Working instruction for the identification of DNA from species of the *Prunus* genus in samples of herbs and spices

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Joint Knowledge Transfer Framework for Food Standards and Food Safety Analysis

Working Instruction

Identification of DNA from species of the *Prunus* genus in samples of herbs and spices

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Preface

In 2017 a framework was agreed for the provision of Knowledge Transfer (KT) of scientific method development to support food standards and food safety analysis in Public Analyst (PA) and industry laboratories. The KT is being delivered through a three year project funded in partnership between the Department for Environment, Food and Rural Affairs (Defra), the Food Standards Agency (FSA), Food Standards Scotland (FSS) and the Government Chemist (GC) programme (funded by BEIS). The framework will provide a more sustainable and cost-effective programme of KT on analytical tools to support food law enforcement for ensuring food authenticity, safety, hygiene and quality.

Under the joint KT project, a workshop titled ‘An analytical roadmap for detecting allergens in spices’ was organised in February 2018. The presentations form the workshop, and the analytical roadmap, are available from the training section of the Food Authenticity Network website (http://www.foodauthenticity.uk/). The Working Instructions contained in this document describe a step-by-step guide to using a real-time PCR and DNA-melting analysis approach for identification of species within the Prunus genus. Using the working instructions, in combination with the analytical roadmap and previously published peer reviewed papers, will provide laboratories with a framework for addressing issues related to identifying the presence of allergens in herbs and spices.

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BACKGROUND

Random testing conducted by the Canadian Food Inspection Agency during October 2014 revealed the presence of almond protein in products containing cumin – a risk for people with associated allergies. This finding culminated in the issuing of alerts on the EU Rapid Alerts System for Food and Feed [1], as well as a widespread recall of cumin products on the international market. However, the source of the almond protein remained unidentified.

One company subject to a recall of their cumin product presented intelligence that suggested another material in the supply chain, *Prunus mahaleb*, had most likely generated the positive reading for almond with screening methods employed at the time. Standard analytical approaches employed for food allergen detection include the use of enzyme linked immunosorbent assays (ELISA), but these can be subject to well characterised cross-reactivity issues, making unequivocal species identification challenging.

These Working Instructions describe a step-by-step guide to using a real-time PCR and DNA-melting analysis approach for identification of species within the *Prunus* genus, first described in the publications of Burns, *et al*., (2016) [2] and Nixon *et al*., 2016 [3]. The methods were developed at LGC for the determination of *Prunus mahaleb* (as opposed to almond) in cumin, and the general determination of a *Prunus* species. To implement these Working Instructions successfully, it is the responsibility of the user to carry out appropriate validation, as per their own internal Quality Procedures, to demonstrate that the method performance is fit for purpose using their own instrumentation and for the test samples (i.e. specific cultivars/varieties) required.

PURPOSE

The purpose of the method described here is the detection and identification of DNA originating from species of the genus *Prunus* in samples of cumin. However, the method may be applied to other herb and spice combinations provided that appropriate method validation is undertaken.

SCOPE

The method has been developed and applied to DNA extracted from samples of the spice cumin (*Cuminum cyminum*), to which gravimetrically determined quantities of ground material originating from either the plant species *Prunus mahaleb* or *Prunus dulcis* (almond) have been added.

ABBREVIATIONS

COSHH – Control of Substances Hazardous to Health

C_q – quantification cycle

DNA – deoxyribonucleic acid

ELISA – enzyme linked immunosorbent assay

HPLC – high performance liquid chromatography

ITS – internal transcribed spacer

MSDS – material safety data sheet

NFQ – nonfluorescent quencher

NTC – no template control

PCR – polymerase chain reaction

qPCR – quantitative real-time PCR

v/v – volume for volume

PRINCIPLE OF THE METHOD

The method employs a combination of real-time PCR and DNA-melting analysis to detect and identify the presence of DNA originating from species within the genus *Prunus* in samples of herbs and spices.

The method employs two PCR-based assays to detect and identify the presence of *Prunus* DNA in a sample. Initially, a hydrolysis probe-based, species-specific, real-time PCR assay is employed to detect the presence of *P.mahaleb* DNA in a test sample. A general *Prunus* DNA binding dye-based PCR assay, incorporating a DNA melt analysis, is then used to identify any additional *Prunus* species that may be present in a test sample.

Both assays utilise identical forward and reverse primer sequences. Both primer sequences are specific to DNA sequences located in the mitochondrial internal transcribed spacer (ITS) region of plant species belonging to the genus *Prunus.* In addition, the specificity of the *P.mahaleb* real-time PCR assay is mediated through homology of the probe sequence to a species-specific target within the ITS region which is flanked by the primer sequences. The speciation capability of the general *Prunus* real-time PCR assay is afforded by use of a DNA-melt assay. Polymorphisms present in the DNA sequence of the *Prunus* ITS region flanked by the primers, result in the generation of species specific melt peaks during DNA-melt analysis. Thermal melting profiles are highly reproducible and may be used to determine the identity of *Prunus* species other than *P.mahaleb* in a test sample.

Overview of method

1. DNA extracted from test sample and normalised to 25 ng per PCR well
   ↓
2. Test sample and control material DNA analysed by *P.mahaleb* specific qPCR assay
   ↓
3. Test sample and control material DNA analysed by general *Prunus* real-time PCR assay incorporating a DNA-melt analysis step
   ↓
4. Data analysed and reviewed
   ↓
5. Report on the presence and identity of *Prunus* DNA compiled

METHOD PERFORMANCE CHARACTERISTICS

Specificity

The *P.mahaleb* real-time PCR assay has been demonstrated experimentally to amplify and detect a specific DNA target sequence within the ITS region of the *Prunus* genome. It has also been confirmed experimentally as exhibiting no cross reactivity with DNA isolated from the specific *Prunus* species that were evaluated which included: almond (*P.dulcis*), apricot (*P.armeniaca*) and peach (*P.persica*). However, to comply with analytical laboratory quality procedure requirements, it is the responsibility of individual laboratories to fully validate the method against a range of test samples (i.e. specific cultivars/varieties) in order to ensure that it is fit for purpose and exhibits no cross reactivity.

The general *Prunus* real-time PCR assay incorporating a DNA-melt analysis has been demonstrated experimentally to amplify a DNA target sequence within the ITS region of the *Prunus*

genus. Application of a DNA-melt analysis to the resulting PCR product has been experimentally demonstrated to enable the differentiation of specific species of the genus *Prunus*, through the generation of diagnostic, species specific, DNA-melt peaks. These included almond (*P.dulcis*), apricot (*P.armeniaca*) and peach (*P.persica*). However, to comply with analytical laboratory quality procedure requirements, it is the responsibility of individual laboratories to fully validate the method against a range of test samples (i.e. specific cultivars/varieties) in order to ensure that it is fit for purpose and exhibits no cross reactivity with species other than those of the genus, *Prunus*.

SAFETY

National and laboratory safety procedures must be adhered to at all times. Analysts should consult laboratory COSHH assessments and MSDS prior to undertaking this method. Laboratories are responsible for their own risk assessments.

MATERIALS AND EQUIPMENT

Reagents: Unless otherwise stated, all reagents are of molecular biology grade quality. All reagents should be sourced from reputable suppliers. Examples of suitable reagents and their respective manufacturers are listed below. Alternative products for use in these working instructions may be sourced but it is the responsibility of the laboratory to validate the use of these products with the method as per the laboratory's internal quality procedures.

- 2 x Fast Plus EvaGreen®qPCR Master Mix, High ROX™ (Cat. No. BT31015-1, Biotium Inc., Hayward, USA).
- 2 x TaqMan Universal PCR Master Mix (Cat. No. 4304437, Life Technologies, Paisley, UK) containing qPCR buffer, hot start Taq polymerase and ROX passive reference dye.
- Nuclease-free water (Cat. No. AM9937, Ambion brand, Life Technologies, Paisley, UK).
- HPLC purity oligonucleotide primers and 6-FAM (reporter)/nonfluorescent quencher (NFQ) labelled hydrolysis probes (**Table 1**).

Assay	Marker	Sequence Name	Sequence (5' – 3')	Labelling
Prunus mahaleb	ITS	PRU_ITS_FWD1	TAG CAG AAC GAC CCG AGA ACT AG	None
		PRU_ITS_REV1	CGC CGG TGT TCG TTT GTA C	None
		PRU_ITS_PROBE1	TTC GCG CAA CCG GCC CTT C	6-FAM/NFQ

Table 1. Primer and probe sequence information

Control materials:

- DNA from species of the genus *Prunus* appropriately certified or verified in-house through the use of appropriate testing methodologies (e.g. species-specific real-time PCR or DNA sequencing-based analysis). Minimum number of species recommended for inclusion: almond, apricot, mahaleb and peach.

Test materials:

- Quantitated DNA extracts with appropriate quality characteristics (A260/280 ~ 1.8, A260/230 ~ 1.8 to 2.2). The utilisation of multiple extracts for each test sample is recommended (e.g. minimum of four extracts from each sample).

PROCEDURE

For the method described here, the *P.mahaleb* real-time PCR assay is used first, followed by the general *Prunus* species real-time PCR-based assay which incorporates a DNA-melt analysis.

- The procedure was developed using an Applied Biosystems 7900HT Fast Real-Time PCR System (Life Technologies, Paisley, UK).
    - Alternative real-time PCR instrumentation may be employed, but requires a full validation of the method to be undertaken, in accordance with the laboratory internal quality procedures, before the method may be considered fit for purpose. In addition, the relevant instrument manufacturer's guidelines for the setting up of real-time quantitative PCR experiments should be followed and the MIQE guidelines (Bustin *et al*., 2009 [4]) should be implemented for recording and documenting experimental information/data.
- A single validated DNA extraction methodology should be applied to the preparation of reference materials and test samples (e.g. Modified CTAB DNA extraction [5]), and multiple independent DNA extractions for each sample is recommended.
- The extracted DNA should be quantified using either conventional spectrophotometric approaches (e.g. Nanodrop™), or through the use of double stranded DNA binding dye assays (e.g. PicoGreen®).

Stage 1. Implementing the *P.mahaleb* real-time PCR assay

1. Prepare a 96-well plate design which should include the following features:
    - A minimum of triplicate PCR technical replication level to ensure data robustness;
    - A no template extraction control, to identify the contamination of extraction reagents;
    - PCR no template control (NTC) where the DNA template has been replaced by nuclease free water, to enable the identification of contaminated PCR reagents.
    - PCR positive control (25 ng of *P.mahaleb* DNA);
2. The assay was developed for a total volume of 25 µl. Combine 12 µl 2 x TaqMan Universal PCR Master Mix with oligonucleotides (forward primer, reverse primer and probe to appropriate final reaction concentration) and make up to 20 µl with DNase/DNA-free water (**Table 2**). Scale reagent volumes to prepare sufficient reaction mixture for 1.2x number of reaction replicates. Transfer 20 µl of reaction mixture into the appropriate wells.

Reagent component	Final concentration
2 x TaqMan Universal PCR Master Mix	1 x
Forward Primer	0.9 µM
Reverse Primer	0.9 µM
Probe	0.2 µM
Water	Make volume to 20 µl
Template DNA (5ng/µl)	25 ng template DNA

Table 2. qPCR reaction composition and final concentrations (25 µl total reaction volume)

3. DNA content of reference and test samples should be quantified prior to dilution to ensure that the required mass of DNA sample is added to each reaction well. Dilute test samples using DNase/DNA-free water.
4. Add 5 µl of the diluted DNA template (5 ng/µl) to each of the requisite wells to provide an input mass of DNA of 25 ng/reaction.

5. Seal the plate and centrifuge to deposit the contents at the bottom of the wells before proceeding with thermal cycling.
6. Real-time analysis should be performed using a FAM/NFQ detector with the instrument configured for real-time data acquisition.
7. Thermal cycling should be performed using a 2-step cycling protocol (**Table 3**).
8. Automatic C_q determination should be performed unless the data requires manual manipulation (as per manufacturer's recommendations).
9. Export the data and analyse using spreadsheet-based software (e.g. Microsoft® Excel or other similar software application).

Temperature	Time	Repetitions
50 °C	120 s	1 x
95 °C	600 s	1 x
95 °C	15 s	45 x
80 °C	60 s	

Table 3. PCR Thermal cycling conditions

Stage 2. Implementing the general *Prunus* real-time PCR assay

1. Prepare a 96-well plate design which should include the following features:
   - A minimum triplicate PCR technical replication level to ensure data robustness;
   - A no template extraction control, to identify the contamination of extraction reagents;
   - PCR no template control (NTC) where the DNA template has been replaced by nuclease free water, to enable the identification of contaminated PCR reagents;
   - PCR positive control materials (25 ng almond, apricot, peach and mahaleb DNA).
2. The assay was developed for a total volume of 25 µl. Combine 12 µl 2 x Fast Plus EvaGreen® qPCR Master Mix, High ROX™, with oligonucleotides (forward primer, reverse primer to an appropriate final reaction concentration) and make up to 20 µl with DNase/DNA-free water (**Table 4**). Scale reagent volumes to prepare sufficient reaction mixture for 1.2 x number of reaction replicates. Transfer 20 µl of reaction mixture into the appropriate wells.

Reagent Component	Final concentration
2 x Fast Plus EvaGreen® qPCR Master Mix, High ROX™	1 x
Forward Primer	0.9 µM
Reverse Primer	0.9 µM
Water	Make volume to 20 µl
Template DNA	5 µl volume (25 ng template DNA)

Table 4. qPCR reaction composition and final concentrations (25 µl total reaction volume)

3. DNA content of control materials and test samples should be quantified prior to dilution to ensure that the required mass of DNA sample is added to each reaction well. Dilute the samples as applicable using DNase/DNA-free water.
4. Add 5 µl of the diluted DNA template (5 ng/µl) to each of the requisite wells to provide an input mass of DNA of 25 ng/reaction.

5. Seal the plate and centrifuge to deposit the contents at the bottom of the wells before proceeding with thermal cycling.
6. Thermal cycling should be performed using a universal 2-step cycling protocol, and followed by a DNA-melt analysis step (**Table 5**).

PCR amplification stage		
Temperature	**Time**	**Repetitions**
50 ^oC	120 s	1 x
95 ^oC	600 s	1 x
95 ^oC	15 s	45 x
80 ^oC	60 s	
DNA-melt stage		
95 ^oC	15 s	1 x
60 ^oC to 95 ^oC	1.75 ^oC incremental increase every 60 s	1 x

Table 5. PCR Thermal cycling conditions

7. Real-time analysis should be performed using a FAM/NFQ detector with the instrument configured for real-time data acquisition.
8. Automatic C_q and DNA-melt peak determination should be performed unless the data requires manual manipulation (as per manufacturer's recommendations).
9. Export data and analyse using spreadsheet based software (e.g. Microsoft® Excel or other suitable software application).

CALCULATIONS AND DATA ANALYSIS

P.mahaleb real-time PCR assay

1. Confirm that no amplification has occurred for the extraction and PCR no template controls (no C_q determined).
   - For any incidence of a positive result being obtained for the negative controls, the experiment should be repeated, sample re-extracted, or PCR reagent aliquots employed discarded.
     - It is recommended that all oligonucleotide and PCR reagents be aliquoted to avoid contamination of an entire batch.
2. Where no amplification in the extraction and PCR no template controls is detected, tabulate the mean and standard deviations for the C_q values obtained for the test and positive control samples.

General *Prunus* real-time PCR assay incorporating DNA-melt analysis

1. Confirm that no significant amplification has been detected below 37 cycles for the extraction and PCR no template controls (no C_q determined).
   - NOTE: Late amplification (> 37 cycles) is frequently observed for DNA-binding dye-based real-time PCR assays due to the presence of primer artefacts (e.g. primer-dimers). These artefacts are frequently absent when target template is present (Wittwer *et al*., 2013 [6]).
2. Tabulate the mean and standard deviations for the C_q values obtained for the test and positive control samples.

3. Tabulate the mean T_m peak values for the DNA-melt profiles generated for each of the test and positive control samples.
4. Determine the mean C_q values for the control materials and test samples.
5. Directly compare the DNA-melting peaks obtained for the control materials with those obtained for the test samples and identify the *Prunus* species present. For this, a positive match of melting peaks is indicated if the T_m of the DNA-melt peak of the test sample matches that of any of the positive controls (± 1°C [7]).

REPORTING

Notes for stage 1 reporting where *P.mahaleb* DNA is detected

If a positive result (C_q determined) is obtained using the *P.mahaleb* specific real-time PCR assay, and assuming:

- C_q not determined for the extraction and PCR No Template Control
- C_q determined for PCR positive control material (25 ng of *P.mahaleb*).

then it may be stated that *P.mahaleb* has been detected in the test sample using a species specific real-time PCR assay for *P.mahaleb*. However, additional species from the genus *Prunus* may be present, for which an analysis using the general *Prunus* real-time PCR assay incorporating a DNA-melt analysis will be required.

Notes for stage 2 reporting

If a positive result (C_q determined) is obtained using the general *Prunus* real-time PCR assay, incorporating a post-PCR DNA-melt analysis, and assuming:

- C_q not determined for the extraction and PCR no template controls.
    - All double-stranded DNA molecules present at the end of each thermal cycle will contribute to the level of fluorescence measured. The Cq values measured for the control reactions therefore provide an indication of the contribution of non-specific PCR products (e.g. primer dimers)
- C_q not determined against negative controls
    - Method only valid against negative controls tested, e.g. peanut, etc.
- C_q determined for PCR positive control material (25 ng almond, apricot, mahaleb and peach)
- C_q determined for 25 ng test samples
- That DNA-melt profiles are highly indicative to each species

then it may be stated that the sample contains DNA sequences which are characteristic of species of the genus *Prunus* being detected in the test sample using a general *Prunus* real-time PCR assay. If the T_m of the DNA-melt profile of the test sample matches that of any of the positive controls (± 1°C [7]), then it may be inferred that the sample contains DNA sequences which are indicative of a species of the genus *Prunus* which has previously been characterised through application of the general *Prunus* real-time PCR assay.

It should be noted that DNA-melt profiles may contain more than one peak. This is indicative of the presence of DNA that originates from more than one species of *Prunus* in the DNA extract of the test sample. However, a low probability exists that a similar melt curve profile could be generated by the presence of non-*Prunus* DNA.

If no match is obtained, and because the assay is specific to the genus *Prunus,* the presence of an uncharacterised *Prunus* species cannot be discounted. In such circumstances, additional rounds

of DNA-melting analysis should be employed which utilise a wider number of reference *Prunus* species(e.g. peach, apricot etc).

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