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# Development of advanced PCR methods for testing the authenticity of herbs and spices

Science  
for a safer world



Gavin Nixon (LGC)  
GC Conference, 21<sup>st</sup> June 2016



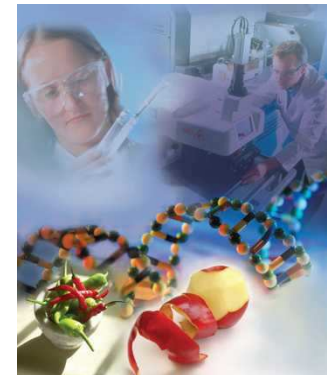
## Development of advanced PCR methods for testing the authenticity of herbs and spices

- Potential adulteration of spices with allergenic nut species

# Overview



- Introduction
  - Food allergies
  - Recent UK Government Chemist cases
- Case 1: Cumin
- Case 2: Paprika
- New technologies
- Summary



# Introduction



- Background

- Food allergies represent a definite threat to the general health and wellbeing of those affected, placing increasing pressure on food producers and regulatory authorities to test for their presence
- In 2015 a number of food products containing cumin were subject to withdrawal from the international market because of alleged adulteration with peanut and almond material
- Two samples (cumin and paprika) of herbs and spices were referred to the Government Chemist for analysis in order to identify the potential presence of nut material



# Introduction continued

- GC response
  - Application of ELISA, mass spectrometry and molecular biology approaches to investigate the potential adulteration of cumin and paprika samples with nut material
  - Development of novel real-time PCR assays for the detection of *Prunus* nut species

## Government Chemist

### **Statutory function**

“an independent and impartial referee analyst, authorised analyst and analyst by reference to or pursuant to certain legislation”

### **Advisory function**

“a source of advice for HM Government and the wider analytical community on the analytical chemistry implications on matters of policy and of standards and of regulations”



# Case1:Cumin



# Cumin case background



**Oct  
2014**

- Oct 2014, Canadian Food Inspection Agency
  - Random tests for allergens revealed undeclared peanut & almond protein in products containing cumin
- EU Labelling Directives (e.g. 1169/2011) – allergenic foods that must be labelled
  - A risk for individuals allergic to almond



**Jan/  
Feb  
2015**

- FSA (UK) issued recalls in Jan/Feb 2015 regarding almond protein in cumin and mixes for ready meals
- Denmark, Sweden & Norway issued alerts / recalls shortly afterwards
- None of the tests detected peanut proteins at levels that would require allergen labelling (FSA)
- Widespread recalls of N. America specific cumin products (Feb 2015)

# Cumin case background continued



**Feb  
2015**

- Sample of cumin referred to the Government Chemist by the FSA in Feb 2015



**Mar  
2015**

- March 2015, UK company subject to recall of the cumin product
  - Mahaleb was possibly the origin of the ELISA almond positives

**April  
2015**

- 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.”



# RASFF notifications



RASFF | Consumers

RASFF Portal

European Commission > RASFF Portal

Notifications list   New search   Export to... ▾

## Search result: 4 notifications

Search criteria   **Notified from** 10/02/2015   **Notified till** 18/02/2015   **Product type** food   **Hazard category** allergens

First   Previous 100   Notifications **1 to 4** of 4   Next 100   Last

|    | Classification            | Date of case | Reference | Notifying country | Subject  | Product Category                             |
|----|---------------------------|--------------|-----------|-------------------|--|--|
| 1. | alert                     | 12/02/2015   | 2015.0152 | United Kingdom    | traces of <u>almond</u> (270 mg/kg - ppm) in fajita meal from Sweden                       | other food product / mixed                   |
| 2. | information for attention | 12/02/2015   | 2015.0149 | Italy             | undeclared gluten in gluten-free milk chocolate bar from Italy                             | cocoa and cocoa preparations, coffee and tea |
| 3. | alert                     | 16/02/2015   | 2015.0181 | Belgium           | undeclared fish and molluscs in beef teriyaki sauce from the Netherlands                   | soups, broths, sauces and condiments         |
| 4. | alert                     | 17/02/2015   | 2015.0187 | United Kingdom    | traces of <u>almond</u> (>18 mg/kg - ppm) in spice mix from Sweden, via the United Kingdom | herbs and spices                             |

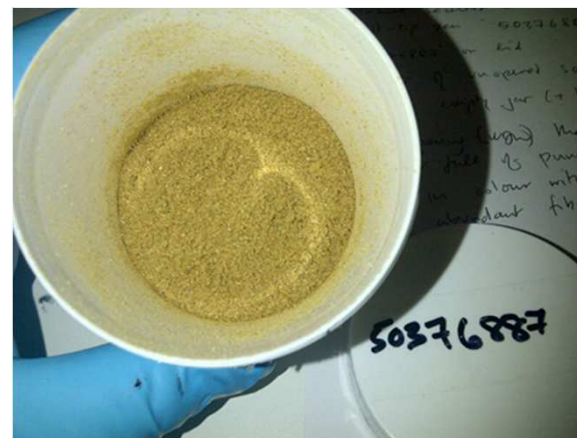
# Almond and mahaleb

- Members of the large ‘Prunus’ genus
- Prunus includes drupes (stone fruit) capable of producing kernels
  - Almond, *Prunus dulcis*
  - Apricot, *Prunus armeniaca*
  - Cherry, *Prunus avium*
  - Peach, *Prunus persica*
  - Mahaleb, *Prunus mahaleb*
- Typical approach for the detection of almond
  - ELISA immunoassay (protein)
  - A number of commercially available “almond” ELISA kits can suffer from significant cross-reactivity issues
- Little known *Prunus mahaleb*
  - Species of cherry tree (native to the Mediterranean)
  - Seeds used for spice



# Analytical strategy

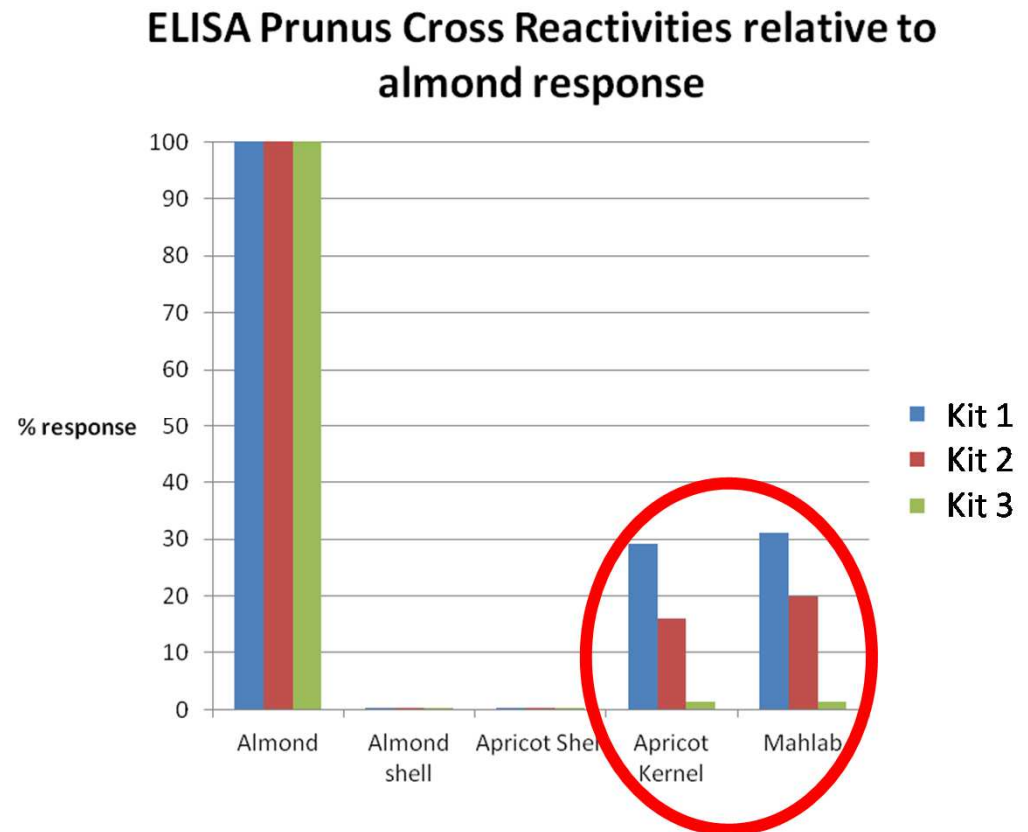
- Referred sample
  - “Ground cumin”
- GC multi-disciplinary approach taken
  - ELISA
  - Mass spectrometry
  - Advanced DNA techniques
- Appropriate control materials
- Develop novel assays/approaches as required



Ground cumin sample

# ELISA and MS approaches

- **ELISA**
  - Three different commercial kits evaluated with common Prunus species
  - Cross reactivity observed
  - A protein indicative of the Prunus genus was detected in referee sample
- **MS**
  - No unique peptides characteristic of almond detected
  - Peptides characteristic of mahaleb detected





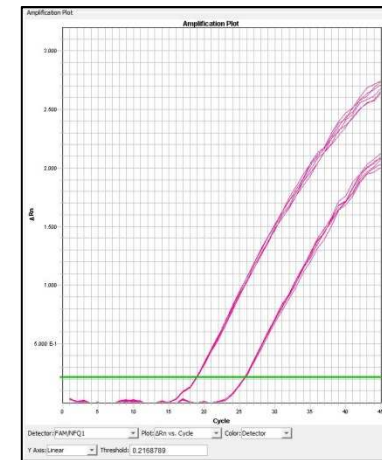
## DNA approach – real time PCR

- Limited available tests
- Search of DNA sequences on publically available DNA databases:
  - National Center for Biotechnology Information (NCBI) GenBank
  - Barcode Of Life Database (BOLD)
- Considerable DNA sequence similarity between all species within the Prunus genus
- Internal Transcribed Spacer (ITS) region identified as a potential target
  - DNA spacer between rRNA subunit genes

# Novel assay development

- *P. mahaleb* species specific real time PCR assay
- Main features
  - Based on common hydrolysis probe real time PCR format
  - Non proprietary
  - Good analytical specificity and sensitivity
- Applied to referee sample

Typical real time PCR results

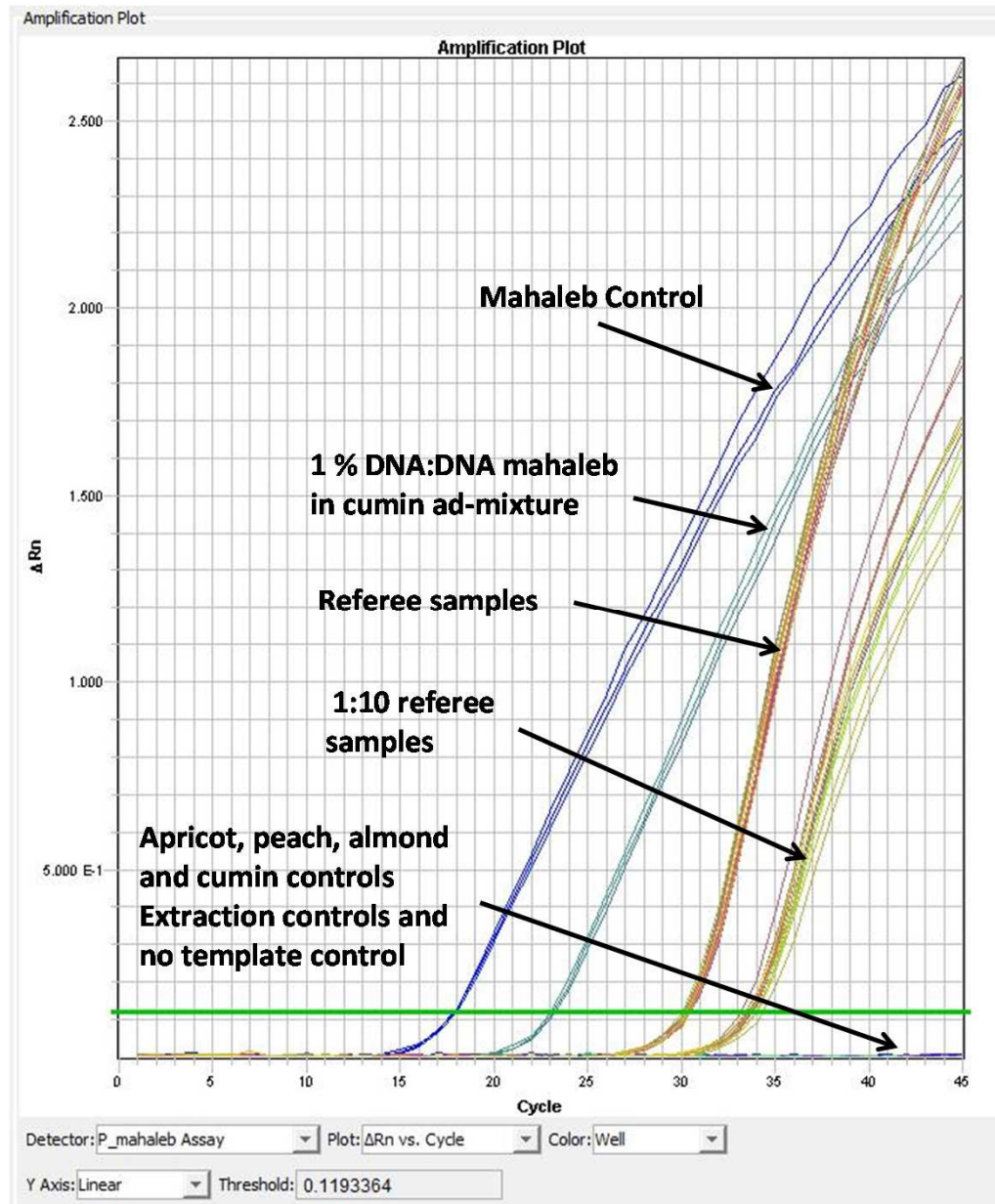


# Real-time PCR results on DNA approach



| Specimen                                       | Amplification    | $C_q \pm SD$   |
|--|------------------|--|
| Almond kernel                                  | No amplification | Not applicable   |
| Apricot kernel                                 | No amplification | Not applicable   |
| Peach kernel                                   | No amplification | Not applicable   |
| Cumin  | No amplification | Not applicable   |
| Extraction blanks (n = 4)                      | No amplification | Not applicable   |
| Mahaleb kernel                                 | Amplification    | $17.87 \pm 0.06$   |
| Referee sample<br>(4 independent extns)        | Amplification    | $30.39 \pm 0.10$ ; $30.14 \pm 0.07$<br>$30.40 \pm 0.04$ ; $30.12 \pm 0.15$ |
| Referee sample (1:10)<br>(4 independent extns) | Amplification    | $33.49 \pm 0.34$ ; $33.58 \pm 0.14$<br>$34.04 \pm 0.22$ ; $33.65 \pm 0.25$ |
| 1% DNA:DNA mahaleb:cumin                       | Amplification    | $23.10 \pm 0.14$   |

- *P. mahaleb* DNA specifically detected
- No cross reactivity observed
- Confirmed the presence of mahaleb







## Case 1 Summary

- In isolation, none of the applied techniques could answer whether almond or mahaleb (or both) were present in the referee sample
- In combination, the techniques provided strong evidence of the presence of mahaleb rather than almond
- DNA approach was crucial in the correct identification of the adulterant ***Prunus mahaleb***
- Conclusion: the referred sample contained Prunus protein and DNA the origin of which was consistent with mahaleb rather than almond
- Method has been submitted as a peer reviewed paper



## Case 2: Paprika



## Paprika case background

- August 2015: a sample of paprika was referred to the Government Chemist by the Food Standards Agency
- Reports/evidence that almond, a species known to be an allergy risk for some individuals, was thought to be present in the sample
- Product did not enter the UK food chain





## Paprika case background

- 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
  - ELISA
  - Mass spectrometry
  - *P. mahaleb* specific real time PCR test





## Analytical findings

- ELISA and MS confirmed the referred sample contain Prunus species proteins
  - No mahaleb specific peptide signatures seen
  - Significant amount of Prunus protein(s) present in the referred sample
- Mahaleb real time PCR assay
  - Mahaleb DNA was not detected (LOD of <1 ppm)
- Prunus protein(s) found: not on label – potential allergen risk
  - Likely to be almond (common Prunus species)
  - Case closed?

# Development of a novel Prunus assay

- Mahaleb real-time PCR assay:
  - Primers (generic to Prunus genus)
  - Probe (provided specificity to mahaleb assay)
- Removal of the probe:
  - Assay will amplify any Prunus species
  - Remove the fluorescent moiety, so how to detect?
- DNA melt analysis approach
  - dsDNA intercalating dye (high sensitivity), e.g. EvaGreen®
  - PCR products identified by melt profile





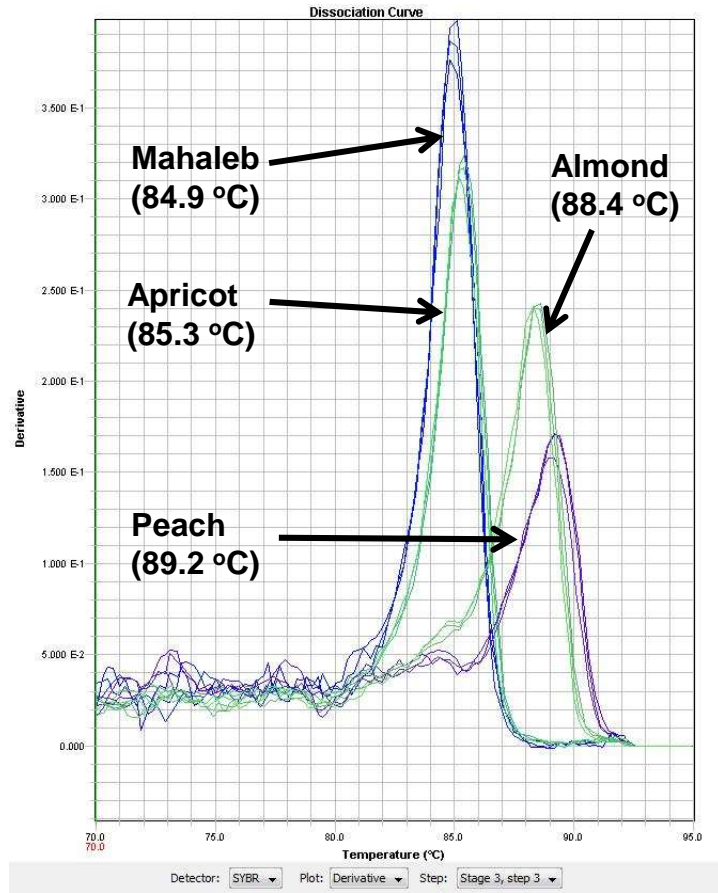
# DNA melt-curve analysis

- Principle
  - PCR amplification of target
  - Slowly heat up dsDNA PCR product, the dsDNA will begin to dissociate (un-wind) into its component ssDNA form
- When the dsDNA “melts” a net-change (drop) in fluorescence is seen when a dsDNA intercalating dye (e.g. EvaGreen®) is present
- The point at which the dsDNA “melts” is dependent upon
  - Size of amplicon
  - Nucleotide composition
- Approach applied to the referred sample

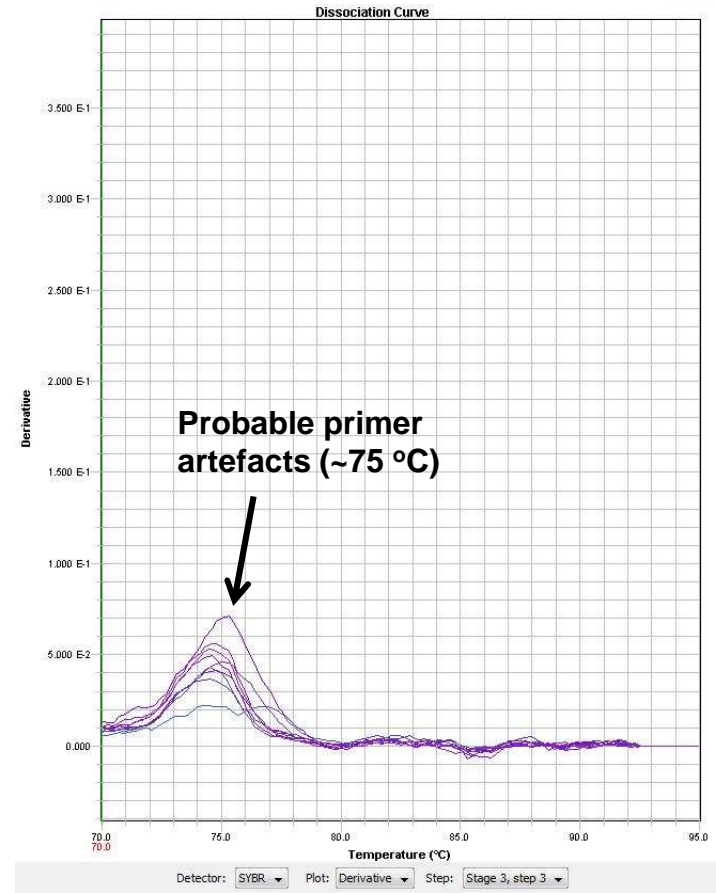




# Results - controls



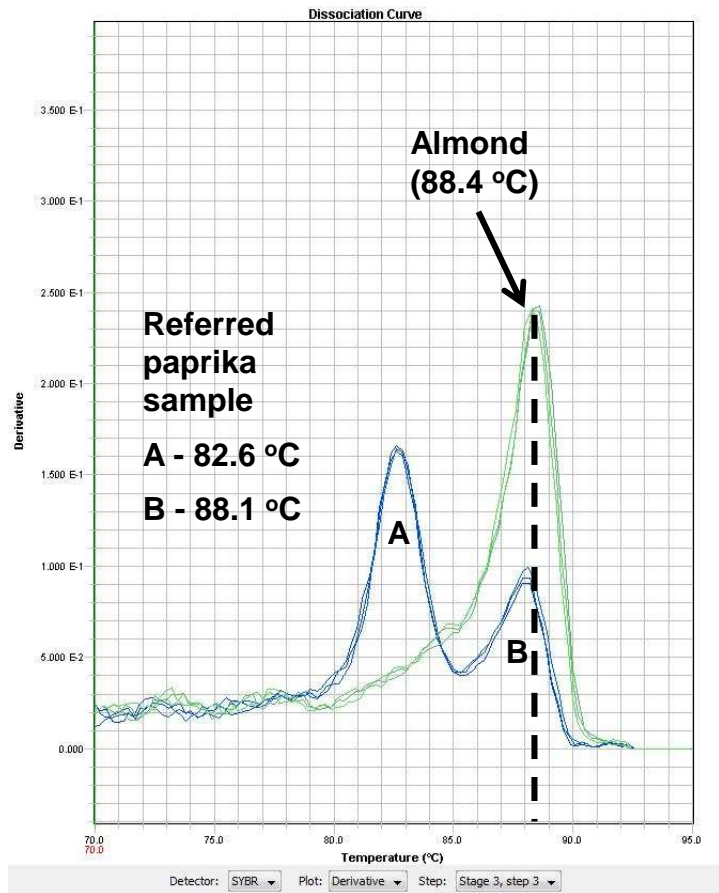
**Prunus species controls**



**Negative and peanut controls**



# Results – test samples



**Paprika-based referred sample and almond controls**

- All of the Prunus species (peach, apricot, mahaleb and almond) amplified
- 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



## Melt curve findings

**Conclusion** - a profile had been generated from the referred sample which was consistent with

- Almond being present
- . . . and a second Prunus like species has been detected
- Sample considered non compliant with relevant UK/EU labelling legislation

## Case 2 Summary

- ELISA - continued application for screening for the authenticity of spices
  - Limitations in terms of cross-reactivity with many members of the Prunus family
- Mass spectrometry
  - No mahaleb specific peptide signatures seen
  - Peptides characteristic of Prunus species (almond) observed
- Real-time PCR
  - No detectable presence of mahaleb; almond confirmed
- DNA melt-curve analysis
  - Demonstrated that it can detect DNA from specific Prunus species
  - Multi-analyte capability
- Non-compliant with relevant food labelling legislation
- In isolation, none of the applied techniques could answer, in a forensically robust manner, the question of whether almond or mahaleb or both were present in the cumin
  - DNA provided best evidence of the particular species present
- Method has been submitted as a peer reviewed paper



# Further information - GOV.UK news stories



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News story

## Cumin analysis: DNA test for mahaleb developed

From: Government Chemist and Food Standards Agency  
First published: 29 June 2015

A novel and pioneering DNA test for the detection of mahaleb has been developed by the Government Chemist programme.

<https://www.gov.uk/government/news/cumin-analysis-dna-test-for-mahaleb-developed>

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## Paprika referee sample: Further testing identifies almond present

From: Government Chemist  
First published: 12 November 2015

Pioneering DNA and mass spectrometry tests for the detection of almond and mahaleb further developed by the Government Chemist programme

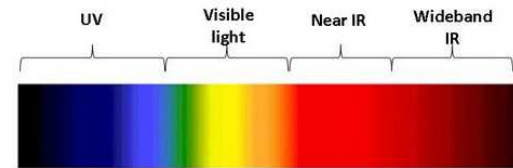
<https://www.gov.uk/government/news/paprika-referee-sample-further-testing-identifies-almond-present>



# Novel technologies

# Multispectral imaging

- Multispectral Imaging (MSI) uses the simultaneous measurement of both:
  - Reflected light across a broad range of wavelengths
  - An order of spatial magnitude

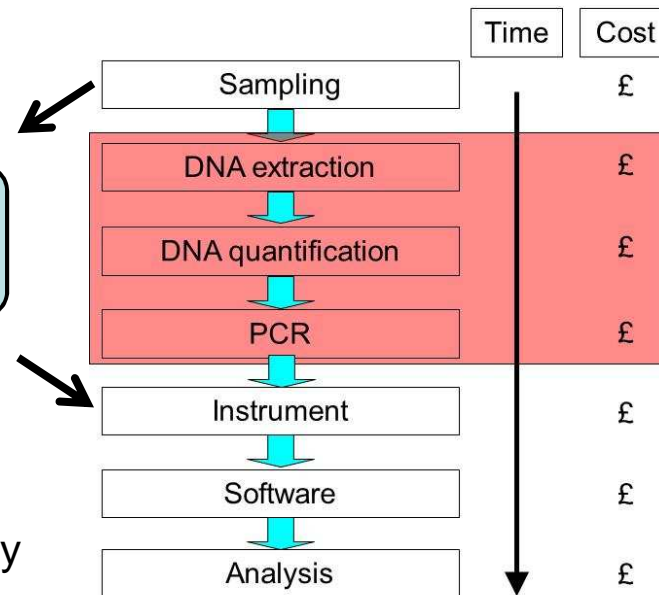


- . . . to allow fast and accurate determination of surface colour, texture and chemical composition of ingredients

- Advantages:

- Rapid
- Non-destructive
- Contact free
- Highly automated
- Limited requirement for specialist training
- Multi-analyte
- Range of applications, e.g. food authenticity

MSI Approach



# Application of MSI to herbs and spices testing - Oregano



Oregano

Myrtle

Olive leaves

1:1:1 Mixture



# Overall summary





# Summary

- Food allergies represent a definite threat to the general health and wellbeing
- In 2015, two samples of herbs and spices were referred to the Government Chemist for analysis
- Multi disciplinary approach (ELISA, MS, DNA)
  - Prunus nut species identified
  - DNA provided species identification
  - Novel molecular tests developed targeting *P. mahaleb* and the Prunus genus
- New analytical approaches and technologies offer enhanced capabilities within the spice and herb testing sector

# Acknowledgements



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  - Malcolm Burns
  - Michael Walker
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