Complementary methods for animal proteins detection in feed

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Legislation methods of detection


- **Commission Directive n° 126/2003** on the analytical method for the determination of constituents of animal origin for the official control of feedingstuffs

- **Commission Regulation No 152/2009** laying down the methods of sampling and analysis for the official control of feed.

- **Commission Regulation No 51/2013** amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed.
Light microscopy and PCR

• Combination edited by SOPs

<table>
<thead>
<tr>
<th>Title</th>
<th>Version</th>
<th>Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>EURL-AP SOP slide preparation and mounting</td>
<td>V.1.0</td>
<td>BINDING</td>
</tr>
<tr>
<td>EURL-AP SOP use of staining reagents</td>
<td>V.1.0</td>
<td>OPTIONAL</td>
</tr>
<tr>
<td>EURL-AP SOP DNA extraction</td>
<td>V.1.0</td>
<td>BINDING</td>
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<tr>
<td>EURL-AP SOP operational schemes</td>
<td>V.2.0</td>
<td>BINDING</td>
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<tr>
<td>EURL-AP SOP Ruminant PCR</td>
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<td>BINDING</td>
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<tr>
<td>EURL-AP File for cut-off determination exact copy number + copies cut-off</td>
<td>V.1.0</td>
<td>BINDING</td>
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</table>
implementation (I)

PAP = prohibited for ruminant
Fishmeal = authorized in artificial milks
implementation (II)

Feed or feed material for aquaculture

Is the feed or feed material known to contain terrestrial PAP?

Light microscopy

Detection of particles from terrestrial animal origin?

PCR

Presence of ruminant DNA?

No prohibited constituents of terrestrial origin detected

Prohibited constituents of terrestrial origin detected

Is the feed or feed material known to contain terrestrial PAP?
Bridging two worlds

Need for new methods

Milk products, Blood product, Blood derivates, Plasma powder

Bones
FISH - Ruminant bones

Fluorescence In Situ Hydridization

Gene
DNAase (random cut)

- Dig-dUTP (or Biotin-dUTP)
- dCTP + dATP + dGTP

Denaturation (75°C)

Fixed Cells (on slides)

Denaturation (Formamid 42°C)

Hybridization (on slides)

Antibodies anti-Dig (or Avidin) linked with a fluorophor

Epifluorescent Microscopy
→ The gene is located

Model can be simplified
FISH - Ruminant bones

Fluorescence In Situ Hydridization
FISH - Ruminant bones

- Home-made bone meals
  - Bovine
  - Ovine
  - Porcine
  - Chicken
  - Salmon
- Bovine and ruminant DNA probe

<table>
<thead>
<tr>
<th>Bone type</th>
<th>BBM</th>
<th>OBM</th>
<th>PBM</th>
<th>CBM</th>
<th>SBM</th>
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</thead>
<tbody>
<tr>
<td># of bone particles examined</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
<td>160</td>
</tr>
<tr>
<td># (%) of bone particles with a positive staining*</td>
<td>156 (97.5)</td>
<td>143 (89.4)</td>
<td>7 (4.8)</td>
<td>1 (0.6)</td>
<td>0 (0)</td>
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<tr>
<td># (%) of bone particles with a positive staining**</td>
<td>140 (87.5)</td>
<td>133 (83.1)</td>
<td>2 (1.2)</td>
<td>1 (0.6)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

* Threshold of 3 co-locations
** Threshold of 10 co-locations
FISH - Ruminant bones

Effect of exogenous contamination:

• Bovine and porcine home-made meals
• Adulteration with milk powder (5g Milk powder/g bone meal)
• Mix and heat

Efficiency of FISH using ATTO 565-conjugated ruminant DNA probe on contaminated meals

<table>
<thead>
<tr>
<th>Bone type</th>
<th>Cont-BBM</th>
<th>Cont-PBM</th>
</tr>
</thead>
<tbody>
<tr>
<td># of bone particles examined</td>
<td>80</td>
<td>80</td>
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<tr>
<td># (%) of bone particles with a positive staining*</td>
<td>79 (98.8)</td>
<td>3 (3.8)</td>
</tr>
<tr>
<td># (%) of bone particles with a positive staining**</td>
<td>76 (95)</td>
<td>1 (1.3)</td>
</tr>
</tbody>
</table>

* Threshold of 3 co-locations
** Threshold of 10 co-locations

No significant ≠ with no-cont. meals
FISH - Ruminant bones

Efficiency on industrial meals:

1. First tests on PAPs produced in a pilot plant (STRATFEED project)
   - Bone surface opaque in brightfield
     ⇒ Co-localisation Hybrids/lacunae impossible

2. Test of various cleaning protocols with different solvents
   - No significant improvement
   - Specific of these meals?

3. Contacts with industries to receive new batches
   - Production of new batches of
     ⇒ Porcine, Chicken, Ovine, Bovine PAPs
FISH - Ruminant bones

Porcine probe?:

• Tested on bovine and porcine home-made meals

⇒ Aspecific hybridization

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<tr>
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<th>PBM</th>
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<tbody>
<tr>
<td>Bovine probe</td>
<td>+</td>
</tr>
<tr>
<td>Ruminant probe</td>
<td>+</td>
</tr>
<tr>
<td>Porcine probe</td>
<td>+</td>
</tr>
</tbody>
</table>
MS - Bovine blood meal/products

➢ **Aim:**
Specific detection of bovine blood meal/blood products

⇒ Identification of bovine blood biomarkers by mass spectrometry MS/MS
  • thermostable
  • ≠ milk products
  • ≠ porcine blood meal/blood products

➢ **Subcontracting for Mass spectrometry analysis:**
URBC (Laboratory of Cellular Biochemistry and Biology)
MS - Bovine blood meal/products

- **Methods** (developed by H. Marbaix on PAPs/University of Namur):
  1. Proteins extraction: TCA/acetone
  2. Proteins precipitation/purification: kit 2-D Clean up
  3. Trypsin digestion
  4. Nanoelectrospray Q-TOF MASS SPECTROMETER
     - HPLC
     - Electrospray
     - Q-TOF: 2 analysers
     - Detector
  5. Data treatment
1st Step: Approach without a priori

Material:

- 1st run (end of 2013): EURL-AP sample bank:
  - 2 porcine plasma powders
  - 1 bovine plasma powder*
  - 1 bovine Hb powder
  - 3 milk products

- 2nd run (March 2014): batches from ≠ industries
  - 6 porcine plasma powder
  - 6 porcine Hb powder
  - 2 porcine blood meal
  - 1 porcine « fresh » plasma
  - 1 bovine plasma powder
  - 1 bovine Hb powder
  - 1 bovine « fresh » plasma
  - 1 blank feed (horse feed)
  - 1 feed + 10 % bovine plasma powder*
  - 1 feed + 1 % bovine plasma powder*

Total:
- 4 Bv blood products
- 1 Bv « fresh » plasma
- 2 Adulterated feed*

-14 Pc blood products
- 2 Pc blood meal
- 1 Pc « fresh » plasma
- 3 Milk products
- 1 Feed
First results:

Proteins only present in bovine blood products (- in porcine blood/milk)

- Alpha -1B-glycoprotein
- Hemopexin
- Apolipoprotein A-II
- Alpha-2-macroglobulin
- Fibrinogen alpha chain
- Fibrinogen gamma-B chain
First results:

- **Hemopexin:**
  - High affinity toward heme (Hb component)
  - => Elimination of hematin (degradation product of heme)

- **Fibrinogen alpha chain and gamma-B chain:**
  - 2 of the 3 components of the fibrinogen (α, β, and γ)
  - => Principal protein of vertebrate blood clotting
Subsequent work:

- Industrial feeds analysis (+ other blood products)
- Peptides (biomarkers) selection
- Immuno-assay? Or Triple Quadrupole Mass Spectrometry?
New sources of animal proteins

- Insects
- Several candidate species
  - *Acheta domesticus*,
  - *Alphitobius diaperinus*,
  - *Drosophila spp*,
  - *assimilis Gryllus*,
  - *Gryllus bimaculatus*,
  - *Hermetia illucens*,
  - *Locusta migratoria*,
  - *Pacnoda butane*,
  - *Tenebrio molitor*,
  - *Zophobas morio*,
Insect meal detection

- Light microscopy?
  - IAG ring test 2014
    - | IAG results   | sens. | spec. |
    - | 2011: feather meal | 0.33  |      |
    - | 2012: salmon meal  |      | 0.70 |
    - | 2013: TCP          | 0.94  | (0.06) |
    - | 2014: insect meal  | 0.19  |      |
  - Morphological markers are lacking!
  - Method not adapted yet!

- PCR?
- NIR?
Insect meal detection

• Light microscopy

EURL-AP micrograph collection already enriched (April 2014)

BF Tegument *Pachnoda butana*  
FL Tegument *Locusta migratoria*
Insect meal detection

- **Light microscopy**

  EURL-AP micrograph collection already enriched (April 2014)

  FL Antenna *Gryllus sp.*

  BF Trachea *Gryllus assimilis*
Insect meal detection

- Light microscopy
  EUROL-AP micrograph collection already enriched (April 2014)

BF Trachea Alphitobius diaperinus
BF Shell fragment Cangon sp.

Insects vs. Fishmeal (crustaceans)
Insect meal detection

• Light microscopy

1. Insects vs Crustacean meals?
2. Larvae meals
   \[ \text{species identification?} \]
   \[ \text{poorly differentiated stage} \]
3. Specific stainings?
Insect meal detection

- **PCR**
  
  first experiments with *Hermetia illucens*

  No DNA amplification when using targets for
  
  - ruminant
  - bovine
  - porcine
  - ovine
  - chicken
  - poultry
  - fish

  - No specificity issues with developed targets
  - Need for dedicated insect targets…

  **At species or at higher taxonomic level?**
## Insect meal detection

### NIR

<table>
<thead>
<tr>
<th>Sample n°</th>
<th>Protein</th>
<th>Moisture</th>
<th>Fibres</th>
<th>Oil A (with hydrolysis)</th>
<th>Oil B (without hydrolysis)</th>
<th>Ash</th>
<th>H</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>48.76</td>
<td>3.76</td>
<td>7.06</td>
<td>31.54</td>
<td>28.82</td>
<td>4.79</td>
<td>13.86</td>
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<tr>
<td>2</td>
<td>49.25</td>
<td>3.69</td>
<td>6.95</td>
<td>32.10</td>
<td>29.07</td>
<td>4.15</td>
<td>14.28</td>
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<tr>
<td>Mean</td>
<td>49.00</td>
<td>3.73</td>
<td>7.01</td>
<td>31.82</td>
<td>28.94</td>
<td>4.47</td>
<td></td>
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</tbody>
</table>

- PAP equation (H>)
- Cross-check with chemical analyses
Insect meal detection

- NIR

Insects vs other PAPs

![Graph showing NIR absorption spectra for different materials](image-url)
Conclusions

1. Promising techniques

2. Extend techniques to industrial material
   • Processes
   • Collaboration with sector

3. Validation

4. I should stop talking and instead go back to the benchwork
Thanks for your attention!