armonk, NY), a statistical software package, was used to determine the significance of the data. A Student's *t*-test was performed in order to determine if

there was a statistical difference between the extractions of fresh or dry samples. The Student's *t*-test determines whether there is a statistical difference between the means of two unrelated groups of the wet and dry samples. The independent variable for this test is whether it was a wet or dry sample and the dependent variable was the DNA concentration. The null hypothesis for Student's *t*-test was that the means for the two groups were equal. The alternative hypothesis is that the two groups were not equal. The alpha significance level to determine this is set at 0.05. This determined if there was significance between the amount of DNA recovered from fresh plants or dried plants.

A one-way ANOVA test was then performed to compare the three extraction methods in order to determine if there was a statistically significant difference between the means of the DNA concentrations and the A260/A 280 ratio of the three extraction methods and where does that difference lies. The dependent variable was the DNA concentration, or the A260/A280 ratio, and the factor was the three extraction methods. Post hoc range test, Tamahane and Dunnett T3 dependent variable test were used when there was no assumption of equal variances. These tests show which method recovered the most DNA, or provided the most pure DNA, and compare the ranges between the three extraction methods. The alpha 0.05 significance level was used.

CHAPTER III

RESULTS

Agarose gel

Three separate agarose gels were run with gel electrophoresis. Each plant species had duplicates of wet and dry plant material. One of the wet and one of the dry duplicate of each plant from each method was used on the gels. The first and second gel had 20 samples and the third had 14. This included a 100 base pair (bp) ladder for the first two gels, 1kb ladder for the third gel, one 9947A cell line control and a wet and dry representative of each plant sample.

The first gel (Fig. 2) contained poinsettia, live oak and oleander. Samples were prepared by adding 2µL of DNA along with 1µL of loading dye along with 5µL of the control (5ng total DNA) and 2µL of 100bp ladder. It was run at 150v for approximately 35 minutes then placed in staining dye (Gel Red) for over an hour. A picture was taken of the gel and the overall quality of the gel bands were poor so the decision was made to let the gel sit in the gel red staining dye for a few more hours. Below is the picture of the gel after it was staining for over 4 hours. The ladder appeared in lane 1 and the control appeared in lane 20. Lanes 2, 3, 6, 7 and 15 showed more intensity than the 5ng control. Lanes 4,5,8,14,18 and 19 showed band intensity approximately equal to the intensity of the 5ng control. The other lanes appeared to have little to no DNA. A few of the bands (lanes 3 and 6) had smearing which indicated degradation.

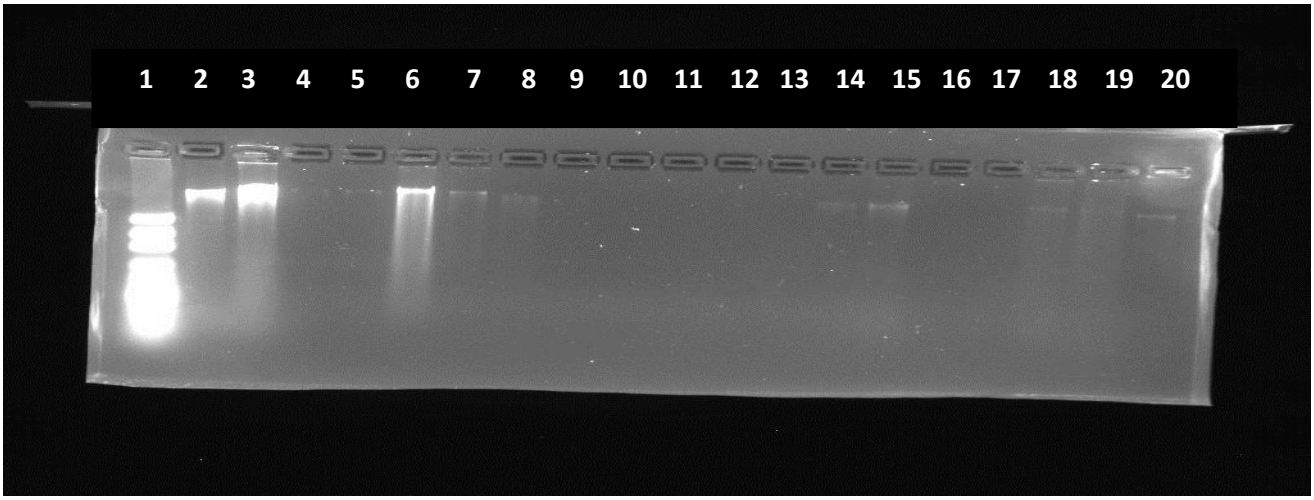

Figure 2. Agarose gel showing bands from poinsettia, oak and oleander. Legend: P=poinsettia, Ok= oak, Ol= oleander, W= wet,D= dry, FP= Fast Prep, BB=Bullet Blender, MP= mortar and pestle Lane 1 -100 base pair ladder. Lane 2- PWFP. Lane 3 PDFP, Lane 4 PWBB, Lane 5 PDBB, Lane 6 PWMP, Lane 7 PDMP, Lane 8 OkFPW, Lane 9 OkFPD, Lane 10 OkBBW, Lane 11 OkBBD, Lane 12 OkMPW, Lane 13 OkMPD, Lane 14 OlFPW, Lane 15 OlFPD, Lane 16 OlBBW, Lane 17 OlBBD, Lane 18 OlMPW, Lane 19 OlMPD, Lane 20 5ng 9947A Control.

The second gel contained elm, African violet and heavenly bamboo. The gel samples were prepared with 5µL of sample DNA and 2µL of loading dye along with 10µL of the control (10ng of total DNA) and 2µL of the 100 bp ladder. The gel sat in the staining solution overnight (Fig. 3).The ladder is in lane 1 and the 10ng control is in lane 20. The DNA bands in lanes 2, 3, 6, 7, 13, 14 and 15 showed more intensity than the 10ng control band in lane 20. Some of these bands had smearing (6, 7, 15 and 18) which indicated degradation. The other lanes showed little or no DNA.

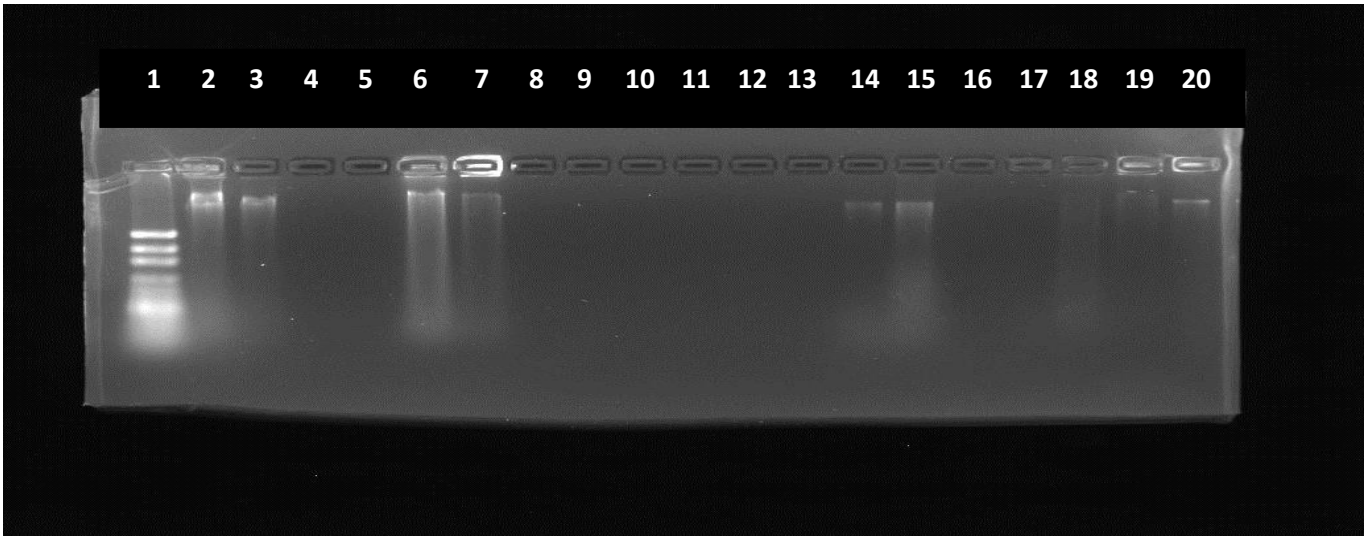

Figure 3. Agarose gel showing bands from Elm, African violet and Heavenly Bamboo. Legend: E=elm, AV= African violet, HB= Heavenly bamboo, W= wet,D= dry, FP= Fast Prep, BB=Bullet Blender, MP= mortar and pestle Lane 1 -100 base pair ladder. Lane 2- EWFP. Lane 3 EDFP, Lane 4 EWBB, Lane 5 EDBB, Lane 6 EWMP, Lane 7 EDMP, Lane 8 AVFPW, Lane 9 AVFPD, Lane 10 AVBBW, Lane 11 AVBBD, Lane 12 AVMPW, Lane 13 AVMPD, Lane 14 HBFPW, Lane 15 HBFPD, Lane 16 HBBBW, Lane 17 HBBBD, Lane 18 HBMPW, Lane 19 HBMPD, Lane 20 10ng 9947A Control.

The third gel contained mistletoe and pine. For this gel 10µL of sample was pepared with 2µL of loading dye along with 10µL of control (10ng total DNA) and 7µL of a 1kb ladder. The gel was stained overnight the in staining soultion. Lanes 2,3,6,7 and 8 produced bands with intensites greater than or equal to the 10 ng control lane. Lanes 6 and 7 had some smearing which indicates some DNA degradation.

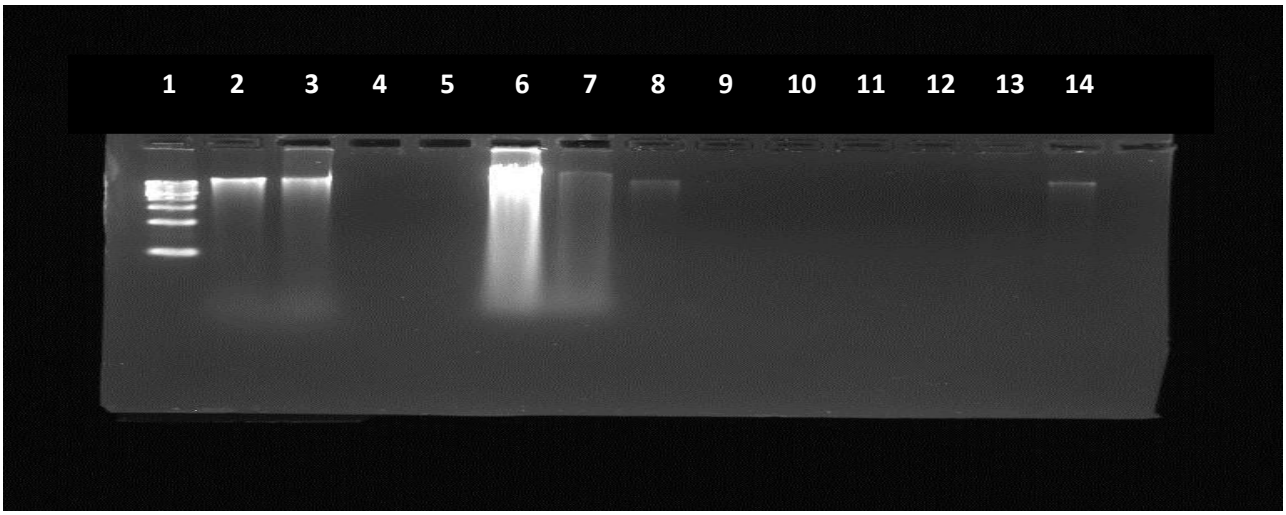

Figure 4. Agarose gel showing bands from Mistletoe and Pine. Legend: M=Mistletoe, P= Pine, W= wet, D= dry, FP= Fast Prep, BB=Bullet Blender, MP= mortar and pestle Lane 1-1kb base pair ladder, Lane 2- MWFP. Lane 3 MDFP, Lane 4 MWBB, Lane 5 MDBB, Lane 6 MWMP, Lane 7 MDMP, Lane 8 PFPW, Lane 9 PFPD, Lane 10 PBBW, Lane 11 PBBD, Lane 12 PMPW, Lane 13 PMPD, Lane 14 10ng 9947A Control.

UV spectroscopy

1ng of each of the 96 samples was placed on the NanoDrop 2000 to determine nucleic acid concentration and DNA purity. Below is a listing of each plant species, whether they were fresh or dried, which method was used the total average DNA concentration and the average A260/A280 ratio. For the concentrations that received negative numbers a zero has been inputted to replace the negative number for that amount. Negative numbers indicate that the DNA concentration was below 2ng and could not be determined accurately.

Plant	Condition	DNA Concentration ng/μL	A260/A280 ratio	Method
Poinsettia	wet	160.1	1.93	Fast Prep
Poinsettia	dry	421.3	3.67	Fast Prep
Poinsettia	wet	2.85	2.72	Bullet
Poinsettia	dry	10.4	2.04	Bullet
Poinsettia	wet	408	1.71	mortar and pestle
Poinsettia	dry	41.7	1.8	mortar and pestle
Live Oak	wet	12.8	.59	Fast Prep
Live Oak	dry	11.2	.895	Fast Prep
Live Oak	wet	.95	32.5	Bullet
Live Oak	dry	.6	0	Bullet
Live Oak	wet	23.3	1.22	mortar and pestle
Live Oak	dry	13.3	1.34	mortar and pestle
Oleander	wet	30.4	1.67	Fast Prep
Oleander	dry	25.7	2.13	Fast Prep
Oleander	wet	1.4	1.38	Bullet
Oleander	dry	1.65	6.65	Bullet
Oleander	wet	77.2	1.71	mortar and pestle
Oleander	dry	74.6	1.61	mortar and pestle
Elm	wet	71.5	1.79	Fast Prep
Elm	dry	14.7	1.72	Fast Prep
Elm	wet	.8	1.34	Bullet
Elm	dry	1	1.25	Bullet
Elm	wet	25.1	2.15	mortar and pestle
Elm	dry	30.8	1.79	mortar and pestle
African violet	wet	0	2.05	Fast Prep
African violet	dry	0	2.80	Fast Prep
African violet	wet	.2	0	Bullet
African violet	dry	0	.58	Bullet
African violet	wet	.85	1.52	mortar and pestle
African violet	dry	.65	1.42	mortar and pestle
Nandia	wet	16.2	1.7	Fast Prep
Nandia	dry	23.5	1.99	Fast Prep
Nandia	wet	1.05	1.68	Bullet
Nandia	dry	.65	1.17	Bullet
Nandia	wet	17.2	1.08	mortar and pestle
Nandia	dry	11.8	1.61	mortar and pestle
Mistletoe	wet	54.4	1.54	Fast Prep
Mistletoe	dry	39.7	1.82	Fast Prep
Mistletoe	wet	91	1.12	Bullet

Mistletoe	dry	106	.665	Bullet
Mistletoe	wet	110	1.53	mortar and pestle
Mistletoe	dry	44.3	1.3	mortar and pestle
Pine	wet	114.6	1.38	Fast Prep
Pine	dry	100.7	.67	Fast Prep
Pine	wet	.65	1.32	Bullet
Pine	dry	0	.77	Bullet
Pine	wet	105.5	1.40	mortar and pestle
Pine	dry	226.15	1.34	mortar and pestle

Table 2. Table summarizing the UV spectroscopy data obtained from the NanoDrop 2000. From left to right: Column 1-Name of Plant; Column 2-Condition of sample; Column 3- Average of the DNA concentration; Column 4- Average of A260/A280 ratio; Column 5- Extraction Method.

An examination of the above data would appear to indicate that the Fast Prep™ method and the mortar and pestle method obtained greater DNA concentrations than those from the Bullet Blender® method. It also seems to indicate that overall there appears to be little difference in recovered DNA between the wet and dry method. The type of plant might also be affecting these results.

SPSS statistical analysis

Independent student *t*-test were used to show if there was any statistically significant differences in the DNA recovered between the wet and dry samples. At an Alpha level of 0.05, p= 0.869 (one-tailed test) or p=0.815 (two-tailed test). Thus, no statistical significance was observed in recovered DNA between the wet and dry storage methods. It is noted that there was a large standard deviation (SD) for both of the samples. Wet SD=119.16 and dry SD= 98.91. Possible explanations and implications for this are discussed in Chapter IV.

A one-way ANOVA test on the three extraction methods was performed. At an alpha level of 0.05 a statistically significant difference in variance was found ($p=0.044$). Two post hoc tests Tamahane and a Dunnett T3 were then employed to further elucidate that significance. These tests demonstrated a statistically significant difference between the Fast Prep-24™ and the Bullet Blender® at an alpha level of 0.05 ($p=0.040$). No significant difference was observed between the Fast Prep-24™ and mortar and pestle ($p=0.994$). No statistically significant difference between the Bullet Blender® and the mortar and pestle method was found ($p=0.067$).

A one-way ANOVA test comparing the A260/A280 ratios was performed in order to determine if there was any statistically significant differences between the three extraction methods regarding the purity of the isolated DNA. At an alpha level of 0.05 ($p=0.375$), no statistically significance differences were found.

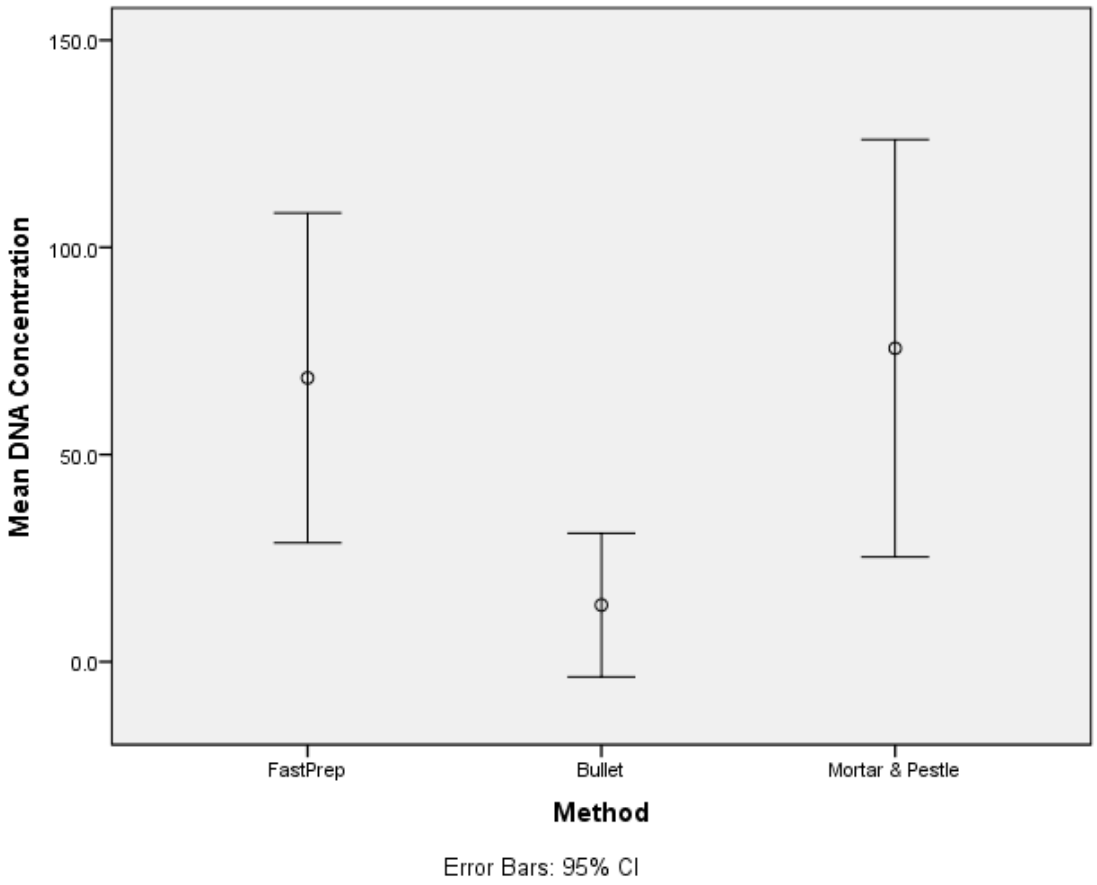

Figure 5. SPSS graph showing the FastPrep24® and mortar and pestle obtaining higher DNA concentrations than the Bullet Blender®. Little to no overlap is observed between the Fast Prep™ and Bullet Blender® method. A high degree of overlap was observed between the Fast Prep™ and mortar and pestle method. Slight overlap was observed between the Bullet Blender® and mortar and pestle. These results correspond to the one-way ANOVA results.

Figure 5 illustrates the one–way ANOVA test results. An examination of the plots reveal overlap between the mean DNA concentrations of the Fast Prep-24™ and mortar and pestle methods thus accounting for the lack of statistically significant differences. There is no overlap observed between the Fast Prep-24™ and Bullet Blender® method which accounts for the significant differences between those two methods. There is a small but evidently still significant enough overlap between the Bullet Blender® and mortar and pestle procedures to remove what would appear to be a significant difference if one were to exam the DNA concentration data without the one-way ANOVA test for significance data.

CHAPTER IV

DISCUSSION & CONCLUSION

In summary, three lysing methods were compared to determine the best method for the use at the Botanical Research Institute of Texas. The Fast Prep-24™ process worked by adding a plant sample and extraction buffer into a Lysing Matrix D tube and placed in the instrument and shaken at 60 meters per second for 2 rounds of 40 seconds. The Bullet Blender® worked in a similar manner in such that the plant sample is added to a tube with the appropriate beads and extraction buffer. The tube is placed on the instrument and shaken. The mortar and pestle is manual and requires grinding the plants before placing in extraction buffer. Although the lysing methods were different the same extraction chemistries were used to keep the project consistent. The Fast Prep-24™ was determined to yield the higher DNA concentration.

Other than the extraction methods themselves, several experimental variables could have also affected this project's outcome. The most troubling aspect in interpreting the significance of this study was lack of a standardized sampling method to prepare the samples for extraction. As mentioned in the Materials and Methods, the samples were first cut into 0.5 mm-1.5 mm squares. Examination of the standard deviations obtained from the wet versus dry Student's *t*-test, (SD=98.91 for the dry and SD=119.16 for the wet) indicate that a more controlled and consistent sample preparation method can be employed. The 95% confidence intervals, P ($1.905 \leq \mu \leq 107.739$), for the Tamhane post hoc test between the FastPrep24™ and the Bullet Blender® method also serve as evidence for this observation. The optimal method would have been to weigh each sample and use the same weight for each method. This alone could have lowered the

standard deviations and produced a smaller and more precise 95% confidence interval. Another possible variable is operator's experience. After the project was completed and the data analyzed, one explanation for some of the variation was that more practice extractions should have been completed before collecting data so as to minimize analytical variation and insure consistency between samples and reduced variation between the duplicate samples.

Though the samples were run in duplicate, due to financial and time constraints, additional samples would have provided better statistically definitive results. At least triplicate or even greater sample numbers would yield additional information that could have provided further strength or clarity to the statistical results.

In addition, both Fast Prep-24™ and Bullet Blender® have optional extraction kits that can be used with the instrument. Using the kits created by the manufacture could have yielded different results than the extraction protocol used in this research project.

Training for both automated instruments is minimal and doesn't require much time. These instruments can process a minimum of 24 samples at once. This could certainly shorten labor hours which will in the long run reduce overall cost. The mortar and pestle is less expensive compared to the instruments but very time-consuming. This could cost more in the long run when the price of labor is calculated into the project. In addition when grinding the plant material with the extraction buffer the liquid was absorbed into the grooves of the mortar which could be problematic. This made it extremely difficult to retrieve the lysed plant sample and greatly increases the chance for cross contamination. Plant material from the previous samples could still be imbedded into the mortar (figure 6).

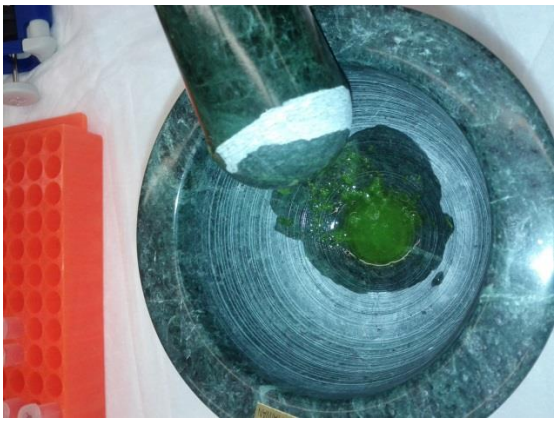

Figure 6. Photograph of sludge created by adding extraction buffer to the mortar and pestle as the sample is being grinded. This could contaminate the next samples being processed.

This could make it difficult when trying to obtain pure PCR products. It would be difficult, time-consuming and labor intensive to clean off enough material, especially DNA to insure that no sample-to-sample contamination would occur. This is particularly true if PCR based tests will be used. The Fast Prep-24™ has individual tubes for each sample, thus reducing the chance for cross-contamination. The Fast Prep-24™ instrument costs $10,000 plus additional fees for matrixes and the accompanying extraction kits. The cost is worth the time saved with processing 24 samples at once. The Fast Prep-24™ is also quieter than the Bullet Blender® which will not disturb the laboratory atmosphere.

Agarose gels were able to provide a limited amount of qualitative data. After staining in the Gel Red solution over night, the gels still had bands and ladders that were not as intense as expected. A practice gel with a staining solution of ethidium bromide had intense bands but because of the hazards of working with that compound it was decided to use a nontoxic solution. The use though of the ethidium bromide might have provided increased sensitivity and thus more information. Because of the low intensity of the gel band the amount of DNA was increased with the second and third gels. This was done because it was thought that addition of DNA would

have stronger bands but in actuality those plants had low DNA concentration which was proven by the UV spectroscopy results.

Observation of the variation among the plant samples suggest that some plants are easier to lyse than others. The plants that received low or no amounts of DNA contain compounds that bind the nuclear DNA or contain compounds such as phenols that degrade the DNA. This could possibly be avoided by using an extraction technique that contains a heated incubation step. Heating the samples with certain reagents could degrade the products that interfere with the DNA being released.

Though a visual examination of the spectroscopic data would appear to demonstrate that the wet samples gave a higher DNA yield than the dry samples, the Student's *t*-test revealed this difference was not statistically significant. While DNA yield may no longer be a factor as to whether to use wet versus dry samples, other factors to be considered are sample transportation and storage time between field collection and laboratory. In forensic casework, sample type is often not a matter of choice but of circumstance and necessity.

Despite the admitted design problems with this study, especially the lack of quantitatively controlling for the amount of input material, an examination of the data can still yield some useful conclusions. The differences in DNA yield between the Fast Prep-24™ and the Bullet Blender® method seem too large to be due to inconsistent sample preparation only and must have some reflection on the method itself. It cannot be contested that the Fast Prep-24™ method is faster, less labor-intensive, less prone to contamination and amenable to automation for large scale operations than the mortar and pestle method. The data and information from this study can be used to design a more complete future study that takes into account weighing the samples

before extraction, use of an experienced analyst and increased sample number with at least three to six repetitions per sample.

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APPENDIX A

The following tables are created by SPSS software. ANOVAs and post hoc test were created using the NanoDrop 2000 data for DNA concentration and A260/A280 ratios.

Group Statistics

	Wet or Dry	N	Mean	Std. Deviation	Std. Error Mean
DNA Concentration	dry	48	49.996	98.9135	14.2769
	wet	48	55.235	119.1598	17.1992

Table A. Group statistics for the wet and dry samples.

ANOVA

DNA Concentration

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	**73527.331**	**2**	**36763.665**	**3.243**	**.044**
Within Groups	**1054330.136**	**93**	**11336.883**		
Total	**1127857.467**	**95**			

Table B. ANOVA for the DNA concentrations for the three methods.

Dependent Variable: DNA Concentration

	(I) Method	(J) Method	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Tamhane	FastPrep	Bullet	54.8219*	21.2869	.040	1.905	107.739
		Mortar & Pestle	-7.1219	31.4754	.994	-84.475	70.231
	Bullet	FastPrep	-54.8219*	21.2869	.040	-107.739	-1.905
		Mortar & Pestle	-61.9437	26.1120	.067	-127.142	3.255
	Mortar & Pestle	FastPrep	7.1219	31.4754	.994	-70.231	84.475
		Bullet	61.9437	26.1120	.067	-3.255	127.142
Dunnett T3	FastPrep	Bullet	54.8219*	21.2869	.040	1.989	107.654
		Mortar & Pestle	-7.1219	31.4754	.994	-84.391	70.147
	Bullet	FastPrep	-54.8219*	21.2869	.040	-107.654	-1.989
		Mortar & Pestle	-61.9437	26.1120	.066	-127.025	3.137
	Mortar & Pestle	FastPrep	7.1219	31.4754	.994	-70.147	84.391
		Bullet	61.9437	26.1120	.066	-3.137	127.025
Games-Howell	FastPrep	Bullet	54.8219*	21.2869	.036	3.120	106.523
		Mortar & Pestle	-7.1219	31.4754	.972	-82.801	68.557
	Bullet	FastPrep	-54.8219*	21.2869	.036	-106.523	-3.120
		Mortar & Pestle	-61.9437	26.1120	.058	-125.611	1.723
	Mortar & Pestle	FastPrep	7.1219	31.4754	.972	-68.557	82.801
		Bullet	61.9437	26.1120	.058	-1.723	125.611
Dunnett C	FastPrep	Bullet	54.8219*	21.2869		2.431	107.213
		Mortar & Pestle	-7.1219	31.4754		-84.589	70.345
	Bullet	FastPrep	-54.8219*	21.2869		-107.213	-2.431
		Mortar & Pestle	-61.9437	26.1120		-126.210	2.323
	Mortar & Pestle	FastPrep	7.1219	31.4754		-70.345	84.589
		Bullet	61.9437	26.1120		-2.323	126.210

Table C. Tamhane and Dunnett T3 Post hoc test comparing the three methods with the DNA concentrations.

ANOVA

260/280 ratio

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	73.633	2	36.816	.992	.375
Within Groups	3452.269	93	37.121		
Total	3525.902	95			

Table D. ANOVA for the 260/280 ratio for the three methods.