Sample Preparation and DNA Extraction for the Detection of Allergenic Nut Materials

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Summary

DNA analysis can be thought of as a series of procedural steps, starting with, for example, sampling, sample preparation, DNA extraction, DNA quantitation, PCR setup, equipment operation, software analysis, manual analysis, and user interpretation. Measurement uncertainty can affect any of these procedural steps, but the upstream stages of sampling and sample preparation are known to have a large effect upon the confidence with which a result is expressed [1].

We describe here an analytical approach for the effective sample preparation of nuts, to maximise the quantity and quality of DNA extracted from raw nut materials. The approach includes the de-shelling of nut material, novel sample treatment and grinding, and subsequent DNA extraction. The "fitness for purpose" of this approach was verified by measuring the quantity and quality of the DNA using UV spectrophotometry and subsequent PCR tests.

***Keywords**: DNA, DNA extraction, nuts, allergens, sample preparation*

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Introduction

Since 1990, records have indicated that there have been an increasing number of people suffering from allergic reactions. 1.5 million people in the UK are currently affected and it is estimated that 10 to 20 deaths (and many more near miss incidents) occur per year due to allergies [2]. Approximately one in four people in the UK population suffer from allergic responses at some stage in their lives, and this figure is increasing on a year by year basis, with many of those affected being children [3]. It is estimated that it costs the NHS over £1 billion in relation to managing allergies within the UK, with affected individuals experiencing a reduced quality of life and possible risk of death. NHS resourcing, food producers, and the UK economy are all affected by this incidence [4].

Currently food avoidance remains the only treatment for allergy and the main strategy in prevention of anaphylaxis in children with acute food allergies [5]. For this reason, food producers and suppliers have seen an increase in demand for "allergen free" product lines with accurate labelling to facilitate avoidance of allergens. Cross contamination of ingredients in food harvesting, storage and production needs to be negated, and crucial to the success of these measures is the development of accurate and effective techniques for detection of allergenic ingredients.

Current methods for allergen detection principally focus on the detection of proteins extracted from the target food matrix, but there appear to be relatively few fully validated protocols in the scientific literature [6]. Additionally, although protein based approaches are generally of high specificity this is not always the case as antibodies used can cross react with other allergenic proteins. They can also be criticised for a lack of sensitivity (low concentrations may not be detected); and the requirement of a large sample size for extraction [7]. Alternative approaches to allergen detection include using DNA as a target analyte. There are several published articles available that describe the use of the Polymerase Chain Reaction (PCR) to amplify sequences from DNA specific to food substances known to cause allergic responses e.g. nut material [8]. DNA has some advantages over proteins in that it tends to be more resistant to degradation during food processing stages, thus potentially enabling

analysis of raw, cooked, and processed food materials. Additionally, PCR has the potential to be more sensitive than more traditional protein approaches.

Whilst DNA approaches may provide alternative and complimentary methods to protein approaches for the detection of nut allergens, the DNA molecular biology field is not as well developed in relation to routine allergen detection. Consequently there are very few published protocols for the effective sample preparation and DNA extraction from nut materials, and there is a need for best practice guidelines to be produced in this area. This document provides initial guidance regarding sample handling and preparation of raw nut materials for effective DNA extraction, including a novel method for material grinding. Traditional methods for sample preparation rely on combined use of liquid nitrogen with a pestle and mortar to grind material, but the current work describes a more efficient method that uses dry ice in combination with a food grinder to achieve better results. This protocol was optimised for the effective extraction of DNA from a variety of nuts including cashew, pecan, peanut, brazil, walnut, hazelnut, pistachio, almond, macadamia, pine nut, chestnut, and is also applicable to sesame seeds.

Protocol for Sample Handling and DNA Extraction from Raw Nuts

Sample Registry and Storage

All samples should be described according to in-house working procedures, recording pertinent details such as a unique sample identification number, description of the sample (including source and nature), analysis required, sample storage location, expiry date and disposal date. It is also good practice to record other pertinent information unique to nut samples if these have been purchased as packaged commodities, such as the disclaimer for presence of other nuts, the ingredient list, best before date, where purchased, and country of origin. If possible the exact botanical name of the species should be ascertained.

Nut materials are highly perishable with exposure to light, heat, moisture and air all contributing to rancidity in samples due to their high oil content. Additionally, proper storage of nuts is extremely important due to the occurrence of aflatoxins, a mycotoxin commonly found on incorrectly stored samples. Nuts-in-shell have a longer shelf life than de-shelled nuts and will keep for up to four months at room temperature.

Storage in vacuum-packed containers is preferable compared to cellophane packaging due to the potential permeability of the latter. At room temperature, nut samples will perish within a few weeks but when refrigerated can be kept for 6 months. Nut samples may also be frozen up to 1 year at -20°C.

Removal of Shells from Nut Material

It is recommended that nut samples have the shells removed before the grinding procedure. This is done to eliminate any potential contamination from other food materials (particularly other nuts) where the outside of the shells may have come into contact with other foods. Contamination can easily occur through the co-mingling of different nut varieties in harvesting, transport, storage and on the same factory production line. Cross contamination is also possible via surfaces, equipment, garments and even hands. Adequate cleaning procedures need to have been introduced to remove the allergens of interest from the work area. During the nut de-shelling process there is a need to minimise any potential cross-contamination, therefore the innovative use of commercially available "glove bags" is recommended.

All the parts of the work bench need to be cleaned using a suitable cleaning agent. One disposable inflatable "glove bag" chamber was used per nut sample type (Figure 1). The glove bag chambers were utilised to avoid cross contamination of samples and work areas. The glove bag was labelled and the rear-mounted gas port-end was sealed with an appropriate clip. The following items were placed inside the glove bag chamber through the entry aperture: one pair of gloves (nitrile or latex powder free); three re-sealable bags; one clean and dry nut cracker; one dry paper towel sheet; one paper towel soaked in cleaning agent; and

an appropriate quantity of nuts-in-shell.

The glove bag opening was folded and sealed with an appropriate food bag clip. Nitrogen gas (or other inert gas) was introduced into the chamber via the gas port and sealed when filled.

Figure 1. Photograph of glove-bag used as part of the sample preparation to minimise chances of cross contamination

The operator's hands were inserted into the glove compartments and additional gloves were placed over the glove compartments to permit adequate sample handling and manipulation whilst working within the glove bag chamber. The nut sample to be processed was then placed into a re-sealable bag in order to enclose it at a later stage. Individual nuts had their shells removed using the nut cracker within the chamber. All waste materials including waste

shells were disposed of within a third re-sealable bag.

All internal surfaces within the glove bag chamber should be cleaned to remove contaminants prior to opening to remove the de-shelled samples. A paper towel soaked in cleaning solution was used to wipe all internal surfaces and was disposed of in the waste re-sealable bag containing the waste shells. All loose shell material was disposed of in the waste shells zip-lock bag. The re-sealable bag containing the de-shelled samples was dried with the dry paper towel. The gloves were removed and also disposed of in the waste bag. All bags enclosed in the chamber were resealed before the glove bag was opened by releasing the clip at the opening. The nut cracker was removed from the chamber, particulate matter removed, and the nut cracker cleaned by allowing it to soak in the cleaning agent for 30 min. The bags containing the samples-in-shell and the de-shelled nuts were removed from the chamber and labelled appropriately. The outside surfaces of both these bags were cleansed with cleaning agent and both stored at 4°C.

Grinding of Nut Material

A single dedicated mini food grinder was used for each nut variety, minimising the potential for cross contamination. All surfaces of the grinder were decontaminated (both inside and outside) by wiping with cleaning agent. All the parts of the grinder were left to dry on the work bench for 10min.

All equipment surfaces, including the top pan balance, were cleaned using an appropriate cleansing solution. All utensils used for the weighing process were decontaminated using the cleaning agent. Duplicate aliquots of 15g of whole de-shelled nut samples were weighed out into 50mL centrifuge tubes. Both tubes were placed inside a re-sealable bag and frozen at -80°C for 2 hours. Prior to commencing the grinding procedure, two 15g aliquots of commercially available dry ice were weighed into appropriate 50mL beakers and placed in a cool box.

A new glove bag (or a previous one used for the same nut variety) was prepared for the

grinding procedure by loading the chamber with the following: one pair of gloves; a stainless steel spatula; one mini food grinder (decontaminated); one dry sheet of paper towel; one paper towel soaked in cleaning solution; two empty re-sealable bags; the two x 15g of de-shelled nut samples in 50mL centrifuge tubes; and two x 15g dry ice in 50mL plastic beakers.

The glove bag opening was folded and sealed as securely as possible with a metal clip to ensure minimal contamination via the opening port of the glove chamber through which the power cable to the mini food grinder was connected. Nitrogen (or other inert gas) was introduced into the chamber to fill the chamber via the gas port. Work continued by inserting hands into the chamber gloves and one pair of gloves placed on top of the bag gloves to permit adequate sample handling and manipulation.

15g of dry ice and the contents of the 50mL centrifuge tube containing the nut sample (15g) were placed into the mini grinder. The mini grinder was closed and the contents pulsed for 10s before inspecting the contents through the translucent lid. Pulsing was repeated for a further 10s if the contents remained bulky or uneven (ground samples produce a fine powder appearance with even particulate size). The sample mixture was transferred back into the 50mL centrifuge tube utilising the metal spatula to aid full recovery of the sample. The lid of each 50mL centrifuge tube was left one quarter turn open to allow CO_2 to escape. The procedure was repeated for the duplicate sample. Prior to retrieving the ground samples, all 50mL centrifuge tubes were closed securely and placed inside re-sealable bags. All surfaces within the glove bag were decontaminated using the moistened paper towel soaked in cleaning solution, then dried with the dry paper towel. All contaminated utensils, gloves and paper towels were bagged and sealed. The inside of the grinder exposed to the sample was not cleaned as only one type of sample would be processed in any one mini grinder. The glove bag chamber was unclipped and the grinder sealed in a bag and labelled for the specific nut species for later use. The sample was stored at 4°C before extracting DNA.

DNA Extraction

A cetyltrimethylammoniumbromide (CTAB) extraction protocol that had been adapted for use with foods with high lipid content was utilised. Each 2 g sample was mixed in a 50mL centrifuge tube with 10mL of CTAB-extraction buffer (2% [w/v] CTAB, 1.4 M NaCl, 20mM ethylenediaminetetraacetic acid, 100mM Tris–OH/HCl) and 30µL Proteinase K (20mg/mL), and incubated at 65°C overnight.

The mixture was centrifuged at 5,000×g for 5min and 1000µL of the supernatant transferred to a 1.5mL centrifuge tube and centrifuged at 14,000×g for a further 5min. 700µL of the supernatant was transferred into a new 1.5mL centrifuge tube and mixed with 500µL of chloroform/isoamylalcohol (24:1) and centrifuged at 16,000×g for 15min. 500µL of the supernatant was transferred to a new 1.5mL centrifuge tube and 500µL cold isopropanol (stored at −20°C) was added. The mixture was kept at room temperature for 30min. The mixture was then centrifuged at 16,000×g for 15min and the supernatant discarded, the pellet was washed with 500µL cold ethanol (70%, stored at −20°C) then centrifuged at 16,000×g for 5min. The ethanol was discarded and the pellet dried at 50°C for an hour before re-suspending in 100µL of molecular biology grade water. The dried DNA pellet did not always dissolve well in water, therefore each DNA extraction was additionally purified twice utilising the Wizard® DNA Clean-Up System to eliminate further impurities. The Wizard® DNA Clean-Up System allowed the pellet to re-suspend and was a critical step, as the DNA extraction procedure often left behind a significant alcohol residue which may have interfered with the downstream manipulation of extracted DNA. The Wizard® DNA Clean-Up System allowed the DNA to be standardised as it was processed twice.

DNA Quantitation

DNA quantitation of the extractions was performed using the NanoDrop® ND-1000 Spectrophotometer according to the manufacturer's instructions. The NanoDrop® ND-1000 uses a surface tension system enabling the analysis of µL sample volumes. A baseline was set on the instrument using the eluate for the DNA purification step, a 1.3µL sample was applied

directly onto the lower measurement pedestal. The sample apparatus was closed and (due to surface tension) a column drawn between the upper and lower measurement pedestals establishing a measurement path. The spectral measurement was recorded then the surface of both measurement pedestals wiped with a tissue to remove the sample. The mean value from three replicate readings were used to establish the concentration of DNA present in ng/µL. UV spectrometry or fluorometric approaches (e.g. PicoGreen®) may also be used to quantitate DNA extractions.

Typical yields of DNA from a 50µl extract were around 50-150 ng/µl, dependent upon the nut variety used. Typical absorption ratios for 260/280 and 260/230 gave a mean and standard deviation of 2.11 ±0.15 and 2.27 ±0.42 respectively, indicating the effective removal of proteins, salts and alcohols that may inhibit subsequent PCR reactions.

The quality of the extracted DNA for use in PCR reactions was confirmed using a real-time PCR assay, that would amplify all eukaryotic DNA sequences and targets the 18s rRNA gene [9]. The "fitness for purpose" of the sample handling and DNA extraction approaches were verified by applying the procedure to a range of nut species including almond, brazil nut, cashew, hazelnut, macadamia, pecan, peanut, pine nut, walnut and in addition sesame seeds. The DNA extracted from this range of nut materials all amplified and gave detectable PCR products (Figure 2). Cycle threshold values (Ct values) are shown in Table 1.

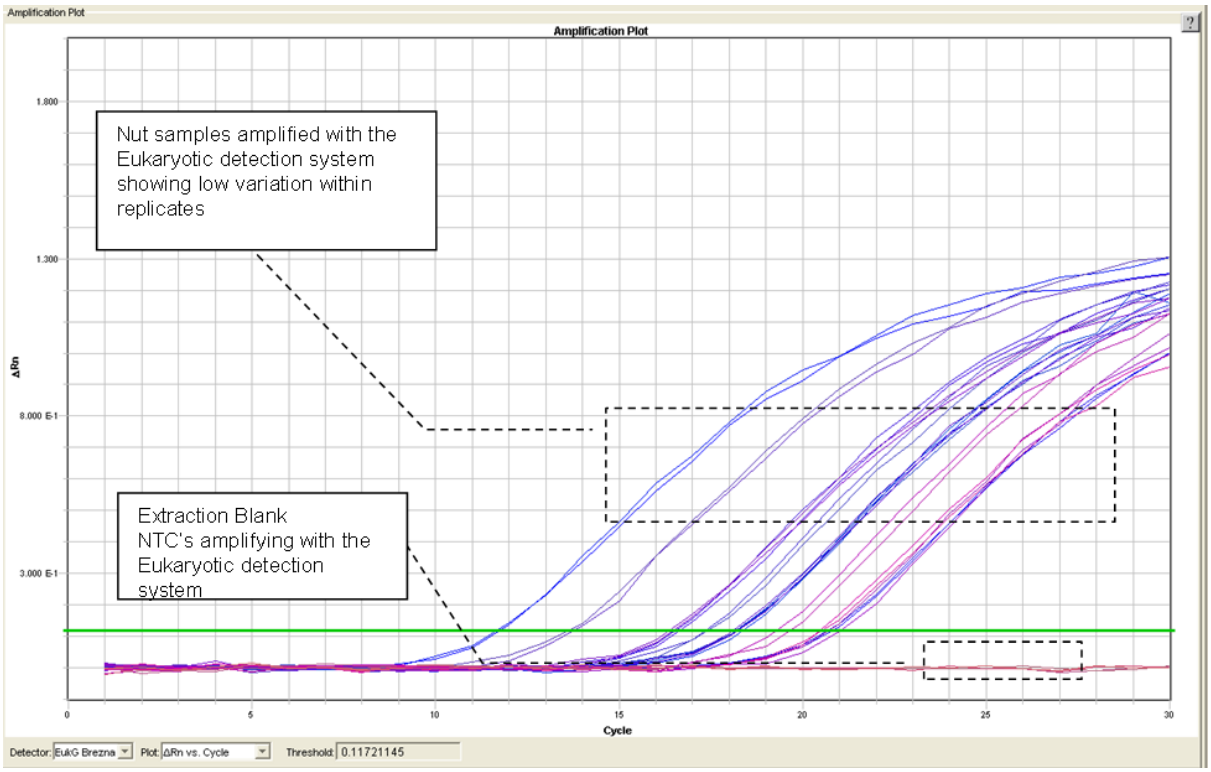

Figure 2 Amplification using Real-time PCR of the nut varieties shown in Table 1 using the eukaryotic detection system. The x-axis represents the number of cycles and the y-axis represents the fluorescent response. The starting DNA template amount was 50ng per PCR reaction, and duplicate reactions were conducted per nut variety.

Nut type	Ct (Mean +/- Standard Deviation)
Almond	11.71 +/- 0.02
Pistachio	18.26 +/- 0.11
Brazil	20.73 +/- 0.06
Sesame	18.15 +/- 0.03
Cashew	16.58 +/- 0.06
Walnut	17.38 +/- 0.12
Hazel	18.14 +/- 0.11
Macadamia	16.40 +/- 0.07
Peanut	20.83 +/- 0.25
Pecan	19.43 +/- 0.22
Pine nut	20.45 +/- 0.05

Table 1. Results of the mean and standard deviation of Ct values associated with the eukaryotic detection system using DNA extracted from nut varieties shown in Figure 2.

Conclusion

We have described here a protocol for the effective shell removal, novel grinding stage, and extraction of DNA that has been shown to be "fit for purpose" for use in downstream PCR reactions across a range of nut varieties. These approaches can form a basis for standardised guidelines for the detection of specific DNA sequences originating from nut varieties known to have allergenic properties in food substances

Acknowledgements

The work described in this paper was funded in part by the UK National Measurement System under the Measurement for Innovators Programme.

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Equipment and Materials

- Chloroform/isoamylalcohol (24:1) (25666 Fluka, Sigma-Aldrich St. Louis, MO 63178)
- Cleaning agent (Microsol^{3+} 1x Anachem MIC-203)
- Coffee grinder (MicroMark MM9894)
- Disposable inflatable "glove bag" chamber (Fisher Scientific GXH-300-010E)
- Dry ice (DruKold UN No1845)
- Molecular biology grade water (Sigma)
- NanoDrop® ND-1000 Spectrophotometer (Thermo Fisher Scientific, NanoDrop products, Wilmington, DE 19810, USA)
- Re-sealable bags (re-sealable, plain, polyethylene storage Minigrip® bags: Fisher Scientific BAJ-360-190U)
- Wizard® DNA Clean-Up System (Promega, Madison, WI 53711 USA)