Peptide biomarkers as a way to determine meat authenticity

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Clear and reliable information about food is demanded nowadays by consumers.

Lifestyle affects individual's choice on food consumption.

Honest and accurate food labeling is essential to assure food safety and choice.

Quantitative Ingredient Declaration (QUID)

Robust and reliable methodologies are needed to assure that fraudulent or accidental mislabeling does not arise.
Meat authenticity problem sources

- Meat origin
  - Geographical origin
  - Feeding/production systems
  - Breed
  - Sex
  - Meat cuts
  - Slaughter age

- Meat substitution
  - Species
  - Tissue
  - Vegetable
  - Animal
  - Organic

- Processing
  - Irradiation
  - Fresh/thawed
  - Preparation

- Non-meat additions
  - Additives
  - Water

Common strategies traditionally used to assess meat authenticity

1) **Analysis of stable isotope ratios:**

- $^{2H}/^{1H}$
- $^{13C}/^{12C}$
- $^{18O}/^{16O}$
- $^{15N}/^{14N}$

Incorporation into animal tissues

**Geographical origin**

**Feed intake**

Incorporation into animal tissues
Common strategies traditionally used to assess meat authenticity

Fraudulent addition of water to meat $\rightarrow$ Increase of size and weight

2) **Determination of the water / protein ratio:**
   - Mass measurement before and after drying of meat $\rightarrow$ Easy to do
   - It can be masked by addition of exogenous proteins

3) **Methods based on magnetic resonance:** NMR, MRI
   - Allow the study of water distribution into meat
   - Non-destructive

Addition of water

Addition of substances to increase water holding properties
Common strategies traditionally used to assess meat authenticity

4) Metabolomics

Identification and quantification of as many Low Molecular Weight Compounds as possible

- Identification of the presence of pasture in animal diets    Sivadier et al. 2010
- Detection of Mechanically Recovered Meat    Surowiec et al. 2011
- Authentication of typical Slavonian Salami    Jerkovic et al. 2010

Examples:

“Non-targeted approach”
Differentiation of Mechanically Recovered pork Meat (MRM) by CG-MS metabolite profiling

Surowiec, Fraser, Pater, Halket & Bramley (2011). *Food Chemistry*, 125, 1468

Clear discrimination of samples is not possible in all cases

Search for *specific* biomarkers
Common strategies traditionally used to assess meat authenticity

- High throughput
- Easy to use
- High sensitivity
- High throughput

Need for specific antibodies
Cross-reactions → False positives
Processing of foods can affect the immunoassay

Identification of meat species in foods

B) Methods based on DNA analysis (PCR):
- High discrimination power (species-specific)
- High sensitivity

Limitations on processed foods:
- Difficulties on DNA extraction
- DNA degradation: pH, heat, hydrolytic enzymes…

Low reliability

Need to develop alternative analytical approaches for species identification

Identification of biomarker peptides

MASS SPECTROMETRY

Proteomics
Proteomic technologies in Meat Science
The study of a genome expression products, with the objective to obtain an integrated and global vision of the cell processes.
Evolution of Proteomics

70's:

- Cell proteins
  - Identification
    - Immunodetection
    - N-terminal sequencing (Edman)
  - 2D-PAGE

Remarkable limitations:
- Not suitable for great scale protein analysis
- Slow and complicated
- Low sensitivity
90's:

- Development of "soft" ionization techniques coupled to mass spectrometry (ESI + MALDI)
- Massive genome sequencing + development of protein databases web-accessible (e.g., SUBSTANT and NCBI)
- Development of high-throughput bioinformatic tools (e.g., MATRIX SCIENCE, SCAFFOLD, Progeny, GenMAPP)

MODERN PROTEOMICS

"Great-scale Proteomics"
Workflows in current Proteomics

A) 1D, 2D-PAGE + MS:

Sample → 2D-PAGE → Digestion ("In-gel") → MALDI-TOF MS → Peptide fingerprint → Identity → Ambiguity → Peptide sequence → Identity

MS/MS fragmentation
Workflows in current Proteomics

B) Gel-free Proteomics:

1. Sample
2. Fractionation
3. Digestion ("In-solution")
4. (LC)-LC-ESI-MS/MS

Proteins:
- Protein 1
- Protein 2
- Protein 3
- Protein 4
Immediately after slaughter

72 h postmortem

In-gel digestion + MALDI-TOF MS

Protein identification

Changes in the pig muscle proteome during postmortem meat storage

Tenderness

Actin & Myosin degradation

Proteomics: Characterisation of proteolysis products in dry-cured ham

Dry-cured ham

Extraction

Deproteinisation

Size-exclusion chromatography

Reverse phase HPLC

Identification of peptide sequences

Mora, L. et al. (2010). Food Chem. 123, 691

Fragments generated from Troponin T degradation

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Identified sequence</th>
<th>Position</th>
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<tbody>
<tr>
<td>1</td>
<td>TAPKIPEGEKVDIQQKRNKD</td>
<td>57-81</td>
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<tr>
<td>2</td>
<td>APKIPEGEKVDIQQKRNKD</td>
<td>58-81</td>
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<tr>
<td>3</td>
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<td>5</td>
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<td>60-77</td>
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</table>
Peptide biomarkers as a reliable and accurate way to reveal meat composition

OBJECTIVE

Development of a methodology capable to overcome the existing limitations on the identification of animal species that can be found in meat products

Avoid fraudulent or accidental mislabelling of meat constituents
Identification of species-specific peptide biomarkers

Workflow:

1. **Extraction of muscle proteins**
2. **SDS-PAGE**
3. **In-gel digestion with proteolytic enzymes**
4. **Selection of target proteins**
5. **MALDI-TOF MS**
6. **ESI-MS/MS**
7. **Identification of species-specific peptide biomarkers**
Detection of chicken in a mix with pork meat

Selection of target protein:

Isoelectric focusing + SDS PAGE of myofibrillar proteins:

100 % pork

100 % chicken

Trypsin digestion

It is possible to detect 1% chicken in pork meat?
In-gel tryptic digestion of myosin light chain 3

**100 % pork**

- m/z: 1284.686, 1514.731, 1723.828, 1993.951, 2163.005, 2289.081, 2383.974, 2562.139, 2760.223

**100 % chicken**

- m/z: 1200.681, 1512.564, 1723.626, 1845.588, 2051.700, 2162.784, 2288.875, 2483.984, 2803.937

**1 % chicken in pork**

- m/z: 1077.382, 1399.499, 1530.482, 1927.700, 2288.875, 2483.984, 2804.020

MALDI-TOF

BRUKER DALTOMICS®

Reflex III
Zoom-in for m/z range 1490-1550

<table>
<thead>
<tr>
<th>M+H⁺</th>
<th>Position</th>
<th>Sequence</th>
<th>Origin</th>
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<tbody>
<tr>
<td>1514,683</td>
<td>81-93</td>
<td>DQGSYEDFVEGLR</td>
<td>Sus scrofa</td>
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<td>1512,6965</td>
<td>81-93</td>
<td>DQGTTFEDFVEGLR</td>
<td>Gallus gallus</td>
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100 % pork

100 % chicken

1 % chicken in pork

m/z range: 1490-1550
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<thead>
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<th>M+H⁺</th>
<th>Position</th>
<th>Modification</th>
<th>Modified mass</th>
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<td>Acetylation</td>
<td>1411.609</td>
<td>ALGQNPTNAEINK</td>
<td>Gallus gallus</td>
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</table>

**Origin**

- **100 % pork**
- **100 % chicken**
- **1 % chicken in pork**

**Acetylation**

- **37-49**

**Sequence**

- ALGQNPTNAEINK

**Acetylation**

- **37-49**

**Sequence**

- ALGQNPTNAEINK
MS/MS of DQGTFEDFVEGLR (peptide 1)

MALDI-TOF
1512,6965 (1+)

Ion trap
757.01 (2+)

Peptide fragmentation (MS/MS):

<table>
<thead>
<tr>
<th>b ions</th>
<th>y ions</th>
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<tbody>
<tr>
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<tr>
<td>244.09</td>
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</tr>
<tr>
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<td>402.16</td>
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<td>549.23</td>
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<tr>
<td>678.27</td>
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<tr>
<td>793.30</td>
<td>D</td>
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<td>940.37</td>
<td>F</td>
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<td>1225.50</td>
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<td>1338.58</td>
<td>L</td>
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<td></td>
<td>R</td>
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</tbody>
</table>
MS/MS for ALGQNPTNAEINK (peptide 2)

MALDI-TOF
1411.609 (1+), Acetyl

Ion trap
685.68 (2+)

Sequence

<table>
<thead>
<tr>
<th>b ions</th>
<th>y ions</th>
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<tbody>
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<tr>
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<tr>
<td>796.39</td>
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<td>1223.60</td>
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<td></td>
<td>K</td>
</tr>
</tbody>
</table>

147.11
Proteomics

Identification of species-specific peptides

Capable to detect the presence of 1% chicken in pork meat

Suitable for quantitation?
Quantitative proteomic approach

**AQUA**: “Absolute QUAntitation”

- Use of stable isotope peptides made from previously selected sequences

L*(\(^{13}\text{C6, }^{15}\text{N}\)) = +7 Da

ALGQNPTNAEINK (Mr 1369.7)

AL*GQNPTNAEINK (Mr 1376.7)

F*(\(^{13}\text{C9, }^{15}\text{N}\)) = +10 Da

DQGTFEDFVEGLR (Mr 1512.7)

DQGTFEDF*VEGL*R (Mr 1522.7)

Quantification of biomarker peptides

Quantification of animal species

Quantitative proteomics

Meat mixes
(0, 0.5, 1, 2, 5 and 10 % chicken in pork)

Protein extraction

Isoelectric focusing

Stable isotope labelled peptides (\(^{13}\text{C, }^{15}\text{N}\))

In-solution trypsin digestion

LC-ESI-MS/MS

Quantification of peptide biomarkers
0.5% chicken \rightarrow MLC-3 \rightarrow Trypsin digestion

Stable isotope labelled peptides

LC-MS

Clipeus C18 (150x0.5 mm)

y = 2.7523x
R² = 0.9921

Suitable for quantitation

ALGQNPTNAEINK (685.532⁺)

Picomol ALGQNPTNAEINK

Suitable for quantitation
Discrimination between closely related species

Prolteomic approach

Capable to differentiate between chicken and turkey meat?

<table>
<thead>
<tr>
<th>Protein</th>
<th>Chicken</th>
<th>Turkey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin Light Chain 3</td>
<td>High sequence homology between chicken and turkey muscle proteins</td>
<td></td>
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</table>
Discrimination between closely related species

OFFGEL separation of chicken and turkey proteins

Trypsin digestion + MALDI-TOF

<table>
<thead>
<tr>
<th>M+H⁺</th>
<th>Position</th>
<th>Sequence</th>
<th>Origin</th>
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<tbody>
<tr>
<td>1010.561</td>
<td>13-20</td>
<td>EAFLLFDR</td>
<td><em>Gallus gallus</em></td>
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<tr>
<td>982.332</td>
<td>13-20</td>
<td>EAFLLFDK</td>
<td><em>Meleagris gallopavo</em></td>
</tr>
</tbody>
</table>

Chicken myosin light chain 3

Turkey myosin light chain 3

Yes, we can!
CONCLUDING REMARKS

- The identification of species-specific peptide biomarkers using a proteomic approach constitutes an interesting and promising alternative to existing methodologies currently in use to assess meat authenticity.

- High discriminating power

- More robustness with respect to actual major limitations of DNA analysis:
  - Food processing
  - Can be applied for both fresh and cooked meats
  - Standardized extraction procedures

- Possibility to develop reliable quantitative determinations

- Possibility to use routine, user-friendly, mass spectrometry equipment
GOOD, GOOD...CHAPS!

Prof. Peter Bramley

Dr. Paul Fraser

Dr. Kaisa Koistinen

Mr. Chris Gerrish

Dr. Quique Sentandreu
Thank you