Government Chemist

Quantitation of meat species DNA

Science
for a safer world

Malcolm Burns
LGC, Principal Scientist and Special Adviser to the Government Chemist
2014 Government Chemist Conference,
The Royal Society, London, Monday 24th November 2014

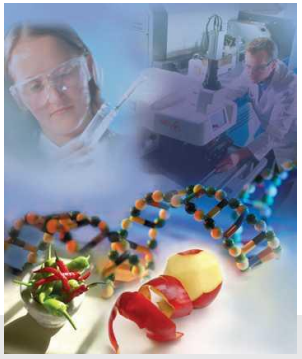
Presentation overview

- 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

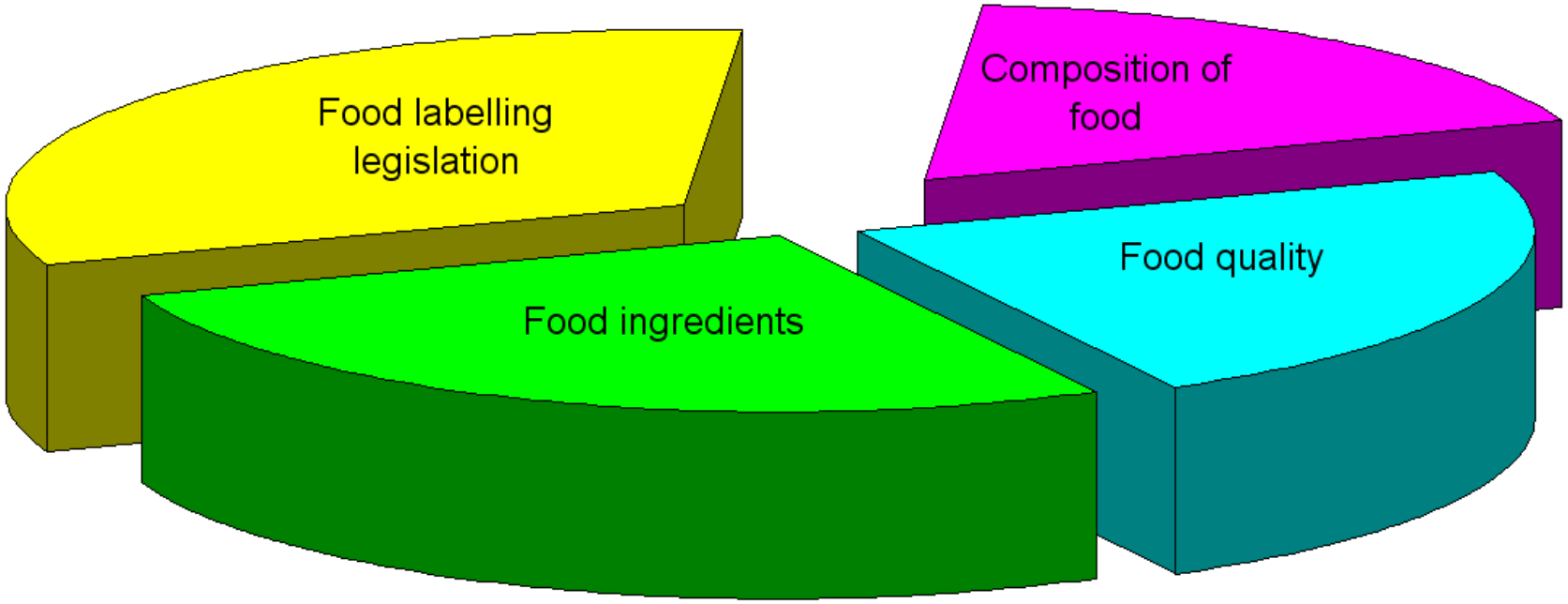
Area of interest

- 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

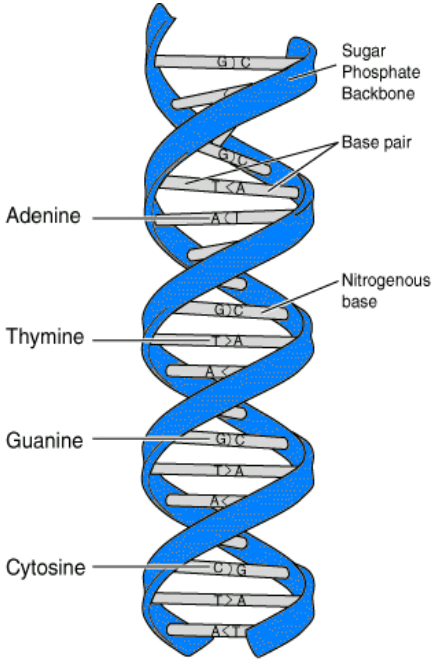
- Food purchased by the consumer must match its description



. . . 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







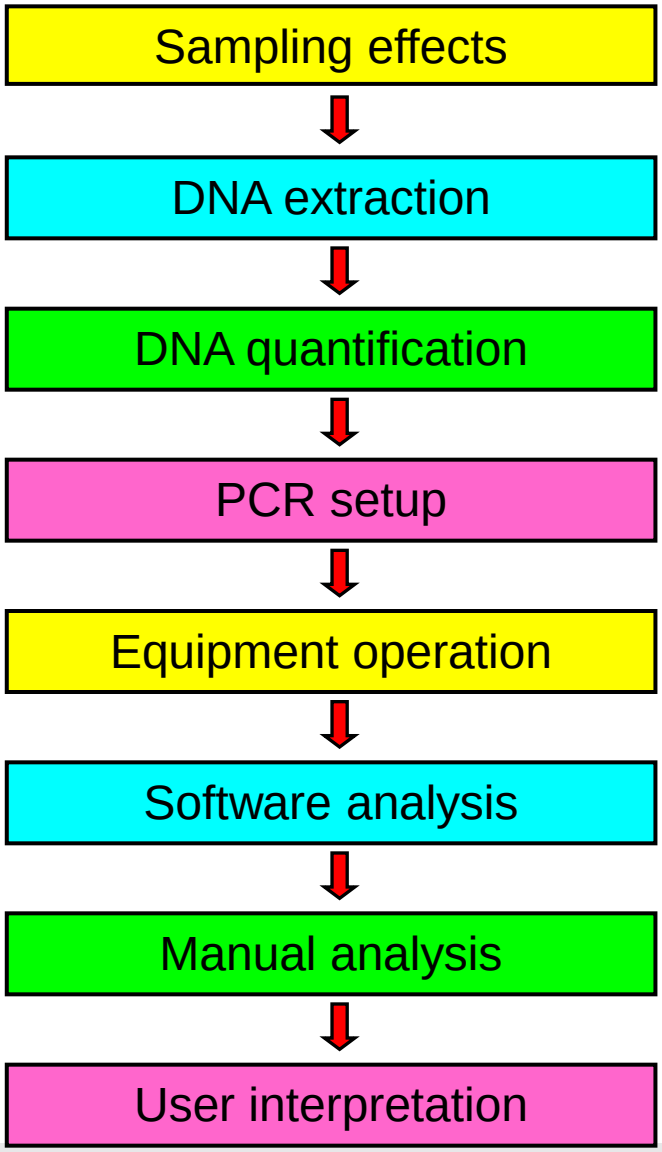

Food Standards Agency
food.gov.uk

FSA Foodbase : http://www.foodbase.org.uk/
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 - ***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 - ***FSA Project Q01069***
  - Extending the Fish Species Lab-on-a-Chip Capillary Electrophoresis PCR-RFLP Database - ***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 - ***FSA project Q01107***
  - The development and validation of DNA marker methods for the verification of meat from wild boar - ***FSA project Q01129***

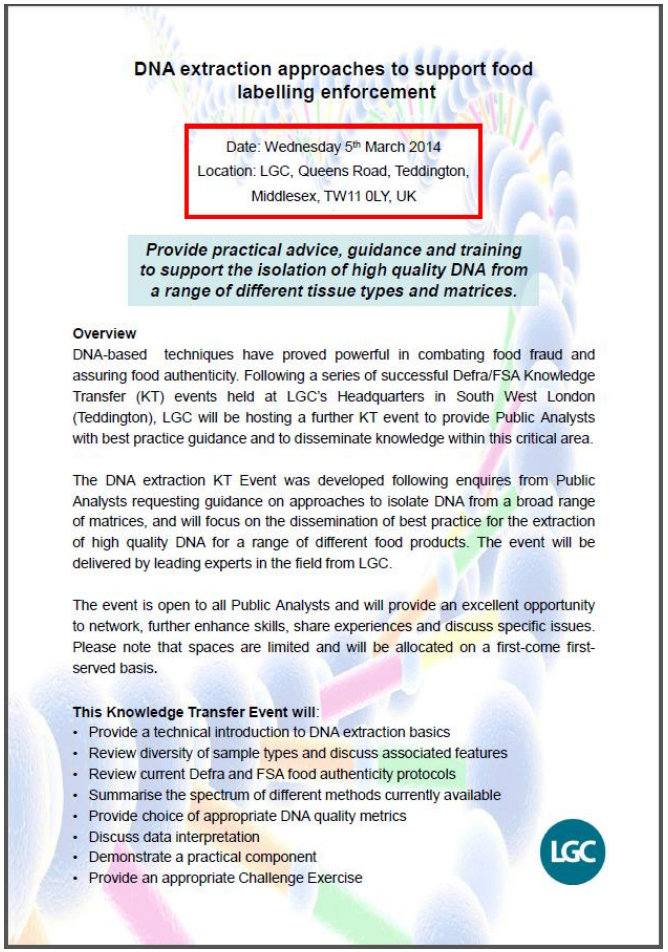
Typical DNA analysis



"A procedural approach for the identification of sources of uncertainty associated with GM quantification and real-time quantitative PCR measurements" M.Burns and H.Valdivia, European Food Research and Technology (2007) 226: 7-18 -DOI: 10.1007/s00217-006-0502-y

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



DNA extraction approaches to support food labelling enforcement

Date: Wednesday 5th March 2014
Location: LGC, Queens Road, Teddington, Middlesex, TW11 0LY, UK

Provide practical advice, guidance and training to support the isolation of high quality DNA from a range of different tissue types and matrices.

Overview

DNA-based techniques have proved powerful in combating food fraud and assuring food authenticity. Following a series of successful Defra/FSA Knowledge Transfer (KT) events held at LGC's Headquarters in South West London (Teddington), LGC will be hosting a further KT event to provide Public Analysts with best practice guidance and to disseminate knowledge within this critical area.

The DNA extraction KT Event was developed following enquires from Public Analysts requesting guidance on approaches to isolate DNA from a broad range of matrices, and will focus on the dissemination of best practice for the extraction of high quality DNA for a range of different food products. The event will be delivered by leading experts in the field from LGC.

The event is open to all Public Analysts and will provide an excellent opportunity to network, further enhance skills, share experiences and discuss specific issues. Please note that spaces are limited and will be allocated on a first-come first-served basis.

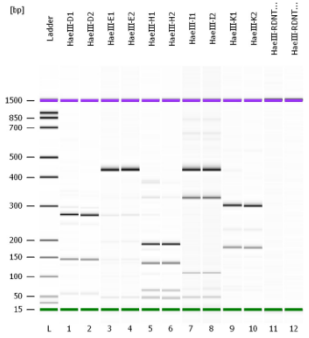
This Knowledge Transfer Event will:

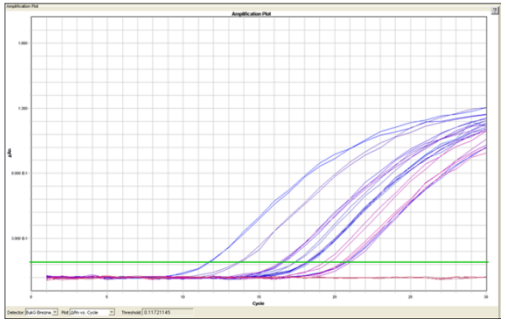
- Provide a technical introduction to DNA extraction basics
- Review diversity of sample types and discuss associated features
- Review current Defra and FSA food authenticity protocols
- Summarise the spectrum of different methods currently available
- Provide choice of appropriate DNA quality metrics
- Discuss data interpretation
- Demonstrate a practical component
- Provide an appropriate Challenge Exercise

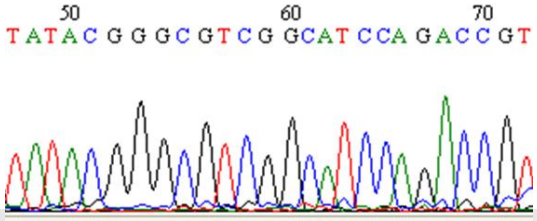
- Feedback from participants: excellent opportunity to network, further enhance their skills, share experiences and discuss specific issues in relation to DNA extraction

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?

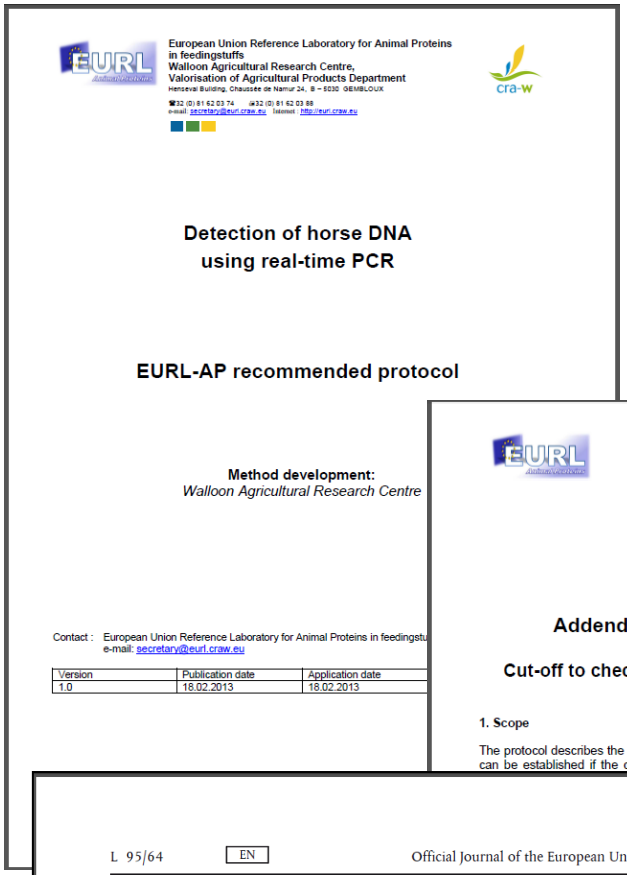
GC assisted Government on all aspects of the four-point plan

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)


European Union Reference Laboratory for Animal Proteins in feedingstuffs
Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval Building, Chaussée de Namur 24, B – 5030 GEMBLOUX

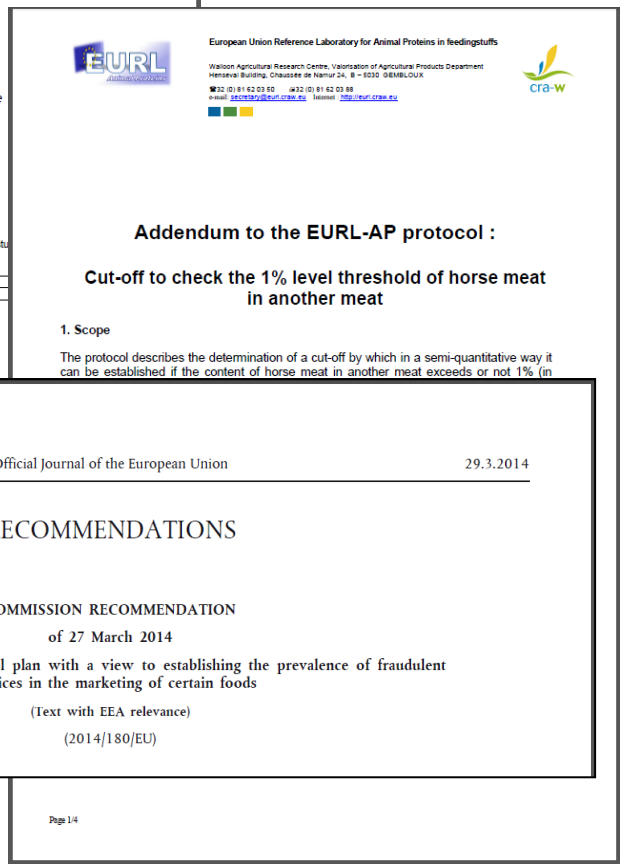
Detection of horse DNA using real-time PCR

EURL-AP recommended protocol

Method development:
Walloon Agricultural Research Centre

Contact : European Union Reference Laboratory for Animal Proteins in feedingstu
e-mail: secretary@eurl.craw.eu

Version	Publication date	Application date
1.0	18.02.2013	18.02.2013


European Union Reference Laboratory for Animal Proteins in feedingstuffs
Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval Building, Chaussée de Namur 24, B – 5030 GEMBLOUX

Addendum to the EURL-AP protocol :

Cut-off to check the 1% level threshold of horse meat in another meat

1. Scope

The protocol describes the determination of a cut-off by which in a semi-quantitative way it can be established if the content of horse meat in another meat exceeds or not 1% (in

L 95/64 EN Official Journal of the European Union 29.3.2014

RECOMMENDATIONS

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
(Text with EEA relevance)
(2014/180/EU)

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European Union Reference Laboratory for Animal Proteins in feedingstuffs

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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

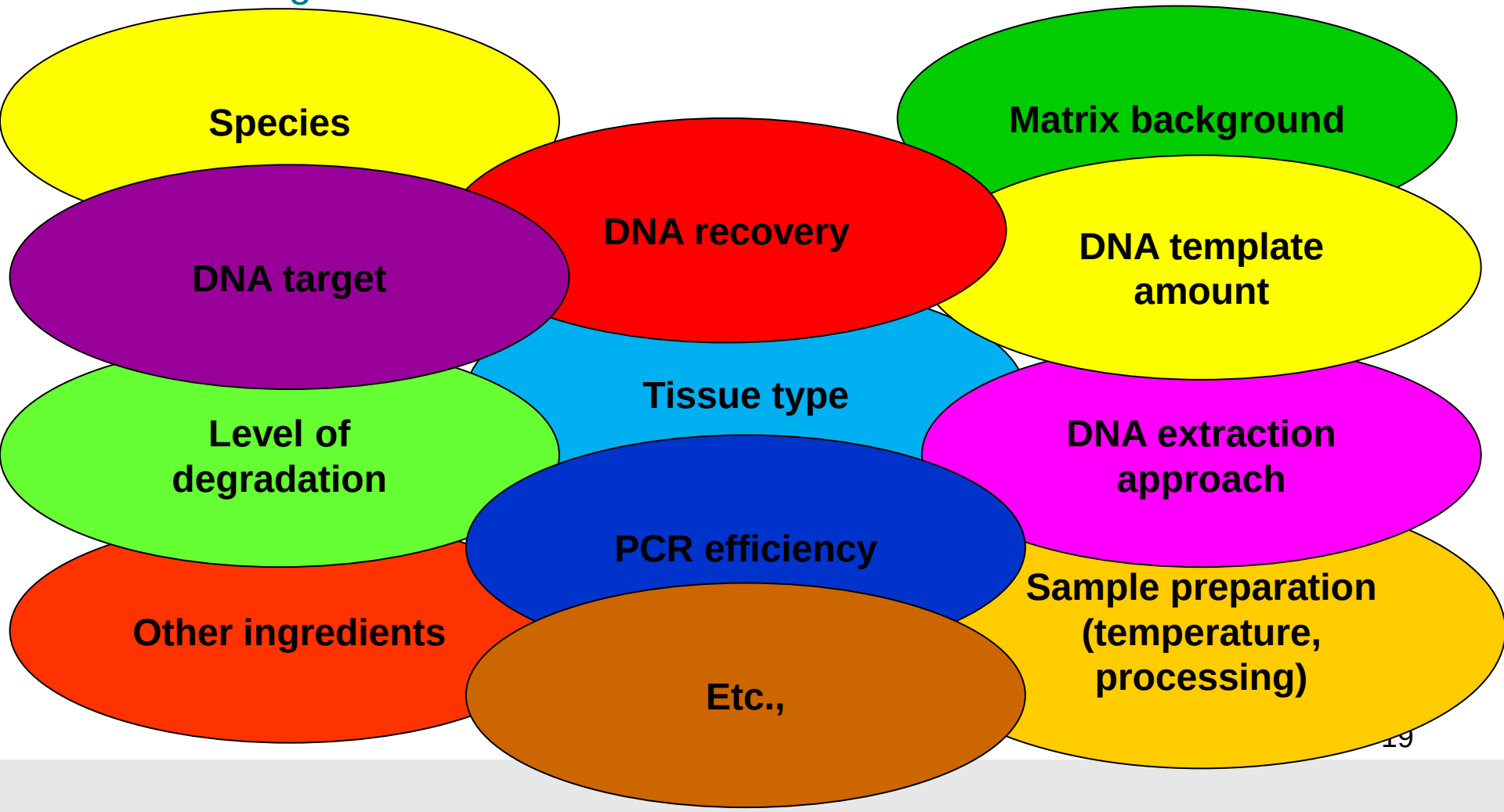
- Current GC programme: Genomic vs. mitochondrial as a DNA target
- Reports in published literature:
  - Mitochondrial target: good for sensitivity studies as target very abundant, but number can be variable
  - Genomic target: stable copy number may give potential for quantitation, but not as abundant 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:



Quantitation

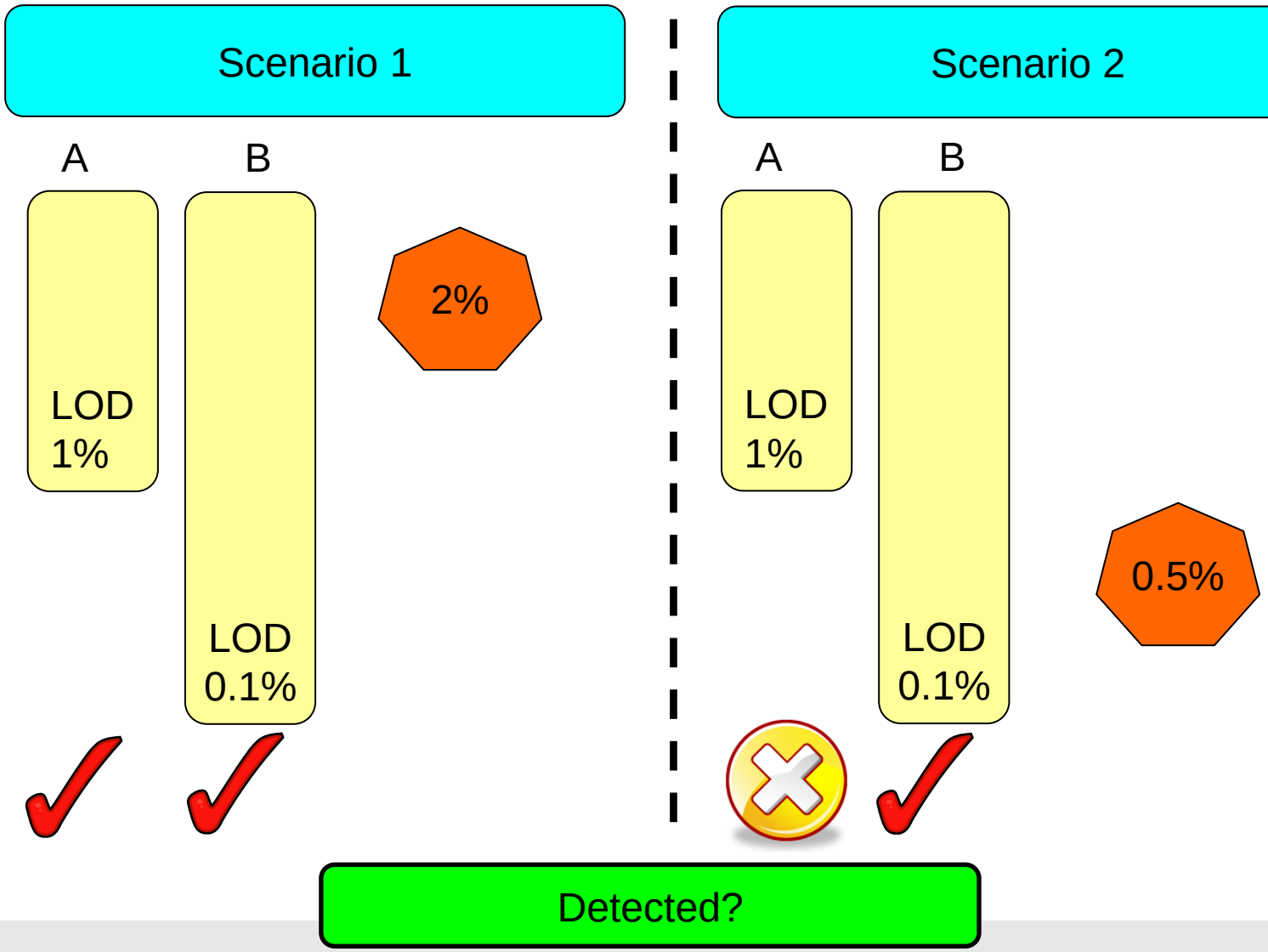
Issue	Meat	GMOs
Expression units	w/w or cp/cp?	Mostly w/w
Target	Mitochondrial vs. nuclear	Nuclear
Relative expression	Total/specific meat? Total/specific DNA?	Relative to taxon specific ingredient e.g. soya
Matrix/processed food	Relative to RM as calibrant	Relative to CRM as calibrant

- 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

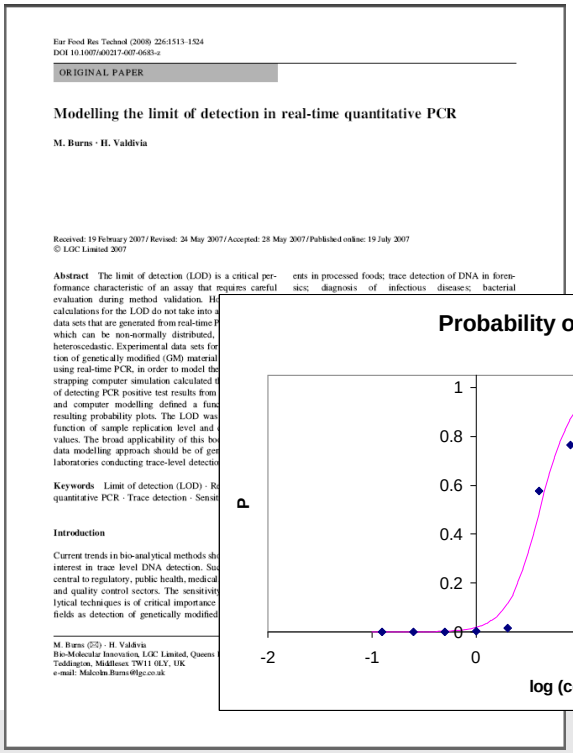
Potential issues: non-detects



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



Eur Food Res Technol (2008) 226:1513–1524
DOI 10.1007/s00217-007-0683-z

ORIGINAL PAPER

Modelling the limit of detection in real-time quantitative PCR

M. Burns · H. Valdivia

Received: 19 February 2007 / Revised: 24 May 2007 / Accepted: 28 May 2007 / Published online: 19 July 2007
© LGC Limited 2007

Abstract The limit of detection (LOD) is a critical performance characteristic of an assay that requires careful evaluation during method validation. H... calculations for the LOD do not take into a... data sets that are generated from real-time P... which can be non-normally distributed, ... heteroscedastic. Experimental data sets for ... tion of genetically modified (GM) material ... using real-time PCR, in order to model the ... strapping computer simulation calculated t... of detecting PCR positive test results from ... and computer modelling defined a func... resulting probability plots. The LOD was ... function of sample replication level and ... values. The broad applicability of this bo... data modelling approach should be of ger... laboratories conducting trace-level detectio...

ents in processed foods; trace detection of DNA in forensics; diagnosis of infectious diseases; bacterial

Keywords Limit of detection (LOD) · Re... quantitative PCR · Trace detection · Sensit...

Introduction

Current trends in bio-analytical methods sh... interest in trace level DNA detection. Su... central to regulatory, public health, medical ... and quality control sectors. The sensitivity ... lytical techniques is of critical importance ... fields as detection of genetically modified ...

M. Burns (✉) · H. Valdivia
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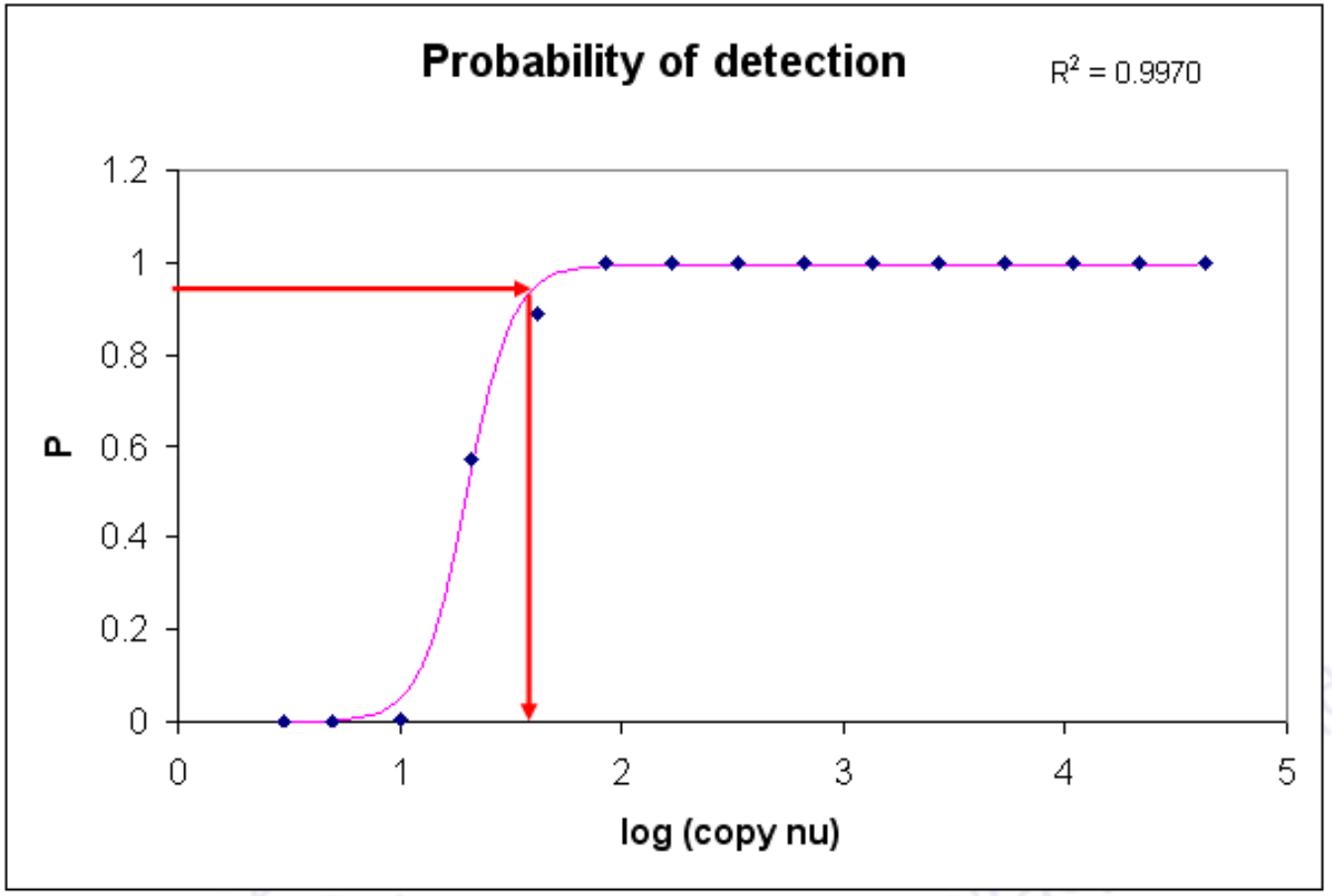
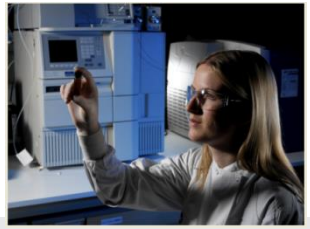
Published GC work



Burns *et al*., (2008) "Modelling the Limit Of Detection in real-time quantitative PCR" European Food Research and Technology 226(6): 1503-1512

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



Science and Research Projects

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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 quantitation 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

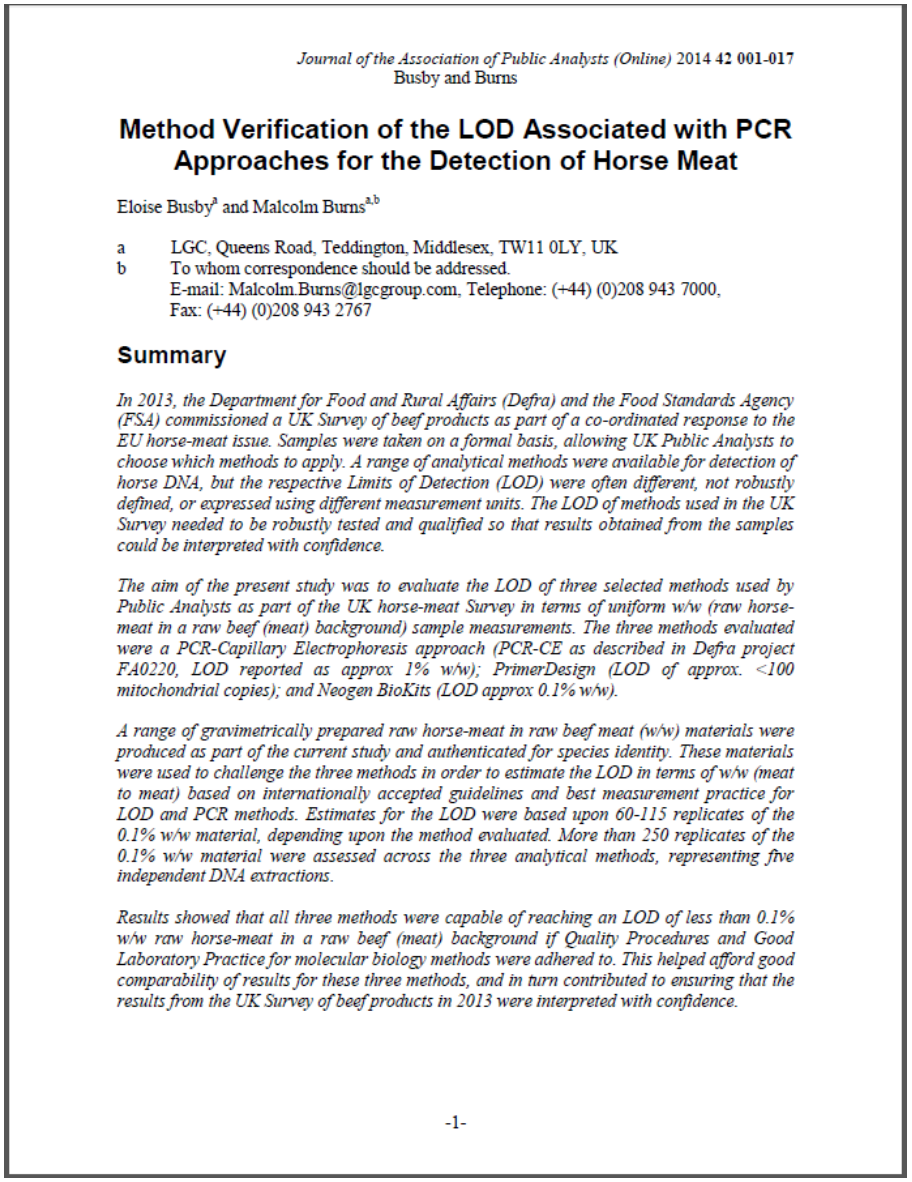
Keywords

Agro Food Quality

- Defra project report
- Defra home page – Science and Research Projects

LOD work

- Publication in Journal of Association of Public Analysts (2014)



Journal of the Association of Public Analysts (Online) 2014 **42** 001-017
Busby and Burns

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

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a LGC, Queens Road, Teddington, Middlesex, TW11 0LY, UK
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Fax: (+44) (0)208 943 2767

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 gravimetrically 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 ensuring that the results from the UK Survey of beef products in 2013 were interpreted with confidence.

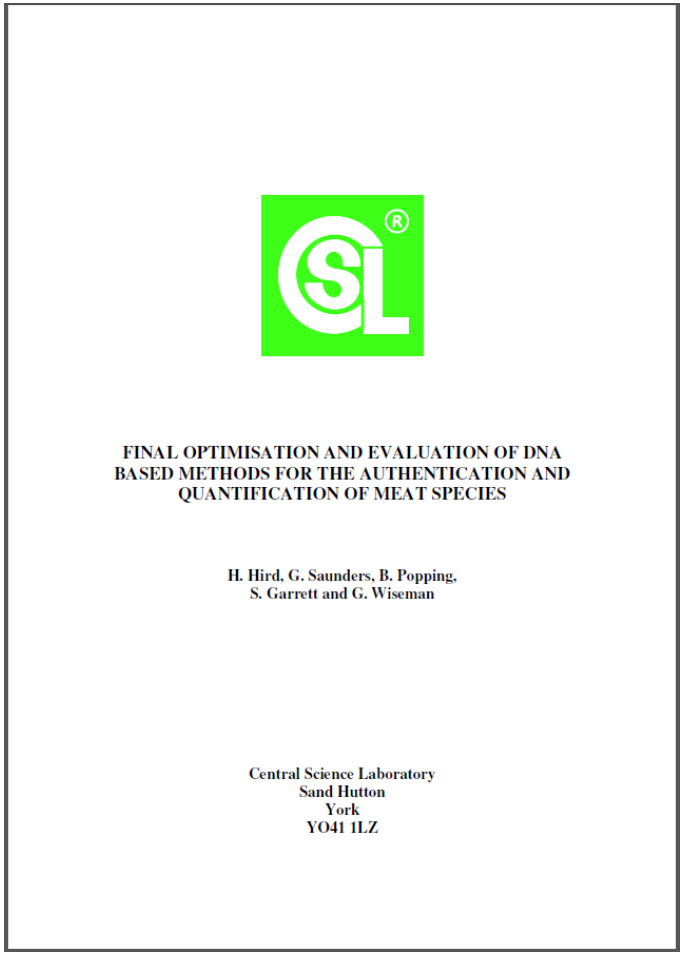
-1-

Eloise Busby and Malcolm Burns (2014) "Method Verification of the LOD Associated with PCR Approaches for the Detection of Horse Meat" Journal of the Association of Public Analysts 2014 (42): 001-017. http://www.apajournal.org.uk/2014_0001-0017.pdf

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

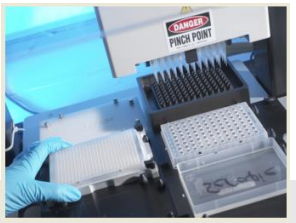

FINAL OPTIMISATION AND EVALUATION OF DNA BASED METHODS FOR THE AUTHENTICATION AND QUANTIFICATION OF MEAT SPECIES

H. Hird, G. Saunders, B. Popping, S. Garrett and G. Wiseman

Central Science Laboratory
Sand Hutton
York
YO41 1LZ

Introduction

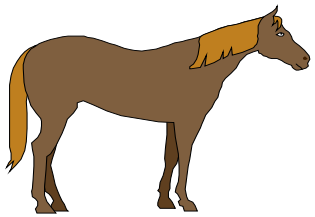
- **Defra Project (FA0135) "Development of a real-time PCR approach for the quantitation of horse DNA"**
- EU horse meat issue:
  - Lack of guidance and scientific standardisation on how the amount of meat adulteration in a sample was expressed
- Highlighted the requirement for a quantitative approach 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



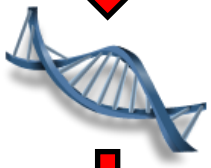
Quantitation of horse DNA

- 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

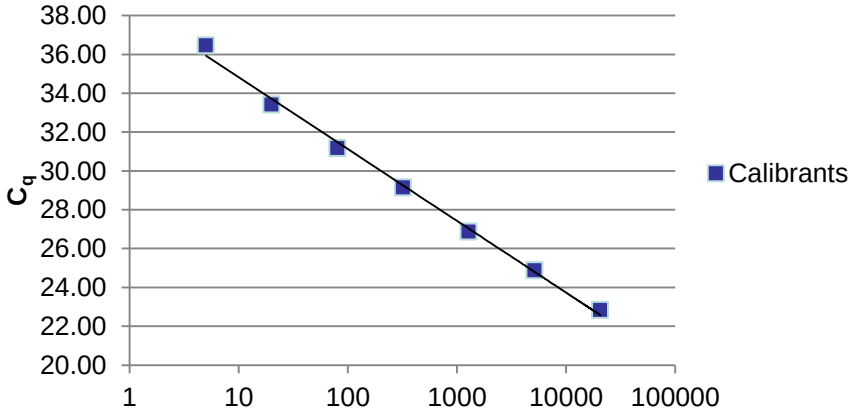


Extract DNA

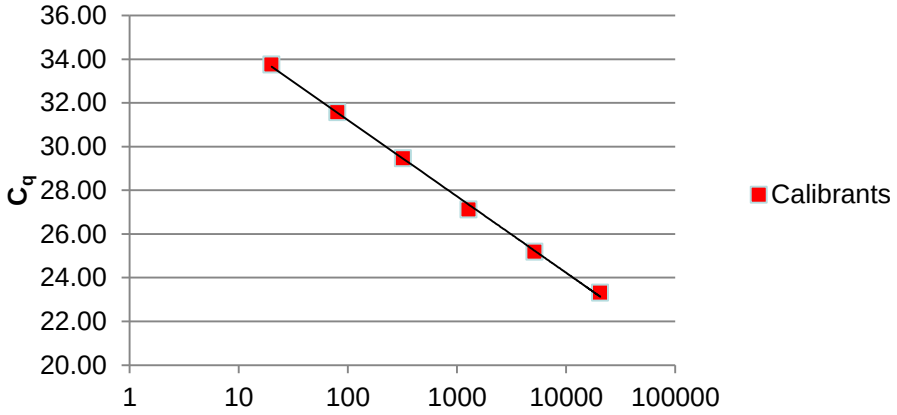
Dilute DNA

qPCR

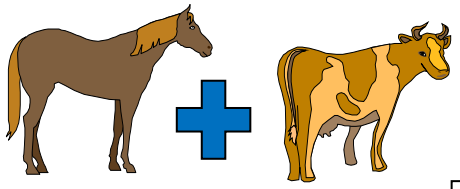
Horse Assay



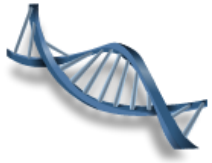
Mammalian Reference Assay



Test sample evaluation

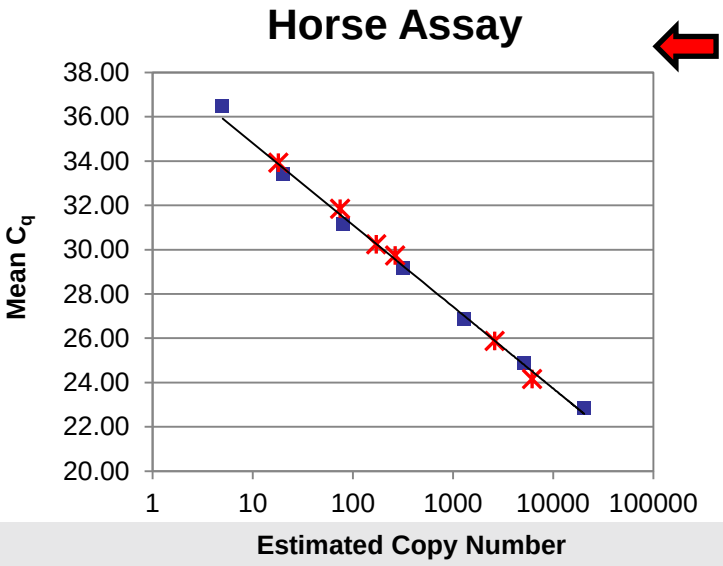


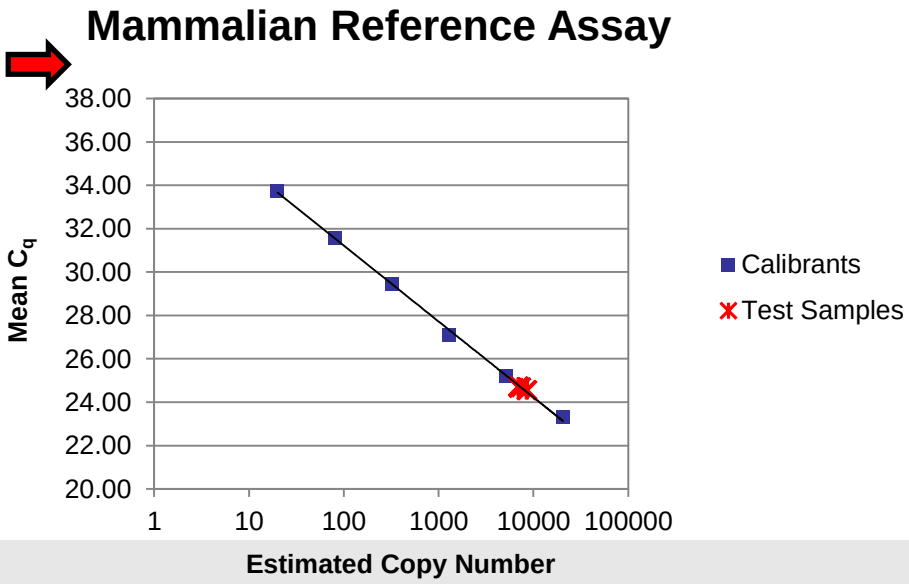
Test Samples
(w/w)



Extract DNA

qPCR





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

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

National Measurement System

Thank you for listening

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National Measurement System

National Measurement Office