DETECTION OF THE PRESENCE OF HAZELNUT DNA IN VIRGIN OLIVE OIL USING REAL-TIME PCR

1. INTRODUCTION

Olive oil is increasingly popular in the UK. The premium prices it attracts may provide unscrupulous producers with the incentive to substitute a part of the olive oil with less expensive vegetable oils. At present, adulteration of olive oil with hazelnut oil is a particular problem and is reportedly widespread in some regions. The detection of such substitution is required to ensure food safety, to protect the interests of consumers and honest traders, and to uphold food law.

Due to the similar chemical compositions of olive and hazelnut oils, the detection of adulteration is not readily achieved by the usual analytical methods (e.g. HPLC) for vegetable oils.

DNA-based methods using the polymerase chain reaction (PCR) have been developed for the qualitative detection of product substitution in a range of foodstuffs, including vegetable oils. The selection of DNA targets is key to the successful outcome of DNA-based tests. The use of multi-copy genes (such as those from chloroplasts) as PCR targets increases the detection sensitivity, thus enhancing the chances of qualitative detection. This method targets a region of the hazelnut 5S rRNA gene. This gene is located on the chloroplast genome, making it a useful multi-copy gene.

2. SCOPE AND FIELD OF APPLICATION

This method is suitable for detecting the presence of hazelnut DNA in DNA extracted from virgin olive oil. Presence of the hazelnut 5S rRNA target sequence in a DNA extract provides a strong indication of the presence of unrefined hazelnut oil in the olive oil sample.

The method is based on real-time PCR (qPCR) amplification of an 84bp DNA target from the hazelnut (*Corylus avellana*) 5S-rDNA gene. The method is suitable for the qualitative detection of DNA extracted from the unrefined hazelnut oils shown in Appendix 1 at a level equivalent to or greater than the amount of DNA present in 2% (v/v) of hazelnut oil H5* in olive oil.

At this LOD, 66% of the hazelnut oils analysed during validation of this method were detected.

3. REFERENCES

Dooley, J.J., Garrett, S.D. and Brown, H.M. (2003) Development of molecular markers suitable for the detection of olive oil adulteration with hazelnut oil. FSA Final Report, Q01060.

Dooley et al. (2006). Validation of a DNA-based method for the determination of hazelnut oil in olive oil. FSA Final Report for Project Q01095.

4. PRINCIPLE OF METHOD

The method uses real-time PCR (qPCR) with TaqMan™ chemistries to detect the presence or absence of hazelnut DNA in DNA extracts from oil samples.

5. PRE-TRAINING REQUIREMENTS

Guidelines for the use of DNA methods.

Operation of the 7700 Sequence Detection (TaqMan™) System.

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* H5 –Cold pressed hazelnut oil, variety Tombul. Hazelnuts originated from Turkey (2004 harvest). Oil was pressed by Statfold Seeds Oils in March 2005.

6. EQUIPMENT

- Gilson pipettes & sterile, aerosol resistant PCR grade filter tips.
- PCR plates (96 well) with ultra-clear lids for qPCR & block for holding plates.
- Eppendorf tubes (clear and amber) and tube rack.
- Bench-top centrifuge.
- A sterile PCR preparation cabinet with UV lights.
- ABI7700 real-time PCR machine (TaqMan)

7. REAGENTS

All reagents should be of a suitable purity defined for molecular biology analysis (e.g. Sigma Molecular Biology products). The water used should be sterile Milli-Q water or equivalent purity.
PCR reagents are stored in a PCR reagents freezer at -15ºC to -22ºC for up to two years, unless otherwise stated.

- TaqMan Universal PCR Master Mix.
  This is available from Applied Biosystems, catalogue no. 4304437.
  Store in the dark at 4°C. Do not freeze this buffer.

- Primer Solutions, 100µM (Stock) and 10µM (Working Stock)

Primers should be of HPSF (high purity, salt-free) quality. Primer specifications are shown in Table 1.

Table 1: Primer and Probe Specifications

Target Sequence	Hazelnut 5S rRNA gene
PCR product size	84 bp

Primer/Probe Name	Sequence	Fluorescent Label
Hazel-5S-For	5'– GGA GAC ACT CGT GCC TTC TTG –3'	–
Hazel-5S-84R	5'– CGA CCG GAG GCA CTC TTT AGT –3'	–
Hazel-5S—P	5' – AAC AAC GAA CCC CGG CGC G – 3'	VIC

Reconstitute the primers specified in Table 1, using sterile Milli-Q water according to the manufacturer's instructions, to produce a primer concentration of 100µM. Transfer half the solution into a labelled, sterile 1.5ml Eppendorf tube.

Use one aliquot of the 100µM solution to prepare a 10µM working stock primer solution. Aliquot this working stock solution into 100µl amounts in 1.5ml Eppendorf tubes.

All primer solutions (100µM and 10µM) can be stored in a dedicated PCR freezer at -15°C to -22°C for up to 24 months.

- Probe Solutions, 50µM (Stock) and 5µM (Working Stock)

Probes are light sensitive and should be stored in the dark. When working with probes, avoid excessive exposure to light and use amber Eppendorf tubes.

Reconstitute the probe (Hazel-5S-P) specified in Table 1 using sterile Milli-Q water to achieve a 50µM stock solution. Detailed instructions for reconstituting and diluting probes are available from the Applied Biosystems website.

Transfer half the stock solution into a labelled, sterile 1.5ml amber Eppendorf tube.

Use one aliquot of the 100µM solution to prepare a 5µM working stock probe solution. Aliquot this solution into 50µl amounts into 1.5ml amber Eppendorf tubes.

All probe solutions (50µM and 5µM) can be stored in a dedicated PCR freezer at -15°C to -22°C for up to 24 months.

8. SAMPLE PREPARATION

- **DNA Samples**

DNA samples are extracted from oils using a suitable method. DNA yields are generally too low to allow DNA quantification.

To avoid PCR inhibition, DNA extracts should be diluted to 1 in 8 using sterile Milli-Q water prior to PCR.

- **Hazelnut DNA spike solution**

A hazelnut DNA spike solution is analysed with every batch to ensure PCR inhibition does not occur.
To prepare a spiked sample, thaw an aliquot of PCR positive control solution (see Appendix 2) and add 2µl of the hazelnut DNA spike solution to 3µl of the 1 in 8 diluted sample DNA.

- **PCR positive**

A PCR positive (see Appendix 2), using DNA extracted from hazelnuts and diluted to 50ng/µl, is analysed with every batch.
To prepare the PCR positive control add 2µl of the control DNA solution to 3µl of Milli-Q water.

- **PCR negative**

A PCR negative (known as No Template Control [NTC]), where Milli-Q water is used in place of sample DNA, is analysed with every plate.

- **Extraction positive control**

An extraction positive is analysed with every batch of samples. The positive is a blend of 5% (v/v) hazelnut oil in olive oil. This is extracted with every batch of samples.

- **Extraction negative control**

An extraction negative control (which was extracted with the batch of samples, using sterile Milli-Q water in place of a sample) is analysed with every batch of samples.

9 PROCEDURE

This method is suitable for the detection of hazelnut DNA in DNA extracts from virgin olive oil. The assay has been used to detect the presence of as little as 0.5% (v/v) unrefined hazelnut oil in olive oil.

Before preparing and setting up the reactions, complete an assay layout sheet with the samples for analysis and all controls that need to be run.

Use the layout sheet to assist with preparation of PCR reactions and sample analysis.

- **Preparation of PCR Reactions**

The following should be performed in the PCR preparation cabinet.

For each sample to be analysed, it is necessary to provide enough PCR Mastermix for three replicate PCRs and a spike in a 1/8 dilution, i.e. four PCRs per sample. In addition, each batch of samples requires a PCR positive, a PCR negative, a DNA extraction positive and a DNA extraction negative to be analysed.

Place a new 96 well PCR plate into the support block and lay out sufficient clear plastic lids.

Label a 2.0ml Eppendorf tube (or suitable vessel for larger volumes) for preparation of hazel mastermix and place in rack.

UV sterilise all equipment and empty tubes in the laminar flow cabinet.

Thaw primers (Hazel-5S-For and Hazel-5S-84R) and probe (Hazel-5S-P) and mix thoroughly. Centrifuge for 10 seconds at 12,000g to collect solution in bottom of tube.

Shake TaqMan Universal PCR Mastermix stock gently to mix.

Prepare a fresh hazel mastermix according to Table 2. To calculate the volumes required, add four (for the controls) to the number of samples to analyse and multiply this by three. Add the number of samples to analyse to this number. Multiply this number by the volumes for one reaction shown in Table 2. Include an additional 10% for pipetting error. An example, using 10 samples, is shown.

Table 2: Hazel Mastermix Preparation

Reagents	Initial Concentration	Volume for One Reaction (µl)	Volume for 10 Samples (µl)[1]	Final Concentration in PCR Reaction
TaqMan Universal MM	2x	12.5	715.0	1x
Hazel-5S-For	10µM	2.25	128.7	900nM
Hazel-5S-84R	10µM	2.25	128.7	900nM
Hazel-5S-P	5µM	0.88	50.05	175nM
Milli-Q water		2.13	121.55	
Total		20.0	1144.0	

[1] Volumes include 10% extra to account for pipette error

Thoroughly mix the hazel mastermix and centrifuge for 10 seconds at 12,000g to collect solution in bottom of tube.

Using the plate layout sheet as a guide, pipette 20µl of hazel mastermix into each well of the PCR plate.

Using the plate layout sheet as a guide, add 5µl of DNA samples, controls and Milli-Q water (blanks) to the appropriate triplicate wells on the plate. Use a fresh tip for every aliquot.

Check sample arrangement against plate layout scheme then seal plate with strip lids. Ensure lids are securely fitted by use of lid fitting tool.

Dislodge any bubbles which may have become trapped in the bottom of the plate wells as these can distort results.

Transfer plate to the Post-PCR laboratory and place into TaqMan with well A1 in back-left position.

Shut and secure heated lid (screw down) and close plastic cover.

- **Preparation of TaqMan**

Start the Macintosh computer and sequence detector software (double click the desktop icon). Set up a new reaction file and ensure the well volume is set to 25µl and that the correct number of cycles (40) is set. Check the PCR profile is as shown in Table 3.

Table 3 : PCR Amplification Profile

Stage	Temperature (°C)	Time
Decontamination	50	2 minutes
Enzyme activation	95	10 minutes
Amplification (40 cycles)	95 60	15 seconds 60 seconds

Set up the plate for use with the VIC layer and select all 96 wells on the plate by highlighting with the mouse.

Change the well sample information to "UNKN" for all 96 wells.

Save the plate information file, using **"SAVE"** (not "Save As") from the "FILE" menu.

Select "SHOW ANALYSIS" button then select "RUN" button. Move mouse cursor away from file area and ensure a distinct 'beep' followed by a 'click' are heard, indicating the laser shield has moved away.

Check temperature on the status readout increases towards 50°C and that the timer starts to count down when 50°C is reached.

Do not use computer until run is complete (approximately 2 hours 30 minutes).

When run is complete, check status is "IDLE". Save the file, using **"SAVE"** from the "FILE" menu.

Remove PCR plate from PCR block and discard. Note that the plate and its contents will be hot (95ºC).

- **Analysis of Data**

With the file open in the VIC layer, change all the unused wells to ‘NOT IN USE’.

Select the “SHOW ANALYSIS” button then analyse the data file following the ABI7700 instruction manual.

Export the analysed data as an Excel file. The Excel file can be examined using the ABI7700 Mac or transferred to a PC.

Open the Excel file and calculate the mean C_T value obtained with each sample (i.e. average the three replicate PCR wells).

Classify those samples where the mean C_T is less than 33 as positive.

Classify those samples where the mean C_T is greater than 33 as negative.

10. EXPRESSION OF RESULTS

Results are expressed in the report as one of the following:

Positive : DNA from either hazelnut or other contaminant species was detected in the sample at or above the limit of detection.

This assay has been shown to cross-react with DNA extracted from 100% walnut oil. No cross-reactivity was detected with DNA extracted from 100% rapeseed or sunflower oil. Other oils have not been tested.

<OR>

Negative : DNA from either hazelnut or other contaminant species was not detected in the sample at or above the limit of detection. It cannot be assumed that the sample does not contain hazelnut DNA.

The limit of detection is the amount of DNA present in 2% (v/v) hazelnut oil H5* virgin olive oil.

At this limit of detection, 66% of the hazelnut oils tested in the validation study were detected.

*H5 – Cold pressed hazelnut oil, variety Tombul. Hazelnuts originated from Turkey (2004 harvest). Oil was pressed by Statfold Seeds Oil in March 2005.

11. QUALITY CONTROL

- **Primer and Probe Quality Control**

New primer sets or probes are available by specifying the primer or probe sequence in Table 1. For probes the fluorescent marker VIC must also be stated.

New primers and probes should be compared with those currently in use. New primers and probes are acceptable if their performance matches that of the primers and probes currently in use.

- **PCR Negative Control**

The PCR negative control, where Milli-Q water is used in place of a sample extract, and known as No Template Control (NTC) is used with every plate to check for contamination during PCR set-up. The PCR negative should be manipulated last at each stage of the process. The NTC control should give a value of $C_T > 37$.

- **DNA Extraction Negative Control**

A DNA extraction negative control is used with every plate to check for contamination during DNA extraction. The DNA extraction negative control should give a value of $C_T > 37$.

- **PCR Positive Control**

PCR positive controls of hazel DNA extracts at 10ng/µl are run in triplicate on each plate. Upon analysis, the average result for the hazel positive control should give a C_T value of between 14 and 20.

- **DNA Extraction Positive Control**

A DNA extraction positive control is analysed with every batch of samples. The DNA extraction positive control should produce a $C_T < 33$.

- **Spiked Samples**

In order to show samples do not contain inhibitors of the PCR reaction that might lead to a false negative result, an aliquot of each sample diluted to 1 in 8 containing a spike of hazelnut DNA is also amplified.

For the sample to show no inhibition, and for a negative result to be valid, the spiked sample should give a C_T value of no more than $2C_T$ units greater than that obtained with the positive control.

12. PRECISION AND ACCURACY

All DNA extracts are amplified in triplicate. Compare each individual C_T, from a sample, to the mean C_T of the three replicates for that sample. The batch is valid if each individual triplicate's C_T is within ±5% of the mean C_T value for that sample. If this is not the case, delete the results for this one well. If more than one well is outside this range the assay should be repeated.

This assay provides a qualitative measure of the presence or absence of unrefined hazelnut oil. Detection is based on the presence of sufficient hazelnut DNA to provide a positive result using the cut-off C_T of 33. This C_T was determined using 2% (v/v) cold-pressed, unrefined hazelnut oil (variety Tombul) in virgin olive oil.

13. RISK ASSESSMENT

Before performing this protocol, ensure you have read and signed the appropriate risk assessment forms.

APPENDIX 1

Hazelnut Varieties	Oil Mills/Producers
White Heart (H1)	
Sivrifindik (H3)	
Karafindik (H4)	Hazelwood Hazelnut, New Zealand
Tombul (H5)	Huilerie Croix Verte, France
Kentish Cobnut (H8)	Statfold Seed Oil, UK
Butler (H9)	

APPENDIX 2

PREPARATION OF PCR POSITIVE CONTROLS

A1 PCR positive control standards are DNA extracts prepared from whole hazelnut reference materials. Reference materials should be stored at room temperature for up to two years.

A2 Reference materials are potential sources of contamination, which may also lead to cross-contamination if not handled with care. Take appropriate steps to reduce the possibility of cross-contamination with these materials.

A3 DNA reference materials are extracted from whole nuts using a CTAB method. The DNA is purified using Wizard purification columns and the concentration determined spectrophotometrically.

A4 Reference materials should be diluted to 50ng/µl then divided into aliquots of at least 50µl in labelled 1.5ml Eppendorf before storage.

A5 All reference material is amplified using the approach detailed in this method, to check the extraction procedure has been correctly performed. The performance of the new PCR positive controls are compared to that of the previous batch.

A6 PCR positive control DNA extracts are stored at -15ºC to -22ºC for up to twelve months.