EURL-AP Interlaboratory Study Microscopy 2010

Validation of a revised version of
Annex VI of EU Regulation EC/152/2009
and proficiency evaluation

Final version

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Summary

The European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised the present interlaboratory study aiming at validating a revision of the official EU method (based on Annex VI of Commission Regulation EC/152/2009) and assessing the proficiency level of the NRL network. This study was also open as a proficiency test to some non-EU participants. These latter participants were asked to use their national methods of reference. Total number of participants was 33 of which 26 NRLs and 7 non-EU participating laboratories. The study was based on a set of 10 blind samples. The sample set consisted of blanks, feed matrices fortified with terrestrial meat and bone meal or fish meal.

Results from the NRLs indicated a very good global performance that was never reached before by this network. The results as well as an in depth analysis of the different parameters from the revised method allowed validating this new protocol notwithstanding the insertion of some minor points for enhanced comprehension. Results from non-EU participants were evaluated as for NRLs. Although no rigorous comparison could be made, the ratio of good performing labs is obviously higher within the NRLs network than among non-EU participants.

The study showed that some participants, NRLs or non-EU, were underperforming. For NRLs in this situation an action plan to remediate to those underperformances was asked.

Keywords:
Meat and bone meals – Processed animal proteins – Light microscopy – Validation study – Qualitative analysis
1. Foreword

European Union Reference Laboratories (EURL) – formerly referred to as Community Reference Laboratories (CRL) – were created in order to ensure a high level of quality and a uniformity of the results provided by European control laboratories. On 29 April 2004, the European Parliament and the Council adopted the Regulation EC/882/2004 [1], improving the effectiveness of the official food and feed controls while redefining the obligations of the relevant authorities and their obligations in the organization of these controls.

On 23 May 2006, the Commission Regulation EC/776/2006 [2], nominated the Walloon Agricultural Research Centre as European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP, http://crl.cra.wallonie.be) for the 2006-2011 period. It has to develop the following priority axes:

(i) To provide National Reference Laboratories (NRLs) with detailed analytical methods, including reference methods for the network of Member State NRLs;
(ii) To coordinate application by NRLs of the methods by organizing interlaboratory studies;
(iii) To develop new analytical methods for the detection of animal proteins in feedingstuffs (light microscopy, near infrared microscopy, PCR, immunology …);
(iv) To conduct training courses for the benefit of NRL staffs from Member States and future Member States;
(v) To provide scientific and technical assistance to the European Commission, especially in cases of disputed results between Member States.

In this framework, the EURL-AP is organising, since 2006, a yearly proficiency test for the assessment of the implementation of the reference microscopic method for the detection of animal proteins in feed as described in Annex VI of Commission Regulation EC 152/2009. In addition the EURL-AP is also organising collaborative studies, or interlaboratory studies supporting validation of method enhancements. The present study report is part of this activity scope.

This final version of the report has been prepared according to a first draft version submitted in March 2011 to the NRL network for comments and review. Results were presented and discussed during the 5th EURL-AP Workshop held in Vienna (Austria) on the 6th and 7th of April 2011.
2. Introduction

Official controls for the detection of animal proteins in feed inside the EU are performed according to the protocol of the microscopic method described in Annex VI of Commission Regulation EC/152/2009 [3]. The existing protocol allows various ways of implementation. With a view to a better harmonization and a standard implementation of the official method, and hence improvement of the detection capabilities, a revision process was undertaken by the EURL-AP and the NRL network. Based on the scientific discussions held during the 4th CRL-AP annual workshop (Turin, Italy, April 2010), a revised protocol was prepared by the EURL-AP.

The objective of the present interlaboratory study is to evaluate the 2010 revised protocol for the detection of processed animal proteins (PAPs) in feed. The modifications brought focus on standardization of the method (i.e. equipment, sequences of observations, and results expressions) and not on the principle of the method itself. Therefore aside the validation goal for the protocol, the present study will also serve for assessing the performance of the NRLs to detect the presence of PAPs for the year 2010.

On proposal of the Commission, invitations to participate to this test were also sent to some official control labs outside the EU. Non-EU participants had to use their own national method of reference as in any classical proficiency test.
3. Material and methods

3.1. Study organisation

Announcement of the study was made on the 1st September 2010 to all participants. The announcement specified the goals of the study which differed according to the status of the participant: either NRL or not.

Participants were the 26 NRLs and 7 laboratories outside this EU network. These seven foreign participants were the Canadian Food Inspection Agency, the Croatian Veterinary Institute, the Servicio Nacional de Sanidad y Calidad Agroalimentaria from Argentina, the China Agricultural University, the Food and Agricultural Materials Inspection Center from Japan, LabNett AS from Norway and the Servicio Nacional de Sanidad Agraria from Peru. A detailed list of the 33 participating labs is included in Annex 1.

On the 15th November 2010, the study set of 10 blind samples has been sent by express shipment to all participants. Simultaneously the instructions and the Excel report forms (Annex 2) were communicated to all participants – downloadable from the EURL-AP intranet for the NRLs or sent to the non-EU participants who do not have access to this intranet.

According to their status (NRL or non-EU participants) the following specific instructions were given:

1. For NRLs
   • Qualitative analysis of the 10 blind samples had to be proceeded by following strictly the dedicated revised protocol – downloadable as from 14th October 2010 from the EURL-AP intranet. Major points of revision were:
     o the use of separation funnels,
     o the use of 10g of sample material for the sedimentation,
     o the use of either sediment plus flotate or sediment plus raw material,
     o suppression of the mandatory use of the stereomicroscope,
     o fixed sequence diagrams for the analysis of the slides,
     o indications on the minimum number of slides to use,
     o harmonised mode of expression of results (present, absent and absent [<LOD]).
   • NRLs were asked to provide additional data such as the fractions on which the analysis was carried out (sediment + flotate / sediment + raw material), the use of a 0.25 mm sieve before slide preparation (yes / no), the use of a stereomicroscope (yes / no), the number of slides observed, the sample and sediment weights, the number of particles they had detected to support their conclusions and to further specify the exact nature of the particles when their number were inferior or equal to 5.
   • NRLs were requested to express their conclusion based on a single analysis (Option A hereafter). Repetitions of analysis were formally prohibited.
   • Each NRL was asked to vote on its preference in case of detection of 1-5 animal particles from a first analysis. Two options were submitted to vote:
     o Option A : no repetition of the analysis and declaration of the sample as “negative” because this number of particles is below the LOD.
     o Option B : up to two repetitions of the analysis (including grinding) until the sample, based on the mean number of particles detected from the analyses, is allowed to be declared as “negative” or “positive”.
The voting bulletin had to be returned by fax by the 29th November 2010 to the EURL-AP.

2. For non-EU participants:

- Qualitative analysis of the 10 blind samples had to be proceeded according to their respective national reference method.
- Result’s expression modes were: present, absent, no results (in case of inconclusive results).
- Non-EU participants were also asked to provide additional data such as the type of method used (light microscopy, PCR, immunoassays, NIR microscopy or other methods). In case of microscopic method: the number of slides observed, the sample and sediment weights, the number of particles they had detected to support their conclusions and to further specify the exact nature of the particles when their number were less or equal to 5.

Some general instructions were delivered to all participants:

- Mention was done that each participating laboratory was itself responsible to reach appropriate homogeneity of the sample sub-portions that had to be taken from the whole sample vial for analysis.
- Results had to be encoded by way of an Excel report form (Annex 2). Participants were asked to carefully read the instructions on how to fill in the result form and to testify they did it prior to encoding their results. No other support for communicating the results was accepted.
- A summarized results sheet was automatically generated. Participants were asked to sign the summarized results sheet and to return it by fax and email to the EURL-AP. Only when both the Excel file and the fax were received by EURL-AP were results taken into consideration.
- The results had to be sent in both forms concomitantly to the EURL-AP by the 10th December 2010. Notification has been done that this date was a deadline and that results arriving later would not be accepted. A shift of the deadline was nevertheless proposed for participants outside EU due to custom related delays in delivery of the samples.

All participants delivered their results and no participant had to be excluded. Results from NRLs and non-EU participants were analysed separately in this report.

3.2. Material

3.2.1. Description of the samples

Nine different materials containing typical feed ingredients and/or processed animal proteins (PAPs) from various animal origins at different concentration levels have been prepared as shown in table 1 (next page).

The composition of the sample set was established taking into account the following considerations:

- The possible emergence of false positive results.
- Target concentrations of mammalian meat and bone meal (referred to as MBM through the text) largely inferior to the classical 0.1% considered for the time being as the adulteration level that the method should be able to detect.
• Presence of fishmeal, or fish feed, that could interfere with the detection of constituents from terrestrial animals when using light microscopy [4] (the so-called “masking effect”).
• A fish meal which presents fish bones with numerous osteocytes resembling mammalian ones.
• Feed matrix conditioning (pelletized) that requires grinding before analysis as requested by Commission Regulation EC/152/2009 [3].

Each participating lab received about 55g of 10 blind samples to which a unique random number was assigned. Details of the samples are indicated in table 1.

Table 1: Composition of the blind sample set used in the EUR-L-AP Interlaboratory Study 2010.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Material</th>
<th>Nr of replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>blank I</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>blank II (pellets)</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>blank III</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>blank IV (pellets)</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>fish feed I</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>fish feed II</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>fish feed II + 0.1% MBM</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>blank I + 0.005% MBM</td>
<td>1</td>
</tr>
<tr>
<td>9</td>
<td>blank II + 0.5% salmon meal (pellets)</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>

3.2.2 Materials used in the preparation of the samples

Different feed matrices were used for the preparation of the sample set:

• A first feed matrix used was a classical commercialized compound feed for pigs. The matrix is composed of wheat glutenfeed, wheat bran, tapioca, soya, rapeseed, palm kernel, beet pulp, barley, molasse, bakery by-products, animal and vegetable fats, minerals and vitamins. Sediment content of this compound feed was about 1.2%. This feed matrix was used for preparing samples 1 and 8.
• A second feed matrix was a pelletized feed supplement for bovines from a local producer. It is composed of rapeseed and palm cattle cake, wheat and wheat glutenfeed, corn, soya bean, barley beat pulp, minerals and vitamins. Its sediment content was about 0.6%. This feed matrix was used for the samples 2 and 9.
• A third feed matrix was a compound feed with milk powder for young ruminants. The matrix is containing barley, extruded corn, soya, whey powder, beat pulp, potato protein, sugar, minerals and vitamins. This feed matrix was only used for sample 3. Its sediment content was about 1.5%.
• Finally a fourth feed matrix was a pelletized feed for bovines from a Belgian producer, with a classical composition. The accurate declaration of composition was nevertheless unknown. It was used for sample 4. Its sediment content was about 0.9%.
Two different fish feeds were used:

- **A first fish feed** was a fish feed for carps. It is composed of soya, wheat, Chilean fish meal, corn gluten, minerals and vitamins. It was used after grinding for sample 5. Its sediment content was about 3.7%.
- **The second fish feed** was a pelletized feed for juvenile sturgeons. It contained Chilean fish meal, soya, corn gluten, fish oil, wheat, vitamins, minerals and inositol. Its sediment content was about 1.3%. This fish feed was used after grinding for samples 6 and 7.

Prior to use, all matrix materials were tested by light microscopy and PCR in order to confirm the absence of any interfering substances from animal origin.

Different processed animal proteins were included in the study:

- The **MBM** utilised for preparing samples 7 and 8 was already used in the 2009 CRL-AP Proficiency Test [5]. It was a mix of 50% ovine-porcine meat and bone meal and 50% pure bovine meat and bone meal treated at least at 133°C, 3 bars for 20 min. Its final bone content was of about 48%. Its purity was controlled by PCR.
- The **salmon meal** used for adulterating sample 9 has been produced by the EURL-AP team. It was prepared from an entire fresh salmon (*Salmo salar* L.) which was steamed, dried, ground and defatted in our laboratory. Its final bone content was of about 8.4%. Purity of the salmon meal was controlled by PCR.

### 3.2.3. Description of the mixing procedures

The stepwise dilution procedure developed by CRA-W and JRC-IRMM was used to produce the following samples: 7, 8 and 9. This procedure has been successfully used in numerous former European interlaboratory studies aiming to evaluate different light microscopy protocols. For preparation of sample 9, it also required a prior grinding of the pellets of the second feed matrix.

### 3.2.4. Pellets production

Sample 9 was pelletized at the EURL-AP facilities with an experimental small-scale pellet mill developed for that purpose. Pelletizing was realised at a temperature of above 60°C and with the addition of a sprayed saturated solution of saccharose. Pellets were dried at 40°C for 24h before conditioning.

### 3.3. Qualitative analysis

Qualitative analysis concerned the presence or absence of terrestrial animal (MBM) and/or fish material. These binary results were analysed by classical statistics: accuracy, sensitivity and specificity. All those statistics were expressed as fractions.

Accuracy is the fraction of correct positive and negative results; it was calculated by the following equation:

\[
\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}
\]
Where $PA$ is the number of correct positive results (Positive Agreements), $NA$ the number of correct negative results (Negative Agreements), $ND$ the number of false negative results (Negative Deviations) and $PD$ the number of false positive results (Positive Deviations).

Sensitivity is the ability of classifying positive results as positive, it was calculated as follows:

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

Specificity is the ability of classifying negative results as negative, it was calculated as follows:

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

The $AC$, $SE$ and $SP$ were calculated separately for each laboratory and for each requested parameter (detection of terrestrial animal material, detection of fish material) for the estimation of its proficiency. A consolidated $AC$ over both parameters was used to rank each participant. Finally a global $AC$ was also calculated for each material in order to estimate the performance of the method.

Statistical comparisons of $AC$, $SE$ and $SP$ between different options from the revised protocol were analysed by applying Fisher’s exact tests of independence on the proportion data. This test is recommended when proportions of at least one class are small (e.g. <5) [6, 7].
4. Results

Gross results from all participants are to be found in Annex 3.

4.1. Homogeneity study

Homogeneity study has been carried out for all materials used. The following table summarizes the results.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Material</th>
<th>Nr of replicates</th>
<th>Light microscopy</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Terrestrial</td>
<td>Fish</td>
</tr>
<tr>
<td>1</td>
<td>blank I</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>blank II</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>blank III</td>
<td>8</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>blank IV</td>
<td>10</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>fish feed I</td>
<td>5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>fish feed II</td>
<td>5</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>fish feed II + 0.1% MBM</td>
<td>5</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>blank I + 0.005% MBM</td>
<td>20</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>blank II + 0.5% salmon</td>
<td>5</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

(Legend: sed = sediment, STD = standard deviation, + = present, - = not present, traces = signals below cut off values)

The homogeneity was studied on 10g of sample material for each replicate. For the homogeneity study, basically only the sediment fraction was analysed; flotate was examined only when needed (e.g. for the blanks).

All blanks (samples 1, 2, 3 and 4) were negative for any presence of animal material by light microscopy. In line with their composition (animal fats, whey powder) samples 1 and 3 showed some positive reactivity to bovine and porcine DNA by PCR.

Fish feeds (samples 5 and 6) were all positive for fish by both light microscopy and PCR. No terrestrial particles were detected by light microscopy. In sample 6 a positive signal for the presence of porcine DNA was detected.
Concerning the fortified samples the following was noted.

In sample 7, the presence of terrestrial bones, in addition of fish particles, was systematically observed. PCR results are in agreement with the sample composition.

In sample 8 the presence of terrestrial bones was systematically detected through the 20 replicates. Fish particles were never observed. PCR confirmed the total absence of material from fish origin.

For the last material, pelletized sample 9, the presence of fish particles was constantly reported as well as the absence of any particle from terrestrial origin. The PCR results showed a presence of pork DNA in addition of the presence of fish. This still remains unexplained as neither blank II nor the pure salmon fish reacted to this DNA target.

Results from the homogeneity study indicated the samples as fit for the purpose.

4.2. Qualitative analyses from the NRLs

4.2.1. On the respect of the instructions

Overall instructions have been respected through the study. Nevertheless some labs did not entirely comply with some recommendations:

- A few labs declared some samples positive for the presence of terrestrial particles although having only observed 5 particles of this nature – instead of using the “Absent (<LOD)” option. Those were labs 6, 14 and 21.

- More than 50% of the labs declared at least once (but sometimes repeatedly) a sample as negative, either for terrestrial animal or fish material, while not having respected the sequence diagrams imposing to observe at least 6 slides before making any decision on the effective absence of animal particles. Those were labs 3, 4, 7, 8, 9, 10, 13, 14, 15, 17, 18, 21, 24, 26 and 27. This reflects a misunderstanding of the sequence diagrams which have to be modified and improved to exclude misinterpretation.

4.2.2. Overview of results and performance of the method

Table 3 summarizes the results submitted by the 26 NRLs for the nine sample types submitted to qualitative analysis.

Table 3: Global results expressed as accuracy (AC) for the nine materials

<table>
<thead>
<tr>
<th>Sample</th>
<th>Material</th>
<th>nr</th>
<th>AC</th>
<th>Terrestrial</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>blank I</td>
<td>26</td>
<td></td>
<td>1.000</td>
<td>0.923 (2)</td>
</tr>
<tr>
<td>2</td>
<td>blank II</td>
<td>52</td>
<td></td>
<td>0.980 (1)</td>
<td>0.942 (3)</td>
</tr>
<tr>
<td>3</td>
<td>blank III</td>
<td>26</td>
<td></td>
<td>0.962 (1)</td>
<td>0.962 (1)</td>
</tr>
<tr>
<td>4</td>
<td>blank IV</td>
<td>26</td>
<td></td>
<td>0.962 (1)</td>
<td>0.923 (2)</td>
</tr>
<tr>
<td>5</td>
<td>fish feed I</td>
<td>26</td>
<td></td>
<td>0.962 (1)</td>
<td>1.000</td>
</tr>
<tr>
<td>6</td>
<td>fish feed II</td>
<td>26</td>
<td></td>
<td>1.000</td>
<td>0.962 (1)</td>
</tr>
<tr>
<td>7</td>
<td>fish feed II + 0.1% MBM</td>
<td>26</td>
<td></td>
<td>0.923 (2)</td>
<td>1.000</td>
</tr>
<tr>
<td>8</td>
<td>blank I + 0.005% MBM</td>
<td>26</td>
<td></td>
<td>0.692 (8)</td>
<td>0.962 (1)</td>
</tr>
<tr>
<td>9</td>
<td>blank II + 0.5% salmon</td>
<td>26</td>
<td></td>
<td>0.769 (6)</td>
<td>0.962 (1)</td>
</tr>
</tbody>
</table>

Accuracy means sensitivity in case of ND and specificity in case of PD. In brackets the number of ND or PD. (Legend: nr = number of observations).
The overall results, expressed in terms of accuracy (AC), reveal a very good global performance for the revised method.

The ratio of false positive results (PD) reported for the blank materials (samples 1 to 4) is of 2% (or 3/130) for terrestrial particles and of 6% (or 8/130) for fish particles. These percentages of PD are the lowest ever observed through EURL-AP studies [8, 9, 10]. Similarly to past studies [9, 10] the percentage of PD for fish is larger than the one noted for terrestrial.

Correct detection of fish feeds (samples 5 and 6) is noted. Only one case of PD for the presence of terrestrial is recorded (1/26 or 4%). Also one case of lack of sensitivity is noted (1/26 or 4%).

The detection of 0.1% MBM in a fish feed matrix (sample 7) generates a lack of sensitivity of 8% (2/26). Considering the low level of adulteration, this score is the best ever obtained through EURL-AP studies when compared to equivalent samples of fish meal adulterated at 0.5% MBM [9, 10].

The disclosure of very low level of MBM at 0.005% within a blank matrix with a high sediment content (1.8%) (sample 8) is more problematical. The number of ND for terrestrial is of 31% (8/26) thus presenting a poor sensitivity. A single PD for fish was also recorded. Nevertheless this result is in agreement with the hypothesis considering that the LOD is influenced by the sediment percentage. The sensitivity obtained is lower than the one experienced by the same network of participants in 2009 [10] on a blank matrix with a low level of sediment (0.6%) but adulterated with the same MBM. This result supports the potential “dilution” effect provoked by the matrix for a MBM of a given f value; the higher the sediment content of the matrix, the higher the dilution of the MBM and hence the increased value for the LOD.

Finally the adulteration of a feed with salmon meal generates a relatively high number of PD for terrestrial presence: 23% (6/26). This lack of specificity for terrestrial particles reflects the difficulty to deal with the somehow differing pattern of the fish bone lacunae from Salmonidae as mentioned in the literature [11]. The usual typical array of canaliculi irradiating from the fish bones’ lacunae does not hold true for salmon fish bones. The EURL-AP on-line micrograph collection illustrates this noticeable situation extensively.

### 4.2.3 Detailed review of results for each sample material

Only details of observations having lead to erroneous results are commented independently from the fact that participants were forced to classify as negative any observations based on 5 or less than 5 particles of a given nature (<LOD).

**Blank I**

Fish particles were detected:

- Lab 18 detected 10 fish bones on a total of 5 slides.
- Lab 26 detected more than 10 fish particles on a total of 4 slides.

**Blank II**

Terrestrial animal particles were detected:

- Lab 19 identified for one replicate 6 terrestrial particles on a total of 20 slides.

Presence of fish was also reported as follows:

- Lab 24 observed for one replicate 5 fish bones and 2 scales on 4 slides.
- Lab 9 reported for one replicate more than 10 fish particles on 4 slides.
- Lab 17 detected for one replicate more than 10 fish particles (2 fish bones and more than 10 muscle fibres) on 4 slides.
Blank III:
Terrestrial animal particles were reported:
  • Lab19 detected 6 bones on a total of 20 slides.
Fish was reported:
  • Lab 18 reported the presence of 7 fish bones on a total of 5 slides.

Blank IV:
Terrestrial animal particles were reported:
  • Lab19 detected 6 bones on a total of 20 slides.
Fish was reported:
  • Lab 19 reported the presence of 9 fish bones and scales on a total of 20 slides.
  • Lab 4 detected 7 fish particles on 5 slides.

Fish feed I:
Terrestrial animal particles were reported:
  • Lab1 detected 6 bones on a total of 8 slides.

Fish feed II:
Only lab13 failed to detect the presence of fish particles on 4 slides.

Fish feed II + 0.1% MBM:
Two labs failed to detect the presence of terrestrial animal particles (Lab 9 and 18) on respectively 4 and 5 slides.

Blank I + 0.005% MBM:
Several labs failed to detect any terrestrial animal particles (Lab 11, 18 and 25).
Other labs declared the sample negative as they found only a few particles:
  • Lab 13 detected 3 bones on 4 slides
  • Lab 23 detected 3 bones on 7 slides
  • Lab 3 detected 2 bones on 3 slides
  • Lab 16 detected 3 bones on 10 slides
  • Lab 15 detected 3 bones on 6 slides
Fish was also reported:
  • Lab 26 reported the presence of more than 10 fish particles on a total of 4 slides.
Blank II + 0.5% salmon:

Only one lab declared this sample as negative for fish as it only identified 2 fish bones (Lab 3).

Some labs reported the presence of particles identified as from terrestrial animal origin:

- Lab 20 detected 8 bones on 4 slides.
- Lab 6 detected 5 bones on 6 slides.
- Lab 3 detected 8 terrestrial particles on 3 slides.
- Lab 9 detected more than 10 terrestrial particles on 4 slides.
- Lab 21 detected 10 terrestrial particles on 4 slides.
- Lab 17 reported 6 terrestrial particles on 4 slides.

4.2.4 Effect of declaring a sample as positive in case of detection of less than 5 particles

From the data collected, a new computation (table 4) presenting the global results was realised but simulating the appliance of a strict “zero tolerance” policy which means that as soon as a single particle of a given animal group is observed the sample is to be declared as positive for that animal group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Material</th>
<th>nr</th>
<th>AC</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Terrestrial</td>
<td>Fish</td>
</tr>
<tr>
<td>1 blank I</td>
<td>26</td>
<td></td>
<td>0.923</td>
<td>0.769</td>
<td></td>
</tr>
<tr>
<td>2 blank II</td>
<td>52</td>
<td></td>
<td>0.808</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>3 blank III</td>
<td>26</td>
<td></td>
<td>0.885</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>4 blank IV</td>
<td>26</td>
<td></td>
<td>0.808</td>
<td>0.769</td>
<td></td>
</tr>
<tr>
<td>5 fish feed I</td>
<td>26</td>
<td></td>
<td>0.885</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>6 fish feed II</td>
<td>26</td>
<td></td>
<td>0.962</td>
<td>0.962</td>
<td></td>
</tr>
<tr>
<td>7 fish feed II + 0.1% MBM</td>
<td>26</td>
<td></td>
<td>0.923</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>8 blank I + 0.005% MBM</td>
<td>26</td>
<td></td>
<td>0.885</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>9 blank II + 0.5% salmon</td>
<td>26</td>
<td></td>
<td>0.654</td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

Accuracy means sensitivity in case of ND and specificity in case of PD. Colour codes refer to either deterioration (red) or improvement (green) compared to actual results from table 3.

When comparing results of tables 3 and 4, a clearly generalised deterioration of the specificity occurs when a strict “zero tolerance” is considered. This is due to a logically expected increase in numbers of PD for both terrestrial and fish. The only improvements noted concerns the detection of MBM at very low concentration (sample 8) by a consistent decrease of the number of ND. The sensitivity is also slightly improved for the detection of salmon (sample 9). The detection capabilities of MBM mixed with fish feed (sample 7) are unaffected.
4.2.5 Influence of the number of slides on the results

The revised protocol fixed, through sequence diagrams, successions of analytical steps. Those diagrams also mention the minimum number of slides that has to be observed before a result can be delivered. This number of slides was deduced from the EURL-AP test series for the determination of the LOD performed over the 2009-2010 period as well as from the agreements made during the 4th CRL-AP Workshop held in Turin in 2010.

As mentioned in the section on the respect of the instructions, many labs have declared some sample as negative while not having observed the minimum number of slides as requested. From the data collected, the influence of the slide number (Figure 1) was analysed in relation to the number of false results (PD and ND).

![Figure 1: Impact of the number of slides on the deviations.](image)

Results of analyses based on the observations of less than 6 slides represented 45% of the total number of analyses while results based on the observation of more than 6 slides represented only 29% of the same total. Among the results based on less than 6 slides, only 17% of them (20/117) were in agreement with the imposed decision diagram (i.e. cases of results yet positive for both terrestrial and fish, thus not requiring further slide observations). In other terms 83% of this bulk of 117 results revealed to be not in line with the instructions.

When looking at the number of wrong answers or deviations in relation with the number of slides, obvious relationships can be noted. A lot of ND, about 58% of all false negative results observed in this study, could have been avoided if participants had observed the requested minimum of at least 6 slides. This does not rule for the PD. On the exception of sample 9 (the salmon adulterated feed), which was not considered because of its known atypical characteristics leading to a high number of false positive results for terrestrial, most of the PD already arose before having observed 6 slides. Hence on the contrary, as
already observed from previous study, results based on a too high number of slides (beyond 6) can lead to a higher frequency of PD – or in other term to a poorer specificity. This is confirmed in the present study by the abnormally high number of PD observed from labs that delivered their results based on excess slide number observations.

4.2.6 Results of vote

NRLs were asked to vote on their preference in case of detection of 1-5 animal particles from a first analysis. Two options were proposed:

- Option A: no repetition of the analysis and declaring the sample as “negative” because this number of particles is below the LOD.
- Option B: up to two repetitions of the analysis (including grinding if this step is required) until the sample, based on the mean number of particles detected from the analyses allow to declare the samples as “negative” or “positive”

Signed voting bulletins were all delivered by fax to the EURL-AP. Results from this vote are:

- 20 NRLs on 26 vote for repeating the analysis (Option B)
- 6 NRLS on 26 vote for not repeating the analysis (Option A)

The revised protocol text will be changed accordingly in its final version before submission to the DG Sanco.

4.2.7 $\alpha$-errors in the present study

Since the 3rd CRL-AP Annual Workshop, held in Gembloux in March 2009, results on the estimation of the limit of detection (LOD) are presented and discussed. The NRL network has demonstrated its capacity of detecting PAPs at very low levels of concentrations: around the 0.0025% of MBM with a $\beta$-error < 5% and an average number of animal particles detected of 4.4 [10]. These results were in line with the experiments realised by the EURL-AP team and presented during the 3rd and 4th CRL-AP Annual Workshops. The experimental model focused on the determination of LOD at an acceptable rate of false negative results – or low level of $\beta$-error. The model used didn’t allow coping with evaluating the associated risk of false positive results from blank matrices – or $\alpha$-error. However defining an acceptable risk of $\alpha$-error is crucial for a highly specific PAP detection.

By using a large numbers of blanks, the organiser of the present interlaboratory study also wanted to outline the ability of the 26 participants to confirm the absence of adulteration. Thus the organiser tried to assess the specificity and to express the results in terms of number of particles being erroneously detected.

| Table 5: Data on animal particles detection from PD on the four blank samples. |
|-------------------------------+--------+--------+--------|
|                               | Terrestrial | Fish   | Consolidated |
| Nr of particles               | 59      | 109    | 168      |
| Nr of slides                  | 203     | 171    | 289      |
| Mean nr of particles on 6 slides | 1.74   | 3.82   | 3.49     |
| Specificity                   | 0.838   | 0.815  | 0.827    |
| Actual specificity*           | 0.977   | 0.938  | 0.958    |

$\ast$Actual specificity is the value observed according the protocol instructions of the present study and classifying as negative results with $\leq$ 5 particles.
The collected data enabled to calculate the numbers of animal particles that have been observed and to report this number on a base of 6 slides which is the minimum slide number that has to be observed before declaring a sample as negative for the presence of animal particles (Table 5).

Values (table 5) show that the mean number of particles detected on PD, based on the analysis of 6 slides, does never exceed 4. The related $\alpha$-errors (1 – specificity), either for terrestrial, fish or both (=consolidated), are nevertheless too high for being acceptable under conditions equivalent to a strict “zero tolerance”. The proposed conditions for declaring a sample as positive only when at least 5 particles are observed from a minimum of 6 slides has the advantage of eliminating the majority of cases of PD with resulting actual $\alpha$-errors which are close to 0.05.

Data collected from previous EURL-AP studies corroborate this assumption. Raw data on the observations of animal particles in blanks from studies organised by the EURL-AP in 2006, 2007, 2008 and 2009 [8, 9, 10, 12] were analysed (details in Annex 4) and enable to prepare table 6.

Table 6: Data on animal particles detection from PD on blank samples through 4 past EURL-AP studies (2006, 2007, 2008 and 2009) representing a data set of 221 analyses.

<table>
<thead>
<tr>
<th></th>
<th>Terrestrial</th>
<th>Fish</th>
<th>Consolidated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum nr of particles</td>
<td>56</td>
<td>77</td>
<td>133</td>
</tr>
<tr>
<td>Nr of slides</td>
<td>79</td>
<td>122</td>
<td>182</td>
</tr>
<tr>
<td>Mean nr of particles on 6 slides</td>
<td>4.25</td>
<td>3.79</td>
<td>4.38</td>
</tr>
<tr>
<td>Specificity</td>
<td>0.937</td>
<td>0.910</td>
<td>0.846</td>
</tr>
</tbody>
</table>

The minimum number of particles detectable on PD and reported on the observation of 6 slides is systematically inferior to 5. This signifies also that samples being declared positive for the presence of animal particles based on observations revealing less than 5 particles actually present a higher risk for $\alpha$-errors and are consequently not acceptable.

4.2.8. Influence of other parameters

Information on several parameters was asked through the result report form with the goal of validating the proposed revised protocol.

Although being more restricting in some aspects (e.g. the sole use of separation funnels and no longer the use of other vials for the sedimentation process), the revised version still offers some degrees of freedom which are options for parameters that can be selected by the operators. Optional parameters which were studied for their potential impact on the accuracy, sensitivity or specificity were:

- The use of either the flotate or the raw material in addition to the observation of the sediment
- The optional use of the stereomicroscope
- The use of sieves for separating gross fractions from fine fractions or a grinding allowing 95% of particle size <500µm
- For fish meals (and other pure ingredients) the opportunity to use sample portions of 3g instead of 10g.

An overview of the impact of those optional parameters is summarised in table 7 (next page).
Table 7: Influence of some parameters on the global accuracy (AC), sensitivity (SE) and specificity (SP).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>nr (%)</th>
<th>AC</th>
<th>SE</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>stereomicroscope</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>used</td>
<td>157 (61)</td>
<td>0.930</td>
<td>0.865</td>
<td>0.959</td>
</tr>
<tr>
<td>not used</td>
<td>102 (39)</td>
<td>0.956</td>
<td>0.910</td>
<td>0.978</td>
</tr>
<tr>
<td>sieving</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>realised</td>
<td>141 (54)</td>
<td>0.926</td>
<td>0.855</td>
<td>0.955</td>
</tr>
<tr>
<td>not realised</td>
<td>118 (46)</td>
<td>0.958</td>
<td>0.913</td>
<td>0.981</td>
</tr>
<tr>
<td>flotate or raw</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>flotate</td>
<td>180 (69)</td>
<td>0.928</td>
<td>0.862</td>
<td>0.959</td>
</tr>
<tr>
<td>raw</td>
<td>79 (31)</td>
<td>0.968</td>
<td>0.936</td>
<td>0.982</td>
</tr>
<tr>
<td>amount of sample for fish feed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3g</td>
<td>17 (22)</td>
<td>0.905</td>
<td>0.889</td>
<td>0.917</td>
</tr>
<tr>
<td>10g</td>
<td>61 (78)</td>
<td>0.984</td>
<td>0.976</td>
<td>1.000</td>
</tr>
</tbody>
</table>

Data treatment by Fisher’s exact tests in order to evaluate statistical differences of AC, SE and SP values between options for the selected parameters did not allow to detect any significant statistical differences for this study. Observed differences therefore should only be interpreted as trends.

The utilisation of the stereomicroscope was chosen only in 61% of the total analyses. Roughly half of the analyses realised were carried out using sieves whereas the other half was carried out after a grinding step insuring 95% of the particle size <500µm. A large proportion of labs were in favour of using the flotate instead of the raw material (69% vs. 31%). The majority of the analyses performed on the fish feeds (78%) were based on the use of a 10g fraction of the sample instead of 3g. This underlines that fish feeds once grinded (cf. section 3.2.2) are confused by many labs with pure fish meals for which 3g of sample portions is authorised by the revised protocol.

Statistical tests demonstrated that both options for each parameter were delivering comparable scores. Moreover considering that even if one can interpret some trends, overall values for AC, SE and SP are still very good. Therefore none of the proposed options has to be rejected.

4.2.9 Individual performances of NRLs in qualitative analysis

Individual performances were assessed for each participant by calculating the accuracy, sensitivity and specificity over the blind samples. This was performed separately for both the detection of terrestrial material and fish material. A ranking of the labs was prepared based on the accuracy.

Results are to be found in tables 8 and 9 (next page).

Concerning the ability to detect terrestrial animal constituents, 15 labs provided incorrect results according to the following details (table 8):

- PD for MBM in blank II : lab 19
- PD for MBM in blank III : lab 19
- PD for MBM in blank IV : lab 19
- PD for MBM in fish feed I : lab 1
- ND for MBM in fish feed II + 0.1% MBM : labs 9 and 18
- ND for MBM in blank I + 0.005% MBM : labs 3, 11, 13, 15, 16, 18, 23 and 25
- PD for MBM in blank II + 0.5% salmon : labs 3, 6, 9, 17, 20 and 21
Tables 8 (left) and 9 (right): NRL proficiencies regarding the detection of terrestrial and fish material. Ranking follows AC values.

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<th>Terrestrial</th>
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<td>SE</td>
<td>SP</td>
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<td>19</td>
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<tr>
<td>26</td>
<td>0.800</td>
<td>1.000</td>
<td>0.667</td>
</tr>
</tbody>
</table>

Concerning the ability to detect fish material, 9 labs provided incorrect results according to the following details (table 9):

- PD for fish in blank I : labs 18 and 26
- PD for fish in blank II : labs 9, 17 and 24
- PD for fish in blank III : lab 18
- PD for fish in blank IV : labs 4 and 19
- ND for fish in fish feed II : lab 13
- PD for fish in blank I + 0.005% MBM : lab 26
- ND for fish in blank II + 0.5% salmon : lab 3
A general ranking of the NRLs was performed on a consolidated evaluation including their proficiency in detecting both terrestrial and fish materials through the set of blind samples (table 10):

**Table 10: General NRL proficiency regarding the detection of terrestrial and fish material. Ranking follows AC values as primary key and SE as second key.**

<table>
<thead>
<tr>
<th>Consolidated</th>
<th></th>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
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<td>SP</td>
</tr>
<tr>
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<tr>
<td>26</td>
<td>0.900</td>
<td>1.000</td>
<td>0.857</td>
</tr>
<tr>
<td>13</td>
<td>0.900</td>
<td>0.667</td>
<td>1.000</td>
</tr>
<tr>
<td>9</td>
<td>0.850</td>
<td>0.833</td>
<td>0.857</td>
</tr>
<tr>
<td>3</td>
<td>0.850</td>
<td>0.667</td>
<td>0.929</td>
</tr>
<tr>
<td>19</td>
<td>0.800</td>
<td>1.000</td>
<td>0.714</td>
</tr>
<tr>
<td>18</td>
<td>0.800</td>
<td>0.667</td>
<td>0.857</td>
</tr>
</tbody>
</table>

The table illustrates the very good level of global performance (= consolidated AC superior or equal to 0.90, i.e. having no more than two false results including a maximum of one ND for terrestrial material) for 22 labs out of 26 NRLs or in other words for 85% of the NRLs.

A second category (cells in blue in table 10) of NRLs having a satisfying global performance is defined (= consolidated AC below 0.90 and having no more than three false results including a maximum of one ND for terrestrial material). Only two NRLs fall into this category. NRLs included in this category are nevertheless asked to report to the EURL-AP on the possible source of these deviations. Attention has to be paid by lab 9 that missed the detection of terrestrial material in the fish feed II + 0.1% MBM (cell in blue underlined in table 10).

A third category (cells in red in table 10) includes NRLs that are underperforming (= consolidated AC below 0.90 and having either at least four false results or two ND for terrestrial). Those labs require
improvement of proficiency. These participants are asked to report on the origin of those multiple errors as well as on the actions they will undertake in order to solve this critical issue.

4.3. Qualitative analyses from the non-EU participants

4.3.1. Individual performances of non-EU participants in qualitative analysis

For reminder foreign participants were requested to realise the test by following their respective national reference method. It was asked to indicate which method was used (cf. Annex 2); all foreign participants used a microscopic based method. “No results” options were always considered as incorrect results because reflecting the inability to deliver a confirmed result.

Individual performances from the 7 participants outside the EU were assessed exactly as in previous section (4.2.9.). A ranking of those labs was prepared based on the accuracy. Results are to be found in tables 11 and 12.

Tables 11 (left) and 12 (right): non-EU lab proficiencies regarding the detection of terrestrial and fish material. Ranking follows AC values.

<table>
<thead>
<tr>
<th>Terrestrial</th>
<th>Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>lab code</strong></td>
<td><strong>AC</strong></td>
</tr>
<tr>
<td>33</td>
<td>0.900</td>
</tr>
<tr>
<td>31</td>
<td>0.900</td>
</tr>
<tr>
<td>30</td>
<td>0.800</td>
</tr>
<tr>
<td>29</td>
<td>0.700</td>
</tr>
<tr>
<td>34</td>
<td>0.700</td>
</tr>
<tr>
<td>35</td>
<td>0.600</td>
</tr>
<tr>
<td>32</td>
<td>0.400</td>
</tr>
</tbody>
</table>

Concerning the ability to detect terrestrial animal constituents, some labs provided incorrect results according to the following details:

- PD for MBM in blank I : labs 32 (“no results”) and 34
- PD for MBM in blank II : labs 29 (one PD and one “no results”) and 32 (two times “no results”)
- PD for MBM in fish feed I : labs 32, 34 and 35
- PD for MBM in fish feed II : labs 32 (“no results”) and 35
- ND for MBM in blank I + 0.005% MBM : labs 30, 31, 32 and 35
- ND for MBM in blank II + 0.5% salmon : labs 29, 30 , 33, 34 and 35

Concerning the ability to detect fish material:

- PD for fish in blank I : labs 29 (“no results”) and 34
- PD for fish in blank III : lab 34
• PD for fish in blank IV: lab 29
• ND for fish in fish feed I: lab 35
• ND for fish in fish feed II: lab 35
• PD for fish in blank I + 0.005% MBM: lab 29
• ND for fish in blank II + 0.5% salmon: labs 33 and 35

Ranking of the non-EU participants was also realized on a consolidated evaluation including their proficiency in detecting both terrestrial and fish materials through the 10 blind samples based on the same criteria as defined for the NRLs (table 13):

Table 13: General non-EU lab proficiency regarding the detection of terrestrial and fish material. Ranking follows AC values as primary key and SE as second key.

<table>
<thead>
<tr>
<th>Consolidated lab code</th>
<th>AC</th>
<th>SE</th>
<th>SP</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>0.950</td>
<td>0.833</td>
<td>1.000</td>
</tr>
<tr>
<td>30</td>
<td>0.900</td>
<td>0.833</td>
<td>0.929</td>
</tr>
<tr>
<td>33</td>
<td>0.900</td>
<td>0.833</td>
<td>0.929</td>
</tr>
<tr>
<td>34</td>
<td>0.750</td>
<td>1.000</td>
<td>0.643</td>
</tr>
<tr>
<td>29</td>
<td>0.700</td>
<td>1.000</td>
<td>0.571</td>
</tr>
<tr>
<td>32</td>
<td>0.700</td>
<td>0.833</td>
<td>0.643</td>
</tr>
<tr>
<td>35</td>
<td>0.650</td>
<td>0.333</td>
<td>0.786</td>
</tr>
</tbody>
</table>

One participant (lab 31) obtained a very good level of global performance.
Two participants (labs 30 and 33) obtained a satisfying result (cells in blue in table 13). For lab 33 an encoding error cannot be excluded.
The other four participants were underperforming (cells in red in table 13) according to EU standards.
5. Conclusions

The present study succeeded at obtaining the best results ever observed since EURL-AP studies are organised for the NRL network. In 2009 [10] the level of very good global performance was obtained by 69% of the NRLs while this year the same level was reached by 85% of the NRLs. Only two NRLs are underperforming and are asked to take actions to make progress in their proficiency.

The sample set design enables to enlighten some difficulties which represent new challenges for PAPs detection in feed, namely the complexity of salmon bones which might be interpreted as terrestrial bones and the influence of the amount of sediment from a matrix on the detection of animal particles at low levels of contaminations. About the detection of MBM at 0.005%, the present results are not as good as those observed in the CRL-AP Proficiency Test 2009 [10] that delivered a sensitivity of 0.962 but based on a matrix with a sediment percentage of about 0.6%. The 0.005% adulteration of a matrix presenting a sediment percentage of around 1.8% is more delicate to disclose. Therefore the LOD seems to be influenced by a matrix factor.

The presence of numerous blanks in the study permitted to investigate on the risk of $\alpha$-errors. In order to reach a specificity of over 0.950 ($\alpha$-error of 0.05 or lower) the mean number of animal particles that has to be found before declaring a sample as really positive must be at least of 5. Consolidated results from past EURL-AP studies [8, 9, 10, 12] confirm this conclusion. The way results are expressed in the revised protocol, i.e. declaring a sample as negative while reporting to have observed less than 5 particles, acts in this sense as shown by the low number of PD for blanks observed in this study.

The present study also aimed at validating the proposed revision of the Annex VI of Regulation EC/152/2009. The overall exceptional performance of the NRL network participants validates the protocol which is intended not only for better performance but also for standardizing the implementation of PAPs detection by means of light microscopy. The few options left over to the operator’s choice are also validated: no statistical differences could be detected among the proposed options. Whatever the options chosen, results were always quite satisfactory. Nevertheless some minor modifications will be brought to the revised protocol. Those modifications will include better graphic representations of the sequence diagrams that have to be followed, a clear mention for a minimum observation of 6 slides before declaring a sample as negative as well as referring to a maximum number of slides per sample. Actually analysis of the detailed data from the result report forms showed that the number of slides impacted both on the number of ND (in case less than 6 slides were observed) and PD (in case of an excess number of slide observations). Finally a last modification will refer to the vote of the NRLs on the alternative of repeating the whole analysis before declaring a sample as negative when from a first sample analysis only 1-5 animal particles are detected. This will once more undoubtedly reduce the risk of PD.

Concerning the non-EU participants, three of them performed satisfyingly or excellently and four of them failed according to the EU standards. Those participants were asked to use their own national protocols. All of them used a microscopic method. Considering the diversity and number of samples as well as the low adulteration levels, the present sample set can be presumed as far more complicated as standard proficiency test composed of solely 3-4 samples.

Acknowledgment

We are grateful to the whole EURL-AP staff and the participants for their fruitful collaboration. Special thanks to Dr Planchon for her advise in statistical analysis.
6. References


Annex 1

List of participants (Laboratories that do not belong to the NRL network are in italics).

<table>
<thead>
<tr>
<th>Country</th>
<th>Institute Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Austria</td>
<td>Austrian Agency for Health and Food Safety</td>
</tr>
<tr>
<td>Argentina</td>
<td>Servicio Nacional de Sanidad y Calidad Agroalimentaria</td>
</tr>
<tr>
<td>Belgium</td>
<td>Federal Agency for the Safety of the Food Chain</td>
</tr>
<tr>
<td>Bulgaria</td>
<td>National Diagnostic Research Veterinary Medical Institute</td>
</tr>
<tr>
<td>Canada</td>
<td>Canadian Food Inspection Agency</td>
</tr>
<tr>
<td>China</td>
<td>China Agricultural University Beijing</td>
</tr>
<tr>
<td>Croatia</td>
<td>Croatian Veterinary Institute</td>
</tr>
<tr>
<td>Cyprus</td>
<td>Cyprus Veterinary Services</td>
</tr>
<tr>
<td>Czech republic</td>
<td>Central Institute of sampling and testing in Agriculture</td>
</tr>
<tr>
<td>Denmark</td>
<td>The Danish Plant Directorate</td>
</tr>
<tr>
<td>Estonia</td>
<td>Veterinary and Food Laboratory</td>
</tr>
<tr>
<td>Finland</td>
<td>Finnish Food Safety Authority</td>
</tr>
<tr>
<td>France</td>
<td>DG for Fair Trading, Consumer Affairs and Fraud Control-Laboratory</td>
</tr>
<tr>
<td>Germany</td>
<td>Federal Institute for Risk Assessment</td>
</tr>
<tr>
<td>Greece</td>
<td>Feedstuffs Control Laboratory</td>
</tr>
<tr>
<td>Hungary</td>
<td>Central Agricultural Office-Directorate Food and Feed Safety-Central Feed</td>
</tr>
<tr>
<td></td>
<td>Investigation Lab.</td>
</tr>
<tr>
<td>Ireland</td>
<td>Department of Agriculture and Food Microscopy Laboratory - Seed Testing Station</td>
</tr>
<tr>
<td>Italy</td>
<td>National Reference Centre for the Surveillance and Monitoring of Animal Feed</td>
</tr>
<tr>
<td>Japan</td>
<td>Food and Agricultural Materials Inspection Center</td>
</tr>
<tr>
<td>Latvia</td>
<td>Institute of Food Safety, Animal Health and Environment &quot;BIOR&quot;</td>
</tr>
<tr>
<td>Lithuania</td>
<td>National Veterinary Laboratory</td>
</tr>
<tr>
<td>Luxemburg</td>
<td>Agroscope Liebefeld-Posieux Research Station (Switzerland)</td>
</tr>
<tr>
<td>Netherlands</td>
<td>RIKILT Institute of Food Safety, Wageningen UR</td>
</tr>
<tr>
<td>Norway</td>
<td>LabNett AS</td>
</tr>
<tr>
<td>Peru</td>
<td>Servicio Nacional de Sanidad Agraria</td>
</tr>
<tr>
<td>Poland</td>
<td>National Veterinary Research Institute</td>
</tr>
<tr>
<td>Portugal</td>
<td>Laboratorio Nacional de Investigacao Veterinaria</td>
</tr>
<tr>
<td>Romania</td>
<td>Hygiene Institute of Veterinary Health</td>
</tr>
<tr>
<td>Slovakia</td>
<td>State Veterinary and Food Institute</td>
</tr>
<tr>
<td>Slovenia</td>
<td>Veterinary Faculty-National Veterinary Institute-Unit for pathology of animal</td>
</tr>
<tr>
<td></td>
<td>nutrition and environmental hygiene</td>
</tr>
<tr>
<td>Spain</td>
<td>Laboratorio Arbitral Agroalimentario</td>
</tr>
<tr>
<td>Sweden</td>
<td>National Veterinary Institute, Department of Animal Feed</td>
</tr>
<tr>
<td>United Kingdom</td>
<td>Veterinary Laboratories Agency</td>
</tr>
</tbody>
</table>
Annex 2

Excel result report form for the NRLs.

<table>
<thead>
<tr>
<th>Laboratory identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laboratory code: 1</td>
</tr>
<tr>
<td>Responsibility agreement: No</td>
</tr>
</tbody>
</table>

*"Yes" means you have read carefully the "Instructions" worksheet and its accurate application through the present study*

<table>
<thead>
<tr>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lab code</td>
</tr>
<tr>
<td>Sample rank</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Qualitative analysis</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Terrestrial animal particles</td>
<td></td>
</tr>
<tr>
<td>Fish particles</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Additional data</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractions used for analysis</td>
</tr>
<tr>
<td>Use of 0.25 mm sieves</td>
</tr>
<tr>
<td>Use of stereo microscope</td>
</tr>
<tr>
<td>Number of slides observed</td>
</tr>
<tr>
<td>Sample weight (W)</td>
</tr>
<tr>
<td>Sediment weight (S)</td>
</tr>
</tbody>
</table>

Number terrestrial particles detected: if ≤ 5 (cf. cell above) please specify (example: bone, hair, muscle, bone, cartilage, feather, egg, scale, blood...)

Number fish particles detected: if ≤ 5 (cf. cell above) please specify (example: fishbone, scale, gill, teeth, cloth...).
### Proficiency Test 2010

**Laboratory identification**
- Laboratory code
- Responsibility agreement

"Yes" means you have read carefully the "Instructions" worksheet and its outreach applications through the present sheet.

**Report**

<table>
<thead>
<tr>
<th>Sample mark</th>
<th>1st</th>
<th>2nd</th>
<th>3rd</th>
<th>4th</th>
<th>5th</th>
<th>6th</th>
<th>7th</th>
<th>8th</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample N*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Qualitative analysis**
- Terrestrial animal particles
- Fish particles

**Additional data**
- Number of slides observed
- Sample weight (W)
- Sediment weight (S)

Number terrestrial particles detected if ≤ 5 (cf. owl above) please specify (example: tooth, hair, muscle, bone, castage, feather, eggshell, blood...)

Number fish particles detected if ≤ 5 (cf. owl above) please specify (example: tooth, scale, gill, teeth, exuviae...)

Excel result report form for the non-EU participants.
Annex 3

A. Gross results of NRL participants (in numerical order of lab ID).

<table>
<thead>
<tr>
<th>Sample N°</th>
<th>Laboratory identification code:</th>
<th>Terrestrial animal part.</th>
<th>Fish part.</th>
<th>Number of slides</th>
<th>W (g)</th>
<th>S (g)</th>
<th>Number of terrestrial part. detected</th>
<th>Comment if number of terr. part. ≤ 5</th>
<th>Number of fish part. detected</th>
<th>Comment if number of fish part. ≤ 5</th>
<th>Fractions</th>
<th>0.25 mm sieving</th>
<th>Stereo-microscope</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1</td>
<td>Absent (&lt; LOD)</td>
<td>Absent (&lt; LOD)</td>
<td>15</td>
<td>10.10</td>
<td>0.122</td>
<td>6 cannot be exactly specified, maybe horn</td>
<td>5 cannot be exactly specified, maybe krill</td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Present</td>
<td>Present</td>
<td>8</td>
<td>3.05</td>
<td>0.123</td>
<td></td>
<td>6 fishbones; no diff between MBM- and FM- muscles possible</td>
<td>&gt; 10 fishbones, scales, muscles, etc. no diff between MBM- and FM- muscles possible</td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Absent</td>
<td>Absent</td>
<td>8</td>
<td>16.04</td>
<td>0.087</td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Present</td>
<td>Present</td>
<td>8</td>
<td>3.13</td>
<td>0.046</td>
<td></td>
<td>&gt; 10 bones; no diff between MBM- and FM- muscles possible</td>
<td>&gt; 10 fishbones, scales, muscles, etc. no diff between MBM- and FM- muscles possible</td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Absent</td>
<td>Absent</td>
<td>8</td>
<td>10.02</td>
<td>0.167</td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Absent (&lt; LOD)</td>
<td>Absent (&lt; LOD)</td>
<td>12</td>
<td>16.05</td>
<td>0.100</td>
<td>1 bone</td>
<td>1 fragment of echinoderm</td>
<td></td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Absent</td>
<td>Present</td>
<td>8</td>
<td>10.08</td>
<td>0.099</td>
<td></td>
<td></td>
<td>&gt; 10 fishbones, scales, muscles, etc. no diff between MBM- and FM- muscles possible</td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Present (&lt; LOD)</td>
<td>Absent (&lt; LOD)</td>
<td>12</td>
<td>10.08</td>
<td>0.162</td>
<td>&gt; 10 bones</td>
<td>1 fishbone</td>
<td></td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Absent</td>
<td>Present</td>
<td>8</td>
<td>3.09</td>
<td>0.044</td>
<td></td>
<td>&gt; 10 fishbones, scales, muscles, etc. no diff between MBM- and FM- muscles possible</td>
<td></td>
<td>Sed. + Flot.</td>
<td>No</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample N°</td>
<td>Terrestrial animal part.</td>
<td>Fish part.</td>
<td>Number of slides</td>
<td>W (g)</td>
<td>S (g)</td>
<td>Number of terrestrial part. detected</td>
<td>Comment if number of terr. part. ≤ 5</td>
<td>Number of fish part. detected</td>
<td>Comment if number of fish part. ≤ 5</td>
<td>Fractions</td>
<td>0.25 mm sieving</td>
<td>Stereo-microscope</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>--------------------------</td>
<td>------------</td>
<td>------------------</td>
<td>-------</td>
<td>------</td>
<td>-------------------------------------</td>
<td>---------------------------------------</td>
<td>-------------------------------</td>
<td>-------------------------------------</td>
<td>------------</td>
<td>----------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Absent</td>
<td>Present</td>
<td>3</td>
<td>10.00</td>
<td>0.221</td>
<td>&gt; 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Absent</td>
<td>Absent</td>
<td>3</td>
<td>10.00</td>
<td>0.125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Absent</td>
<td>Absent</td>
<td>3</td>
<td>10.00</td>
<td>0.097</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Absent (&lt; LOD)</td>
<td>Absent</td>
<td>3</td>
<td>10.00</td>
<td>0.156</td>
<td>2</td>
<td>2 bones</td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Present</td>
<td>Present</td>
<td>3</td>
<td>10.00</td>
<td>0.132</td>
<td>&gt; 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Absent</td>
<td>Absent</td>
<td>3</td>
<td>10.00</td>
<td>0.091</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Absent (&lt; LOD)</td>
<td>Absent</td>
<td>3</td>
<td>10.00</td>
<td>0.148</td>
<td>2</td>
<td>2 bones</td>
<td></td>
<td></td>
<td></td>
<td>Sed. + Flot.</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Absent</td>
<td>Present</td>
<td>3</td>
<td>10.00</td>
<td>0.125</td>
<td>&gt; 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
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|-----------|--------------------------|------------|------------------|-------|------|------------------------------------|----------------------------------------|                                |                                |                |                 |                |
| 2         | Present                  | Absent     | 20               | 10.02 | 0.090 | 6 bone                            |                                        |                                |                          |                |                 |                |
| 5         | Absent                   | Present    | 15               | 10.00 | 0.419 | > 10                               |                                        |                                |                          |                |                 |                |
| 3         | Present                  | Absent     | 20               | 10.01 | 0.231 | 2 bone                            |                                        |                                |                          |                |                 |                |
| 1         | Absent (< LOD)           | Absent (< LOD) | 20              | 10.01 | 0.152 | 2 bone                            |                                        |                                |                          |                |                 |                |
| 2         | Present                  | Absent     | 20               | 10.02 | 0.068 | > 10                               |                                        |                                |                          |                |                 |                |
| 6         | Absent                   | Present    | 15               | 10.14 | 0.160 | > 10                               |                                        |                                |                          |                |                 |                |
| 4         | Present                  | Present    | 20               | 10.05 | 0.097 | 9 fishbone, scale                 |                                        |                                |                          |                |                 |                |
| 9         | Absent                   | Present    | 20               | 10.03 | 0.064 | > 10                               |                                        |                                |                          |                |                 |                |
| 7         | Present                  | Present    | 6                | 10.03 | 0.115 | > 10                               |                                        |                                |                          |                |                 |                |

<p>| Sample N° | Terrestrial animal part. | Fish part. | Number of slides | W (g) | S (g) | Number of terrestrial part. detected | Comment if number of terr. part. ≤ 5 | Number of fish part. detected | Comment if number of fish part. ≤ 5 | Fractions | 0.25 mm sieving | Stereo-microscope |
|-----------|--------------------------|------------|------------------|-------|------|------------------------------------|----------------------------------------|                                |                                |                |                 |                |
| 2         | Absent                   | Absent     | 9                | 10.00 | 0.078 |                                    |                                        |                                |                          |                |                 |                |
| 3         | Absent                   | Present    | 9                | 10.00 | 0.391 | &gt; 10                               |                                        |                                |                          |                |                 |                |
| 5         | Absent                   | Absent (&lt; LOD) | 10       | 10.00 | 0.168 | 3 fish bone + 2 scales            |                                        |                                |                          |                |                 |                |
| 2         | Absent                   | Absent     | 9                | 10.00 | 0.082 |                                    |                                        |                                |                          |                |                 |                |
| 4         | Absent                   | Absent     | 6                | 10.00 | 0.112 |                                    |                                        |                                |                          |                |                 |                |
| 6         | Absent                   | Present    | 8                | 3.00  | 0.036 | &gt; 10                               |                                        |                                |                          |                |                 |                |
| 1         | Absent                   | Absent     | 9                | 10.00 | 0.167 |                                    |                                        |                                |                          |                |                 |                |
| 7         | Present                  | Present    | 3                | 10.00 | 0.129 | &gt; 10                               |                                        |                                |                          |                |                 |                |
| 8         | Absent (&lt; LOD)           | Absent (&lt; LOD) | 9        | 10.00 | 0.350 | 2 fish bone                       |                                        |                                |                          |                |                 |                |
| 9         | Present                  | Present    | 4                | 10.00 | 0.176 | 8                                  |                                        |                                |                          |                |                 |                |</p>
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Laboratory identification code: 27

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### B. Gross results of non-EU participants (in numerical order of lab ID).

Note: an error of automatic transcription of the report summary for the non-EU participants was noted. Instead of “No results” the mention “Absent (<LOD)” appeared on the report summary. This was corrected in the tables hereunder.

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<th>S (g)</th>
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Laboratory identification code : 33

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Laboratory identification code : 34

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