Presentation overview

• Food authenticity
  – Area of interest
  – DNA as a target analyte

• Food authenticity testing
  – Methods for DNA analysis

• Identification and analysis of horse meat
  – EU Guidance on detection of horse meat
  – Aspects associated with quantitation of DNA from meat species
  – GC involvement
  – LOD work

• Development of a real-time PCR approach for quantitation of horse DNA
  – Quantitation of horse DNA
  – Results
  – Scope of work and limitations

• Other approaches
  – DNA sequencing; Multispectral imaging; dPCR; Mass-spec

• Summary
Food authenticity
Area of interest

- Malcolm Burns – Principal Scientist
- Molecular and Cell Biology Group (LGC)
- Area of specialisation: food authenticity testing (DNA)
  - e.g. GMOs; meat & fish speciation; allergens; Basmati rice; durum wheat; etc.,
- Manager for the UK National Reference Laboratory for GMOs in Food and Feed
Food authenticity

- Food purchased by the consumer must match its description

... all aspects require development of analytical methods for correct identification of food materials
Why use DNA?

- DNA molecule relatively resistant to degradation
- Potentially enables analysis of following samples:
  - Raw
  - Cooked
  - Processed
- Ubiquitous: copies of the DNA target sequence will be present throughout all tissues of an organism/food
- Choice of DNA targets cf. antibodies for proteins
- Specificity / sensitivity aspects
- Qualitative/Quantitative assay
- Alternative and confirmatory approach to protein
Methods for food authenticity testing
GC involvement in protocols for food authenticity testing

- Examples
  - Pasta (Durum wheat)
  - Basmati rice
  - Fish speciation
  - Allergens
  - GM
  - Meat speciation
  - Fruit juice

Food Standards Agency's open access repository
Defra website “GOV.UK”
Example fish and meat speciation protocols

- Rob Ogden, TRACE Wildlife Forensics Network
  - Adaptation of DNA analysis techniques for the identification of illegally imported bushmeat for use on the Agilent 2100 bioanalyser - \textit{FSA Project Q01109}

- John Dooley and Steve Garrett, Campden BRI
  - Application of a Chip-based Capillary Electrophoresis System to Enable Simple PCR-RFLP identification of Fish Species - \textit{FSA Project Q01069}
  - Extending the Fish Species Lab-on-a-Chip Capillary Electrophoresis PCR-RFLP Database - \textit{FSA Project Q01099}

- Hez Hird, Fera
  - The adaptation and validation of real-time PCR methods for the identification of exotic meat species, for analysis on a capillary electrophoresis chip system - \textit{FSA project Q01107}
  - The development and validation of DNA marker methods for the verification of meat from wild boar - \textit{FSA project Q01129}
Typical DNA analysis

- Sampling effects
- DNA extraction
- DNA quantification
- PCR setup
- Equipment operation
- Software analysis
- Manual analysis
- User interpretation

“A procedural approach for the identification of sources of uncertainty associated with GM quantification and real-time quantitative PCR measurements”
Knowledge Transfer event

- Knowledge Transfer event “DNA extraction approaches to support food labelling enforcement” for Public Analysts
- Jointly sponsored by Defra, the FSA and the GC
- Held at LGC
  - Provided technical introduction to DNA extraction basics
  - Reviewed current Defra and FSA food authenticity protocols
  - Summarised the spectrum of different DNA extraction methods currently available
  - Provided DNA quality metrics to adhere to
  - Discussed data interpretation
  - Practical component
  - Appropriate follow-up Challenge Exercise

- Feedback from participants: excellent opportunity to network, further enhance their skills, share experiences and discuss specific issues in relation to DNA extraction
Methods for Nucleic Acid analysis

- PCR and Capillary Electrophoresis
- Real-time PCR
- Digital PCR
- Rapid DNA testing (Point-of-test/on site)
- DNA sequencing and Next Generation Sequencing (NGS)
Identification and analysis of meat species
Background

• 15th January 2013 the Food Safety Authority of Ireland (FSAI) published a report:
  – A total of 27 beef burger products were analysed with 10 of these products (37%) testing positive for horse DNA and 23 (85%) testing positive for pig DNA
  – In one instance when analysing a beef burger on sale at supermarket, results were reported that stated “the level of horse DNA indicated that horsemeat accounted for approximately 29% relative to the beef content”

• 16th January 2013 – Food Standards Agency (FSA) issues four-point plan for the investigation:
  1. Urgent review of the traceability of the food products identified in the FSAI survey
  2. Explore methodology used
  3. Consider whether any legal action is appropriate
  4. Work with Defra on a UK-wide survey

• Global issue - Illegal substitution of beef with horse
How did GC help?

GC assisted Government on all aspects of the four-point plan
Advice, Analysis and Research

- GC worked with FSA/Defra and provided advice on methods for determination of horse meat as part of the UK survey of beef products
- Advised on issues associated with threshold labelling (1%)
- Attended EU consultative meeting for 2nd round of horse-meat testing (Brussels) with FSA representing UK expert laboratory
- Members of Defra’s AMWG and AMWG-TSG
- Defra Project: establish LOD of methods used in the UK survey of beef products for horsemeat
- Defra Project: develop a real-time PCR approach for quantitation of horse DNA
- Project to establish whether species cross contamination occurs in UK meat processing plants during the GMP production of mince meat
- Analysed 7 referee cases related to meat speciation in 2013
  - Horse, beef, pork, lamb
EU Guidance

- EURL for Animal Proteins in feedingstuffs
  - Recommendations for detection of horse DNA
  - Expressing amount of horse meat in relation to other meat species on a w/w basis using a DNA approach
  - Published guidance on how to implement and test a threshold level (1% w/w)
- Commission Recommendation of 27 March 2014 “On a second coordinated control plan with a view to establishing the prevalence of fraudulent practices in the marketing of certain foods” (2014/180/EU)
Quantitation of meat species

Example issues:

- Lack of “standardisation”
- Agreement on expression units (w/w or cp,cp)
- Mitochondrial vs. nuclear DNA?
- Quantitation: relative term. Relative to what?
  - Total meat?
  - Total DNA?
  - Specific meat?
  - Mammalian DNA?
- Relationship between DNA copy numbers and actual meat content
- Evaluation and assessment of impact of food processing on DNA measurement
- Requires full appreciation of factors
Current GC work

• Current GC programme: Genomic vs. mitochondrial as a DNA target
• Reports in published literature:
  – Mitochondrial target: good for sensitivity studies as target very abundant, but number can be variable
  – Genomic target: stable copy number may give potential for quantitation, but not as abundant as mitochondrial targets
• Current GC work:
  – Application of genomic and mitochondrial assays (kits / published literature)
  – Assessing potential for quantitation in a range of raw and processed meat materials
Example factors that can affect quantitation

- Accurate quantitation is dependent upon a number of factors, including:

  - Species
  - DNA target
  - Level of degradation
  - Other ingredients
  - Matrix background
  - DNA template amount
  - DNA extraction approach
  - Sample preparation (temperature, processing)
  - PCR efficiency
  - Tissue type
  - DNA recovery
  - DNA target
  - Species

Etc.,
Quantitation

<table>
<thead>
<tr>
<th>Issue</th>
<th>Meat</th>
<th>GMOs</th>
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<td>Expression units</td>
<td>w/w or cp/cp?</td>
<td>Mostly w/w</td>
</tr>
<tr>
<td>Target</td>
<td>Mitochondrial vs. nuclear</td>
<td>Nuclear</td>
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<tr>
<td>Relative expression</td>
<td>Total/specific meat?</td>
<td>Relative to taxon</td>
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<td></td>
<td>Total/specific DNA?</td>
<td>specific ingredient e.g.</td>
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<td></td>
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<td>soya</td>
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<td>Matrix/processed food</td>
<td>Relative to RM as calibrant</td>
<td>Relative to CRM as</td>
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<td>calibrant</td>
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• There are a number of questions to answer . . . but it is not impossible to resolve this
  – e.g. Quantitation of GMOs using DNA

• Harmonisation of approaches and expression/interpretation of results as well as understanding of some of the key factors that can affect the reliability of a result

• Communication between EU member states and EU guidance
Defra Project: FA0134

- Defra Project (FA0134) “Method verification of the LOD associated with the UK Survey on Horse-meat”
- Horse-meat issue 2013: Defra/FSA commissioned a UK Survey of beef products
- Samples taken on a formal basis by Public Analysts
- Range of analytical methods/kits available but respective Limits of Detection (LOD) often different, not robustly defined, or expressed using different measurement units e.g.
  - DNA copy numbers (Approx. <100 copies mitochondrial genome)
  - Gravimetric meat preparations (Approx. <0.1% w/w)
  - Amount of DNA (25pg of mitochondrial DNA)
  - DNA:DNA ratios (<0.1% DNA/DNA basis)
- LOD of a method:
  - Critical performance characteristic that represents the lower limit of applicability of the method
  - Needed to be robustly defined so results can be interpreted with confidence
Potential issues: non-detects

Scenario 1

A
LOD 1%

B
LOD 0.1%

2%

Scenario 2

A
LOD 1%

B
LOD 0.1%

0.5%

Detected?
Aim of LOD work

- **Aim**: Evaluate LOD of specific methods used by PAs as part of the UK horse-meat Survey in terms of uniform w/w (raw horse-meat in a raw beef (meat) background) sample measurements
- Range of gravimetrically prepared raw horse-meat in raw beef meat (w/w) materials produced
  - Authenticated for species identity (real-time PCR, ELISA and DNA sequencing)

- **Previous GC work**
  - “Modelling the limit of detection in real-time quantitative PCR”
  - European Food Research and Technology
  - Novel approach for assessing sensitivity limits in real-time PCR
Published GC work

Results

• All three methods had the capability of reaching a LOD of less than 0.1% w/w raw horse-meat in a raw beef (meat) background
  – if Quality Procedures and Good Laboratory Practice for molecular biology methods were adhered to
• Likelihood of not detecting the presence of the target was similar between those methods
• Helped afford good comparability of results from the methods
• Gave added confidence in the interpretation of the results from the UK survey on beef products
Method verification of the LOD associated with the Defra/FSA Study protocol for detection of horse DNA in food samples - FA0134

Description

Food authenticity and food fraud are becoming increasingly problematic owing to pressures on food production and the current climate of financial constraint. The recent findings of horse DNA present in beef burgers sold in a UK supermarket chain has highlighted the need to provide support for rapid and reliable appraisal of the meat supply chain by developing standardised approaches for the detection and quantification of different meat products.

Defra/FSA propose to conduct a UK wide study for identification of equine DNA in food samples using an FSA validated screening approach. This approach has an LOD associated with it that was based on approximate calculations. This limit of sensitivity needs to be robustly tested and qualified so that results from the study can be interpreted with confidence.

Objective

The aim of the proposed work is to use a statistically robust design to fully validate the LOD associated with the Defra/FSA study screening method, in terms of w/w (meat to meat) sample measurements. This will allow the results from the Defra/FSA Study on detection of equine DNA in food samples to be qualified and standardised in terms of a robust limit of detection.

Project Documents

- EVID4 - Final project report: Final report FA0134 (288k)

Time-Scale and Cost

From: 2013
To: 2013
Cost: £33,315

Contractor / Funded Organisations

LGC Limited

Keywords

Agro Food Quality
Method Verification of the LOD Associated with PCR Approaches for the Detection of Horse Meat

Eloise Busby and Malcolm Burns

Summary

In 2013, the Department for Food and Rural Affairs (Defra) and the Food Standards Agency (FSA) commissioned a UK Survey of beef products as part of a co-ordinated response to the EU horse-meat issue. Samples were taken on a formal basis, allowing UK Public Analysts to choose which methods to apply. A range of analytical methods were available for detection of horse DNA, but the respective Limits of Detection (LOD) were often different, not robustly defined, or expressed using different measurement units. The LOD of methods used in the UK Survey needed to be robustly tested and qualified so that results obtained from the samples could be interpreted with confidence.

The aim of the present study was to evaluate the LOD of three selected methods used by Public Analysts as part of the UK horse-meat Survey in terms of uniform w/w (raw horse-meat in a raw beef (meat) background) sample measurements. The three methods evaluated were a PCR-Capillary Electrophoresis approach (PCR-CE as described in Defra project FA0220, LOD reported as approx. 1% w/w), PrimerDesign (LOD of approx. <100 mitochondrial copies); and Neogen BioKits (LOD approx. 0.1% w/w).

A range of genetically prepared raw horse-meat in raw beef meat (w/w) materials were produced as part of the current study and authenticated for species identity. These materials were used to challenge the three methods in order to estimate the LOD in terms of w/w (meat to meat) based on internationally accepted guidelines and best measurement practice for LOD and PCR methods. Estimates for the LOD were based upon 60-115 replicates of the 0.1% w/w material, depending upon the method evaluated. More than 250 replicates of the 0.1% w/w material were assessed across the three analytical methods, representing five independent DNA extractions.

Results showed that all three methods were capable of reaching an LOD of less than 0.1% w/w raw horse-meat in a raw beef (meat) background if Quality Procedures and Good Laboratory Practice for molecular biology methods were adhered to. This helped afford good comparability of results for these three methods, and in turn contributed to asuring that the results from the UK Survey of beef products in 2013 were interpreted with confidence.
Development of a real-time PCR approach for quantitation of horse DNA
Previous FSA work

• FSA project (Q01084): “Final optimisation and evaluation of DNA based methods for the authentication and quantification of meat species” (2005)
  – H. Hird - (CSL)
  – G. Saunders (VLA); B. Popping (Eurofins); S. Garrett (CCFRA); G. Wiseman (RHMT)
• FSA Foodbase
• Laid foundations for a better understanding of meat speciation and quantitation and additional development of approaches
• One of the areas investigated: quantitation of meat species using real-time PCR
• Some of the most promising results:
  – Plasmid absolute copy number calibrants combined with Ct measurements
  – Use of a “GM” model approach for analysis based on dilutions of a calibrant
Introduction

• Defra Project (FA0135) “Development of a real-time PCR approach for the quantitation of horse DNA”

• EU horse meat issue:
  – Lack of guidance and scientific standardisation on how the amount of meat adulteration in a sample was expressed

• Highlighted the requirement for a quantitative approach to be developed to accurately measure the amount of horse DNA present in samples

• Aim of the work: Develop a real-time PCR approach for the quantitation of horse DNA

• In line with current scientific thinking and sharing synergy with EU guidance on approach, two nuclear DNA targets were chosen: one target specific to horse DNA, the other as a general reference target for any mammalian DNA
Quantitation of horse DNA

• Meat samples:
  – Authenticated for species identity using real-time PCR, ELISA and DNA sequencing

• Authenticated meat samples used to produce a range of w/w tissue gravimetric materials
  – Raw horse meat in a background of raw beef (meat) on a gravimetric w/w basis

• The quantitative approach for horse DNA was validated in terms of:
  – Specificity
  – PCR efficiency and linearity
  – Limit of Detection (LOD)
  – Trueness and precision

• Tested on raw meat samples
Calibration

Sample

Extract DNA

Dilute DNA

qPCR

Horse Assay

Mammalian Reference Assay

Estimated Copy Number

Estimated Copy Number

Calibrants

Calibrants
Test sample evaluation

Horse Assay

Mammalian Reference Assay
Conclusion to Quant work

• **Limitations:**
  – Conducted using ideal controlled conditions
  – Measures relative amount of DNA
  – Results can be expressed in relation to a gravimetric w/w meat basis **BUT** only in terms of relative amount of raw horse meat in a raw beef (meat) background

• **But:**
  – This is the current state-of-the-art of the science
  – Similar approaches used for other meat species (e.g. commercial kits)
  – Significantly added value to the science

• **Further work:**
  – Applicability to different meats and samples
  – Characterise precision around 1% level
  – Assessment of processed foods
  – Potential Knowledge Transfer event for Public Analysts and Industrial stakeholders
Additional approaches

• Other approaches being investigated for their potential for meat quantitation:
  • DNA sequencing
    – NGS for massively parallel sequencing and relative abundance of different PCR amplicon populations
  • Multispectral imaging
    – Successfully applied for meat spoilage testing
  • dPCR
    – Absolute single molecule detection; calibration curve
  • Protein mass spectrometry
    – Potential for quantitatively determining specific meat species (e.g. species-specific peptide biomarkers)
Summary

• The EU and UK horse meat incident highlighted a number of important issues
• There is a lack of harmonised approaches for quantitating the level of meat adulteration
• Traceability of sources of materials/ingredients used in foods is a prerequisite
• A demonstrable need to invest in analytical techniques and strategies for the detection and quantitation of meat species:
  – Development (R&D)
  – Maintain these approaches and adapt as necessary
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Defra

FSA
Thank you for listening

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