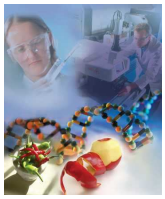
Spice authenticity – DNA-based approaches for the detection of adulterating nut material

Timothy Wilkes
28 February 2018

Overview

- **Introduction**
- **Case study 1: Mahaleb in Cumin,**
  - Development of a *Prunus mahaleb* specific real-time PCR assay
- **Case study 2: Almond in Paprika**
  - Development of a *Prunus* DNA melting curve method
- **Acknowledgments**



Cumin & Paprika recalls – Referee Cases

1. 31 Jan '15 FSA recall cumin - almond not listed on the label
2. 10 Feb '15 FSA refer cumin sample to Government Chemist
3. Elisa Testing
4. March '15 supplier "…mahleb gives positive ELISA for almond"
5. Protein by Mass Spectrometry
6. 30 April '15 Canada rescinds recalls " … mahleb false positives"
7. **DNA Testing**
8. 26 June '15 Govt Chemist confirms cumin contains mahleb
9. 29 June '15 FSA rescinds cumin recalls "mahleb present; not almond"
10. 13 Aug '15 FSA refer sample of paprika to Government Chemist
11. 9 Nov '15 Govt Chemist confirms paprika contains almond

Introduction

- **In 2015, a number of spice products were subject to recall from the international market due to the suspected unlabelled presence of almond**
- **Samples of the suspected spices, cumin and paprika, were referred to the UK Government Chemist in 2015 for further analysis**
- **Government Chemist scientists developed novel DNA-based techniques which enabled the conclusive identification of the adulterant species**
- **In the first case, a novel real-time PCR assay was developed which definitively identified *Prunus mahaleb as* the adulterant *Prunus* species present in samples of cumin**
- **In a second study, a commercial paprika sample was proven to be adulterated with almond following analysis using a novel DNA melting analysis**

Case study 1: Mahaleb in Cumin,

Development of a novel qPCR assay specific for *Prunus mahaleb*

Background

- **October 2014, Random testing conducted by the Canadian Food Inspection Agency suggested presence of almond protein in products containing cumin**
  - > Widespread product recall and issuing of alerts on the EU Rapid Alerts System for Food and Feed
- **March 2015, UK company subject to recall of the cumin product:**
  - > Mahaleb was possibly the origin of the ELISA almond positives
- **April 2015, Canadian Food Inspection Agency rescinded ~ 25 product recalls**

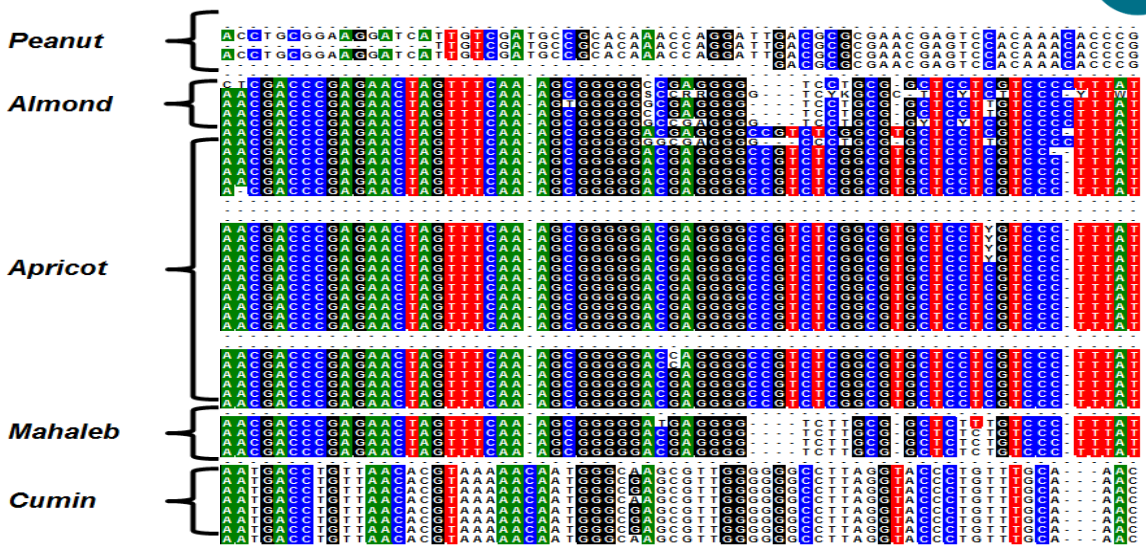
  "New evidence regarding the cross-reactivity of mahaleb, a spice obtained from a specific species (*Prunus mahaleb*) of cherry seeds, with the almond allergen test kit. It is highly likely that the positive sample results for the ground cumin and cumin-containing products were due to mahaleb contamination and not almond"
- **Therefore a requirement to provide a definitive identification for the *Prunus* species present in the samples and responsible for the initial result**

Almond

- **Almond: Member of the large and commercially important '*Prunus*' genus**
- **Genus also includes drupes (stone fruit) capable of producing kernels :**
  - > Almond, *Prunus dulcis*
  - > Apricot, *Prunus armeniaca*
  - > Cherry, *Prunus avium*
  - > Peach, *Prunus persica*
  - > Plum, e.g. *Prunus domestica*
  - > Mahaleb, *Prunus mahaleb*

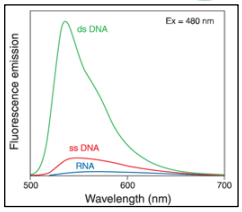
- **Biological relatedness of *Prunus* species is very high**
  - > Multiple cultivars developed for commercial fruit production
    - Almond and peach are able to form hybrids
    - Almond and peach crosses regularly utilised as root stock for grafting
- **Phenotypic similarity mirrored by similarity of homologous gene DNA sequences**

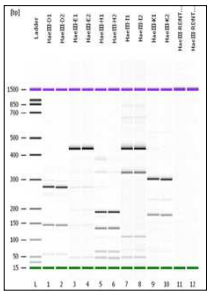
Sequence homology – 18S ribosomal RNA gene



DNA-based quantitation approaches

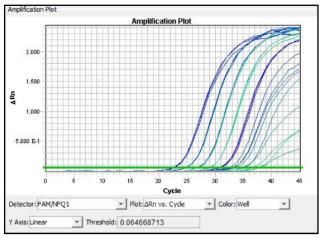
- A number of methods exist for the analysis of nucleic acids
- Examples include: electrophoresis, spectrophotometry, and the Polymerase Chain Reaction (PCR)
- PCR enables the amplification of a specific DNA target sequence to be achieved
- PCR reaction products can be visualised by DNA staining following electrophoretic separation on an agarose gel or capillary-based system
- This endpoint approach can provide a qualitative indicator for the detection of specific DNA targets, but is limited in terms of providing a quantitative estimate

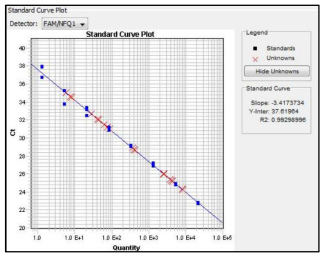




Quantitative PCR

- Quantitative real-time PCR (qPCR) is a modification of the basic PCR method
- Widely employed throughout the field of food analysis, including: food authenticity testing, GMO presence, Microbial contamination
- The modification employs the measurement of specific fluorescent signals generated as a consequence of the amplification process
- The fluorescent signals are directly proportional to the amount of PCR amplicon being synthesised
- The number of target copies present in a sample can be determined by reference to a calibration curve
- Calibration curves can be produced from standards of a known target concentration





Examples of non-commercial, *Prunus* real-time PCR assays

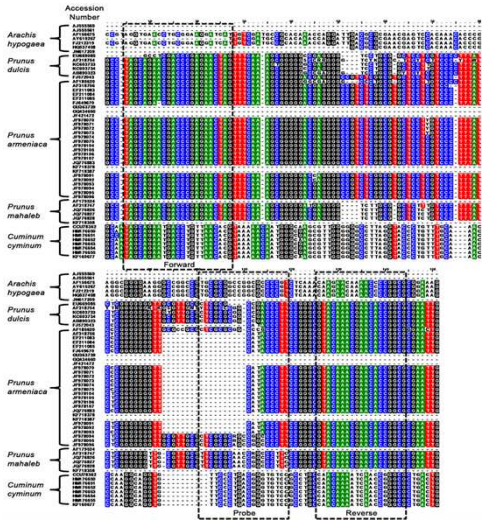
- **Number of real-time PCR assays have been developed for use in the detection of *Prunus* species**
- **Employ a range of different DNA sequences as targets**
- **Majority of existing assays reported to exhibit some degree of cross-reactivity between *Prunus* species**
- **No real-time PCR assay available for the specific detection of *Prunus mahaleb***

Publication	DNA Target	Assay
Leidinger *et al*., 2009	11s globulin (prunin, Pru 1)	TaqMan® real-time PCR
Pafundo *et al*., 2009	11s globulin (prunin, Pru 1)	SYBR® Green real-time PCR
Koppel *et al*., 2010	Pathogenesis related protein (PR10)	TaqMan® real-time PCR
Röder *et al*., 2011	Non-specific lipid transfer protein (LTP1)	TaqMan® real-time PCR
Costa *et al*., 2012	60s acidic ribosomal protein (AL60SRP)	Evagreen® real-time PCR and DNA high resolution melting
López-Calleja *et al*., 2012	ITS1 (Intergenic spacer)	TaqMan® real-time PCR
Costa *et al*., 2013	11s globulin (prunin, Pru 1)	TaqMan® real-time PCR

Identification of PCR target DNA sequence

- **Identified relevant publicly available DNA sequence data**
  - Barcode of Life (BOLD)
  - GenBank (NCBI)
- **Imported relevant DNA sequence data into sequence analysis software**
  - BioEdit (Ibis Therapeutics, California)
- **Performed Multiple Sequence Alignment analysis**
  - Clustal W (EMBL-EBI)
- **Inspected alignments to identify regions of homology and polymorphism**
  - DNA sequence for the ITS1 region between 5.8s and 18s rRNA genes selected as the PCR target

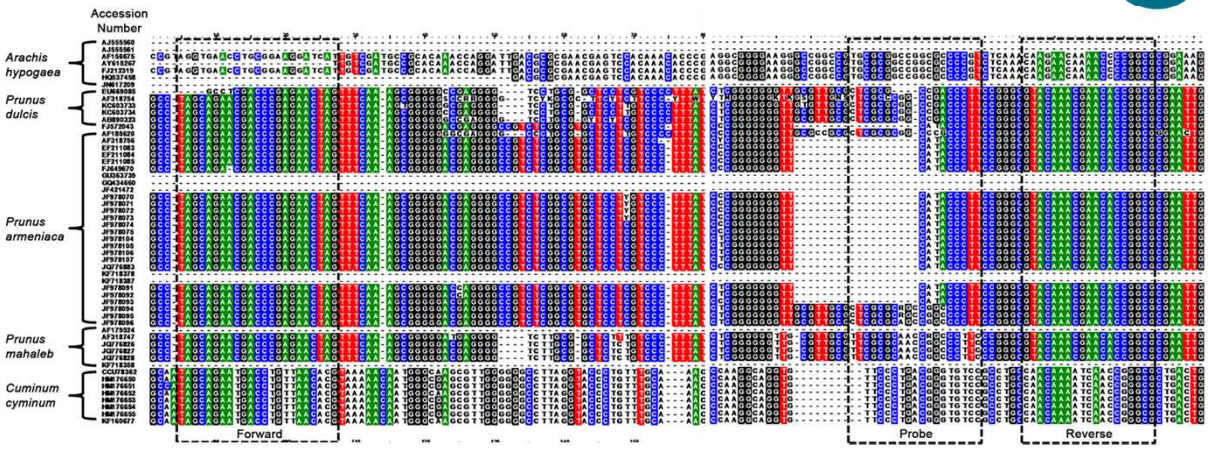
Alignment of the *Prunus* and cumin ITS1 regions



Identify potential PCR target DNA sequences

- **Search for publicly available *Prunus* DNA sequence data**
  - Barcode of Life (BOLD)
    - 904 records representing 188 species (March 24, 2015)
  - GenBank (NCBI)
    - 236,565 nucleotide sequences for *Prunus* species
      - 99,941 derived from *P.armeniaca* (apricot)
      - 1747 derived from *P.dolcis* (almond)
      - 49 derived from *P.mahaleb (St Lucie cherry)*
- **Import DNA sequence data into sequence analysis software**
  - BioEdit (Ibis Therapeutics, California)
- **Perform Multiple Sequence Alignment analysis**
  - Clustal W function
    - Clustal Omega (EMBL-EBI)
- **Inspect alignments to identify regions of homology and polymorphism**
  - ITS1 region located between the 5.8s and 18s rRNA genes selected as the PCR target

Alignment of DNA sequence reads for *Prunus* and cumin ITS1 regions.



The positions of the LGC *P. mahaleb* specific real-time PCR assay forward primer, reverse primer and probe are indicated by the hatched black boxes

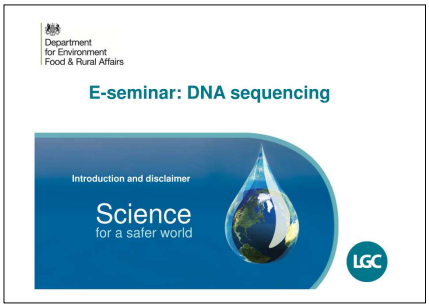
Design of a real-time PCR assay

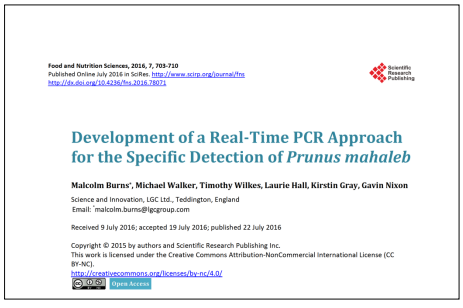
- **Import candidate DNA sequence into primer design software**
  - > Primer BLAST (NCBI)
  - > DNA sequence *Prunus* ITS1
- **Specify the type of assay required design**
  - – Real-time hydrolysis probe
  - – *Prunus mahaleb* specific
  - – Used the default software settings
- ***Evaluate assay* specificity *in silico***
  - – Primer-BLAST (NCBI)
    - Used the default software settings
    - Screened primers and probe DNA sequences against all GenBank entries

***LGC Prunus mah*aleb real-time PCR assay**	
Assay	qPCR
Target	*Prunus mahaleb*
Marker	ITS1
Forward Primer	[TAG] [CAG] [AAC] [GAC] [CCG] [AGA] [ACT] [AG]
Reverse Primer	[CGC] [CGG] [TGT] [TCG] [TTT] [GTA] [C]
Probe	[TTC] [GCG] [CAA] [CCG] [GCC] [CTT] [C]
Amplicon	~ 133 bp

Additional sources of information

- **For further information delegates are referred to:**
  - > Burns *et al*., (2016) "Development of a Real-Time PCR Approach for the Specific Detection of *Prunus mahaleb*". Food and Nutrition Sciences, 7, 703-710
  - > The LGC e-seminar, "DNA sequencing to support food labelling enforcement", which is available from the Food Authenticity Network at : http://www.foodauthenticity.uk





Experimental design

- **Control materials employed**
  - > Control materials were shared between the multidisciplinary teams at LGC
  - > For use with the DNA methodologies, species identities for control materials were independently confirmed by PCR amplicon sequencing[1]
- **DNA extraction[2]**
  - > DNA was isolated and purified using a modified CTAB/Proteinase K extraction method
    - Two independent DNA extractions were performed for each of the control samples
    - Four independent DNA extractions were performed for the referred sample
  - > DNA concentration and purity were determined spectrophotometrically
- **Experimental design**
  - > Two DNA concentrations were employed,
    - 25 ng/reaction
    - 2.5 ng/reaction (1:10 dilution)
  - > Triplicate PCR replicates performed for each sample

For further information delegates are referred to [1]DNA sequencing to support food labelling enforcement and [2]DNA extraction to support food labelling enforcement, available at the Food Authenticity Network: http://www.foodauthenticity.uk/

Experimental conditions

Component	Concentration
Forward primer	900 nM
Reverse primer	900 nM
Probe	200 nM
2 x TaqMan® Universal PCR Master Mix	1 x
DNA template	25 ng/reaction
Reaction volume	25 µl

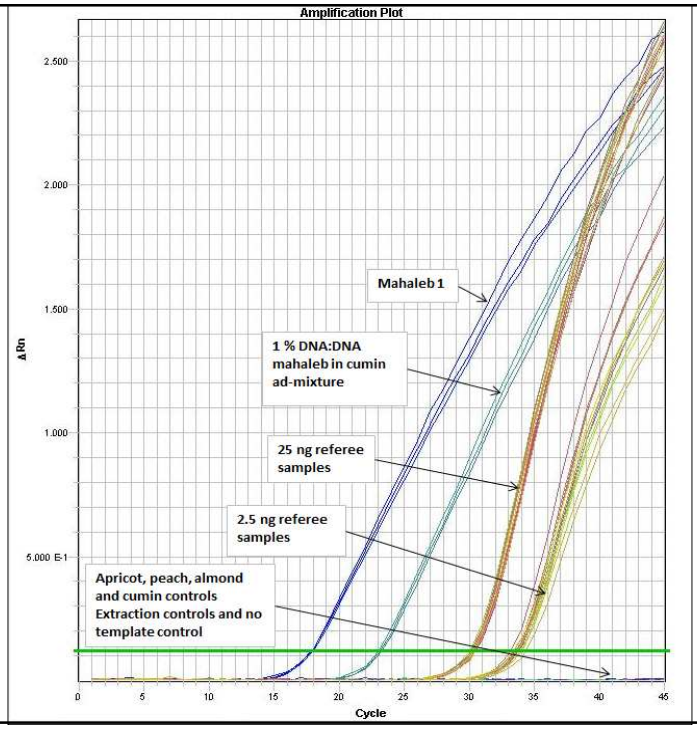
Recommended composition of the LGC *Prunus mahaleb* real-time PCR reaction

- **HPLC purified primers and probe**
- **Consumables and biological reagents from reputable distributers**

Step	Temperature	Duration	Cycles
UNG incubation	50°C	5 minutes	1 cycle
Hot start Taq activation	95°C	10 minutes	1 cycle
Template denaturation	95°C	15 seconds	45 cycles
Anneal + extend	60°C	60 seconds	

Recommended thermal cycling profile for use with the LGC *Prunus mahaleb* real-time PCR assay

- **Conditions employed with use of an ABI 7900HT Fast PCR System**



Real-time PCR results

Specimen	Amplification	Ct ± SD
Almond kernel	No amplification	Not applicable
Apricot kernel	No amplification	Not applicable
Peach kernel	No amplification	Not applicable
Mahaleb kernel	Amplification	17.87 ± 0.06
Cumin	No amplification	Not applicable
Referee sample (4 independent extractions)	Amplification	30.39 ± 0.10; 30.14 ± 0.07 30.40 ± 0.04; 30.12 ± 0.15
Referee sample (1:10) (4 independent extractions)	Amplification	33.49 ± 0.34; 33.58 ± 0.14 34.04 ± 0.22; 33.65 ± 0.25
1% DNA:DNA mahaleb:cumin	Amplification	23.10 ± 0.14
Extraction blanks (n = 4)	No amplification	Not applicable

Non-standard approach for estimating the abundance of *Prunus mahaleb* n the referred sample

This is not the standard method for estimating the amount of target present for qPCR which should utilise gravimetric standards to generate a calibration curve. Alternatively, the use of a digital PCR based approach could be evaluated

- **A 1 in 10 dilution of the 25 ng (DNA) reference sample resulted in a 2.5 ng PCR reaction**
- **The difference in Cq values between the two PCR's was approximately 3.4 and which is in agreement with what would be expected for a 1:10 dilution of a target**
- **A difference of 7.2 Cq was observed between the 1% mahaleb in cumin sample (normalised to 25 ng) and the referee sample (25 ng)**
- **Equates to 150 fold difference in target template concentration**
- **Infers that the concentration of mahaleb in the referee sample may be around 0.001% DNA:DNA (less than 10 ppm) based on a large number of assumptions**

Caveats associated with the estimation of *P. mahaleb* abundance

> Assumes similar PCR efficiency between samples

> Assumes no other *Prunus* DNA present in the referee sample, which could interfere with the results

> Genome size differences between cumin and mahaleb mean that a 1% DNA:DNA volumetric dilution may not correspond to a 1% w/w mahaleb powder in cumin powder

> Volumetric dilution based on spectrophotometric readings do not take into account double stranded and single stranded DNA

Advantages of the qPCR method for *P. mahaleb* detection

> Rapid technique

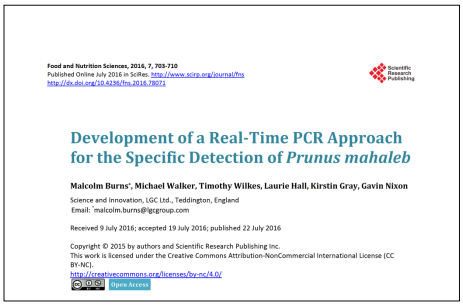
> Uncomplicated setup and workflow

> Sensitive and robust

> Applicable for use on a wide number of real-time capable instruments

Summary

- **Conclusion: The referred sample contained *Prunus* protein and DNA, the origin of which was consistent with the presence of *Prunus mahaleb***
- **The DNA approach provided unequivocal species identification that showed that *Prunus mahaleb* was present**
- **Method has been published as a peer reviewed paper**
  - Scope:
    - Method evaluated with use of commercially sourced samples as controls
      - Several species/cultivars of apricot, plum, cherry and peach
    - *In-silico* database searches indicated that the method real-time assay unlikely to cross react with untested *Prunus* species, but additional experimental validation advocated



Food and Nutrition Sciences, 2016, 7, 703-710
Published Online July 2016 in SciRes. http://www.scirp.org/journal/fns
http://dx.doi.org/10.4236/fns.2016.78071

Development of a Real-Time PCR Approach for the Specific Detection of *Prunus mahaleb*

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Case study 2: Paprika

Development of a novel *Prunus* DNA melting curve method

Case History

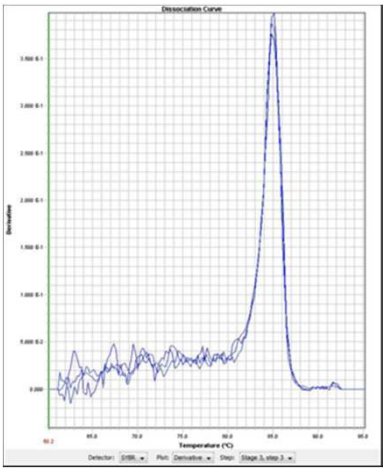
- **August 2015: a sample of paprika was referred to the Government Chemist by the Food Standards Agency**
- **Reports/evidence that almond, was thought to be present in the sample**
- **Product did not enter the UK food chain**
- **The Government Chemist was asked if it was possible to tell whether almond or mahaleb (or both) was present in the referred sample of paprika**
- **This required further investigation of the analytical methods previously developed for mahaleb in cumin to ensure they were applicable in paprika**

Initial screening approach

- **DNA Specificity tests**
  - The *Prunus mahaleb* real-time PCR assay developed at LGC was revisited:
  - Did not cross react with paprika
  - 10% mahaleb spiked into background of paprika was easily detected (no inhibition from paprika background)
- **Sensitivity tests:**
  - Using a dilution series (mahaleb in paprika, DNA/DNA) the LOD was estimated as <1 ppm (comparable to ELISA)
  - Using six independent extracts of the referred sample:
    - Mahaleb DNA was not detected (LOD of <1 ppm)
- **Conclusion**
  - Mahaleb DNA was not detected (LOD of <1 ppm)
  - Likely to be almond (common *Prunus* species)
  - Further analysis required to determine the species present

Development of a novel DNA assay for screening for *Prunus* species

- **Existing *P. mahaleb* real-time PCR assay:**
  - > Primers (generic to *Prunus* family)
  - > Probe (provided specificity to mahaleb assay)
- **Removal of the probe:**
  - > Assay will amplify any *Prunus* species
- **Incorporate dsDNA intercalating dye**
  - > EvaGreen® assay (high sensitivity)
  - > SYBR® Green assay
- **Use PCR to amplify *Prunus* species present in the sample**
- **Perform post PCR DNA melt-curve analysis**



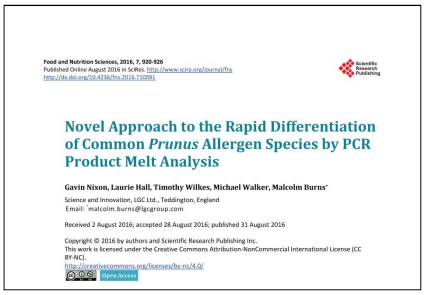
Example of a DNA melt curve

DNA melt-curve analysis

- **DNA melt curve analysis is a post-PCR analysis method**
- **Used in a range of applications including**
  - > Genotyping
  - > Current EU-RL GMFF assay for detection of Chinese GM rice
- **DNA melt curves are generated by slowly denaturing (melting) a double stranded DNA (dsDNA) sample through increasing temperatures in the presence of a dsDNA binding fluorescent dye (e.g. EvaGreen®)**
  - > While in the dsDNA bound confirmation, the dyes fluoresce
  - > When the dsDNA "melts" a net-change in fluorescence signal is observed
- **The point at which the dsDNA "melts" is dependent upon:**
  - > Size of amplicon
  - > Total GC content
- **DNA melt curve analysis can discriminate related DNA sequence targets**

Additional resources

- **For further information delegates are referred to:**
  - > Nixon *et al*., (2016) "Novel Approach to the Rapid Differentiation of Common *Prunus* Allergen Species by PCR Product melt Analysis". Food and Nutrition Sciences, 7, 920-926
  - > Applied Biosystems High Resolution Melting Getting Started Guide, available at the URL: *https://tools.thermofisher.com/content/sfs/manuals/cms_050347.pdf*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http://dx.doi.org/10.4236/fns.2016.710091

Novel Approach to the Rapid Differentiation of Common *Prunus* Allergen Species by PCR Product Melt Analysis

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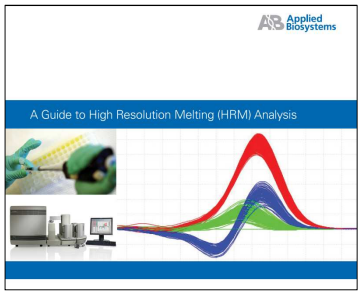

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  - > All species identities for control materials were independently confirmed by PCR amplicon sequencing[1]
- **DNA extraction[2]**
  - > DNA isolated and purified using a modified CTAB/Proteinase K extraction method
    - Two independent DNA extractions were performed for each of the control samples
    - Four independent DNA extractions were performed for the referred sample
  - > DNA concentration and purity determined spectrophotometrically
- **Experimental design**
  - > One DNA concentrations of 50 ng/reaction employed
  - > Triplicate PCR replicates performed for each sample

[1] For further information delegates are referred to [1]DNA sequencing to support food labelling enforcement and [2]DNA extraction to support food labelling enforcement, available at the Food Authenticity Network: http://www.foodauthenticity.uk/

Experimental setup

Component	Concentration
Forward primer	900 nM
Reverse primer	900 nM
2 x Fast Plus EvaGreen® qPCR Master Mix	1 x
DNA template	50 ng/reaction
Reaction volume	25 µl

Step	Temperature	Duration	Cycles
Hot start Taq activation	95°C	10 min	1 cycle
Template denaturation	95°C	15 sec	45 cycles
Anneal + extend	60°C	60 sec	
DNA melt analysis	95°C	15 sec	1 cycle
	60°C to 95°C	1.75°C/min	

Recommended composition of the LGC *Prunus* PCR reaction

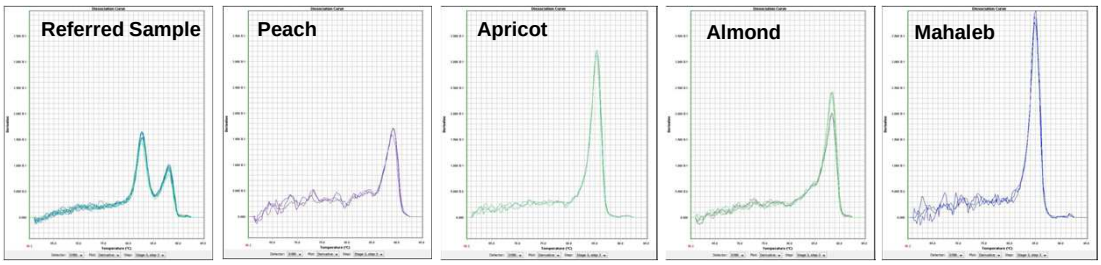
- **HPLC purified primers and probe**
- **Consumables and biological reagents from reputable distributers**
- **Appropriate experimental and contamination controls**

Recommended thermal cycling profile for use with the LGC *Prunus* PCR assay and DNA melt analysis

- **Conditions employed with use of an ABI 7900HT Fast PCR System**

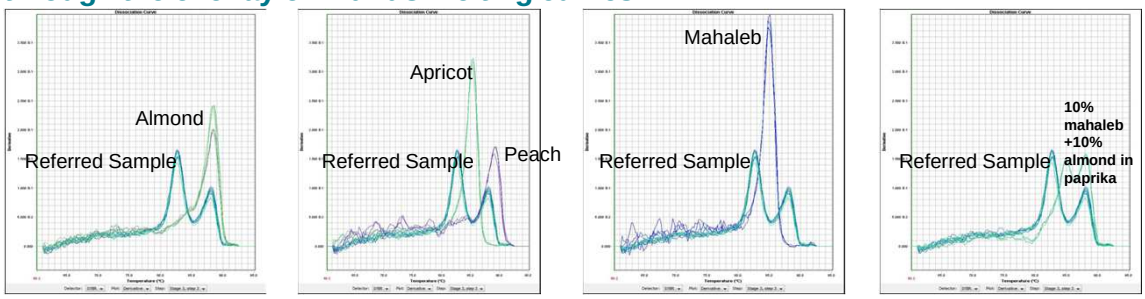
DNA melting curves observed for single *Prunus* species samples and the referred sample

- **All samples generated fluorescent profiles**
- **Single *Prunus* species samples characterised by a single major peak**
- **Referred sample profile exhibited two distinct peaks**
- **Temperature at which maximum difference in fluorescence occurred was specific to *Prunus* species**



DNA melting curves observed for combinations of *Prunus* species and the referred samples

- All of the mixed *Prunus* samples generated fluorescent profiles
- Individual *Prunus* species present in the mixed sample could be distinguished through reference to their respective melting peak
- Identity of *Prunus* species present in referred sample was partially identified through the overlay of *Prunus* melting curves



DNA melt curve findings

- All of the *Prunus* species (peach, apricot, mahaleb and almond) amplified
- Two distinct peaks were present in the dissociation plots of 10% mahaleb and 10% almond in paprika
- Two distinct peaks were present in the dissociation plots of the referred sample
  - > One peak consistent with almond
  - > Other peak not consistent with any of the control samples currently studied

Conclusion: a profile had been generated from the referee sample which was consistent with:

- Almond being present
- A second unidentified *Prunus* like species was detected

Overall conclusion

- **DNA:**
  - > No detectable presence of mahaleb
  - > Confirmed presence of almond
- **Impact:**
  - > Non-compliant with relevant food labelling legislation
  - > Although limitations still remain in the state of the science the referred sample contains *Prunus* protein(s) and DNA the origin of which is consistent with almond rather than mahaleb

Acknowledgements

LGC Molecular Food Authenticity Group

- **Malcolm Burns, Principal Scientist and Special Advisor to the Government Chemist**
- **Gavin Nixon**
- **Claire Bushell**
- **Michael Walker**

Department for Environment Food & Rural Affairs

Food Standards Agency food.gov.uk

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Any Questions?

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