# Combined microscopy-PCR
# EURL-AP Proficiency Test 2021

*Final version*

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## Summary

The European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP) organised the present proficiency test for assessing the ability of the NRL network with respect to the detection of processed animal proteins (PAPs) in feed using both light microscopy and PCR according to current legal requirements.

The total number of participating laboratories was 35 (26 NRLs and 9 labs outside the NRL network). All laboratories delivered results. The study was based on a set of six samples (to be analysed both by light microscopy and PCR) consisting of blank feed matrices or feed materials fortified or not with processed animal proteins from terrestrial vertebrates and/or from fish.

Regarding the detection of PAPs by light microscopy the overall results indicated an excellent and satisfactory level of global performance for 65 % of the NRLs. Nine NRLs out of the 26 (35 %) are underperforming. Problems of sensitivity for the detection of terrestrial vertebrates, mainly in the case of blood products, remains important. The complete description and reporting of the particles detected is crucial and is still to improve.

Concerning the PCR results, 77 % of the NRLs (20 out of 26) performed excellently. The six remaining NRLs (23 %) returned satisfying results. No laboratory was considered as underperforming. The instruction to skip the application of the SOP on the combination of microscopy and PCR and to analyse all the samples by PCR is most probably responsible of the globally good performance of the network.



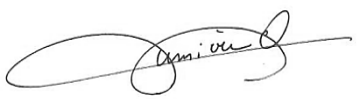
**Keywords :**

Processed animal proteins – Light microscopy – PCR - Proficiency test – Qualitative analysis


*This report identified by an ISBN has been prepared from a draft version sent for revision and comments to the participants on the 9th February 2022. After reception of the comments on the 25th February 2022, it was amended accordingly and approved by the signature of the organisers.*

*ISO 17043 coordinators signature for approval:*

*Olivier Fumière* *Pascal Veys*



# 1. Foreword

European Union Reference Laboratories (EURL) were created in order to ensure a high level of quality and a uniformity of the results provided by European control laboratories. On 15th March 2017, the European Parliament and the Council adopted Regulation EU/625/2017 [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 March 2011, Commission Regulation EC/208/2011 [2] renewed the nomination of the Walloon Agricultural Research Centre as European Union Reference Laboratory for animal proteins in feedingstuffs (EURL-AP, https://www.eurl.craw.eu). 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 has been organising yearly since 2006 proficiency tests for the assessment of the implementation of the reference methods for the detection of animal proteins in feed as described by current Annex VI of Commission Regulation EC/152/2009 [3]. Since 2016, the proficiency tests conducted by the EURL-AP are organised under the ISO17043 standard.

The present study report is part of the activity scope of the EURL-AP annual programme.

# 2. Introduction

According to modified Annex VI of Commission Regulation EC/152/2009 [3] official controls for the detection of animal proteins in feed inside the EU have to be performed by light microscopy and/or PCR since June 2013. Standard Operating Procedures (SOP) are supporting the implementation of the two methods.

The objective of the present proficiency test was strictly to evaluate within the network of 26 NRLs the analytical performance to detect processed animal proteins (PAPs) in feed by light microscopy and PCR. Participation of the NRLs is mandatory.

In addition, and on proposal of the Commission, invitations to participate to this test were also sent to a limited number of official control labs outside the EU. Non-EU participants were asked to apply also light microscopy and PCR although strict following of Annex VI of Commission Regulation EC/152/2009 and related SOPs was not imposed to them.

# 3. Material and methods

## 3.1. Study organisation

Twenty-six NRLs and nine laboratories outside this EU network participated to the study. A detailed list of the 35 participating labs is included in Annex 1.

Official announcement (Annex 2) of the study was made on the 10th September 2021 to all invited participants.

On the 15th October 2021, the sample sets were shipped to the participants. On the same day the Excel report forms containing the instructions (Annex 3) were communicated to all participants – downloadable from the EURL-AP intranet for the NRLs or sent by email to the non-EU participants who have no access to this intranet.

The deadline for the delivery of the results was fixed in the announcement and in the instructions at the 15th November 2021.

Within the instructions, some general recommendations were delivered to the participants:

- Laboratories participating to the proficiency test were themselves responsible to reach appropriate homogeneity of the sample sub-portions that had to be taken from the whole sample vial for analysis. Precautions to avoid laboratory cross-contamination were also highlighted.
- Results had to be encoded by way of an Excel report form (Annex 3). 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.
- Participants were asked to sign the summarized results sheet that is automatically generated when filling the form and to return it by email to the EURL-AP. Results were taken into consideration only when both the Excel file and a copy of the summarized results sheet were received by the EURL-AP.
- Participants were notified that results arriving later would not be accepted.

All results were delivered on time to the organiser. The proficiencies of NRLs and other participants were evaluated separately.

## 3.2. Material

3.2.1. Description of the samples

Six different blind test materials were prepared for the study. The composition of the sample set was established considering the following factors:

- Use of feed and feed materials intended to different farmed animals ;
- Use of pure fishmeals intended for aquaculture for assessing the detection capabilities of terrestrial PAPs because since the 1st June 2013 non-ruminant PAPs are authorized in aquafeeds according to Commission Regulation EU/56/2013 [4] ;
- Use of materials prepared from blood and milk known to deliver no sediment (or very limited quantities) ;
- Use of adulterants intended to deliver positive ruminant signals by PCR.

Each participating lab received about 40g for each of the six blind samples to which a unique random number was assigned. Details of the sample set are indicated in Table 1.

**Table 1: Composition of the sample set**

| Sample | Material | Nr of replicates | Expected results * Microscopy: Terrestrial particles | Expected results * Microscopy: Fish particles | Expected results * PCR: Ruminant DNA |
|---|---|---|---|---|---|
| 1 | milk replacer | 1 | - | - | + |
| 2 | tuna meal | 1 | - | + | - |
| 3 | salmon meal + 1% pig PAP | 1 | + | + | - |
| 4 | pig feed + 1% haemoglobin powder | 1 | + | - | - |
| 5 | mixed blood meal | 1 | + | - | + |
| 6 | plasma powder + 1% collagen | 1 | + | - | + |
| **Total** | | **6** | **4** | **2** | **3** |

**(* Explanations on expected results are described in section 3.4)**

Expected results were internally determined based on the known composition of the samples (presence or absence of PAP) and the results obtained during the homogeneity study.

3.2.2. Materials used in the preparation of the samples

Six commercially available feed materials or feed were used as matrices:

- The **milk replacer** was a complete feeding for calves used in a 2019 study [5] made of skimmed milk powder, lactoserum, palm oil, copra oil, dextrose, calcium carbonate, magnesium sulphate, sodium bicarbonate. Its sediment was of 0.3 %. Rumiant DNA was detected by PCR.
- The **tuna meal** was a pure fishmeal with a sediment content of 25.8 %. PCR analyses revealed it free from ruminant, porcine and poultry DNA.
- The **salmon meal** was a pure fismeal with a sediment content of 22.3 %. PCR analyses revealed it free from ruminant, porcine and poultry DNA.
- The **pig feed** was a complete feed for fatteners made of wheat, maize, barley, wheat gluten, pealed soybeans, sunflower meal, rapeseed meal, beet pulp, molasses, calcium carbonate, soy oil and natrium chloride. Its sediment was of 0.8 %. PCR analyses revealed it free from ruminant, porcine and poultry DNA.
- The **blood meal** of mixed origin was prepared at the laboratory with two ruminant blood meals. Its sediment content was very low and not measured. Ruminant DNA was detected and one of the blood meals presented traces of porcine DNA.
- The **plasma powder** was a mixture of three porcine plasma powders prepared at the laboratory. It had no sediment. Their porcine origin was confirmed by PCR.

Adulterant material used:

- A **porcine PAP** was used for preparing sample 3. It had a high bone content with a sediment of about 50 % and its purity was checked by microscopy and PCR. It presented traces of ruminant DNA.
- An **haemoglobin powder** from porcine origin was used to fortify sample 4. No sediment could be obtained and its porcine origin was confirmed by PCR.
- A **collagen powder** from bovine bone origin was used for preparing sample 6. Probably due to a strong PCR inhibition, no trace of DNA presence could be detected. Nevertheless, in low concentration in sample 6, a ruminant PCR signal is always present.

3.2.3. Description of the mixing procedures and pelleting

To avoid presence of interfering material, a cleaning of the rooms where the samples were handled was performed prior to sample preparation, mixing of the materials and filling of the vials.

The milk replacer (sample 1) was conditioned first in order to avoid contamination.

The samples containing fish materials (samples 2 and 3) were then prepared. Samples 4 and 6 were prepared and finally sample 5, which was the dustiest, was conditioned.

All adulterations (samples 3, 4 and 6) were proceeded by direct spiking with the adulterant.

## 3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [6].

### 3.3.1. Light microscopy

Qualitative analysis concerned the detection of terrestrial animal and/or fish material.

Results are expressed by the participants in three formulations according to regulation EU/56/2013 [4] amending regulation EC/152/2009 [3]:

- Positive (= presence of microscopically detectable animal material)
- Negative (= absence of any microscopically detectable animal material)
- Below LOD (= low level presence of microscopically detectable animal material with a risk of false positive result)

Considering the risk of false positive results, all results expressed as below LOD have to be assimilated to negative ones as by definition they cannot be certified as positive *sensu stricto*. This allows an on-off, or binary result analysis.

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 network.

### 3.3.2. PCR

Qualitative analysis concerned the detection of ruminant DNA.

The participants delivered Ct values (in cycles) to compare to a cut-off value (in cycles) set at 15 copies of the target and validated by a quality criterion (the cut-off Ct value must correspond to a number of copies of the target > 9.00 copies). For each sample, DNA is extracted from 2 test portions. The results obtained from the 2 test portions must be consistent, in the sense that both Ct values should be close enough to each other and on the same side compared to the cut-off value. A Ct value < cut-off value corresponds to a

positive result. Respectively, a Ct value ≥ cut-off value corresponds to a negative result. Results are expressed by the participants in two formulations:

- Present (= presence of ruminant DNA detected)
- Absent (= no ruminant DNA detected)

As for light microscopy, these binary results were analysed by classical statistics (accuracy, sensitivity and specificity) with the same formulae as presented in 3.3.1.

## 3.4. Performance criteria

Evaluation of the performance and scoring were applied as recommended by ISO 13528 [6].

This year again, since all samples had to be analysed by both light microscopy and PCR, no assessment of the correct implementation of the legislation (i.e. choice of method to apply in accordance with the SOP on operational protocol) was realised. The only evaluation done was on the reported analytical results.

### 3.4.1. Light microscopy

Considering the sample set composition, the expected results are indicated in Table 1.

Sample 1 was to be declared negative for both terrestrial vertebrates and fish material presence. It has nevertheless to be pointed out that the recent modification of Annex VI of Commission Regulation EC/152/2009 by Commission Implementing Regulation EC/2020/1560 [7] refers to milk globules and lactose crystals which can be found in dairy products. Results that would accordingly be declared as positive for terrestrial vertebrates' presence by referring to such observed structures have been considered as correct too.

Samples 2 had to be declared negative for terrestrial vertebrates' presence and positive for fish material presence.

Sample 3 had to be declared positive for both terrestrial vertebrates and fish presence.

Samples 4, 5 and 6 had to be declared positive for terrestrial vertebrates and negative for fish material presence.

Based on these considerations, the following performance criteria were decided for the light microscopy:

- **Excellent** level of global performance = consolidated AC superior or equal to 0.90 with no ND for terrestrial material.
- **Satisfying** level of global performance = consolidated AC superior or equal to 0.90 with one ND for terrestrial material OR a consolidated AC superior to 0.83 with no ND for terrestrial material.
- **Underperforming** level of global performance = consolidated AC equal or inferior to 0.83 OR superior to 0.83 with one ND for terrestrial material

### 3.4.2. PCR

As for light microscopy, the expected results are indicated in Table 1.

Samples 2, 3 and 4 were considered to be declared negative for the presence of ruminant DNA.

Samples 1, 5 and 6 were considered to be declared positive for the presence of ruminant DNA. Sample 1 contained skimmed milk powder and lactoserum. Sample 5 was a mix of two ruminant blood meals. Sample 6 was spiked with a collagen powder prepared from bovine bones.

Concerning the PCR, the performance criteria were decided as:

- **Excellent** level of global performance = no wrong result for the detection of ruminant DNA.
- **Satisfying** level of global performance = no more than 1 wrong result for the detection of ruminant DNA.
- **Underperforming** level of global performance = 2 wrong results or more for the detection of ruminant DNA.

## 3.5. Homogeneity study

Homogeneity study has been carried out for all materials used. Table 2 summarizes the results.

**Table 2: Homogeneity study – Results**

| Sample | Material | Light microscopy | | | PCR | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Nr of replicates | Terrestrial | Fish | Nr of replicates | Ruminant | Porcine | Poultry | Fish |
| 1 | milk replacer | 10 | - | - | 10 | + | - | - | ND |
| 2 | tuna meal | 10 | - | + | 10 | - | - | - | + |
| 3 | salmon meal + 1 % pig PAP | 10 | + | + | 10 | - | +* | - | + |
| 4 | pig feed + 1 % haemoglobin powder | 10 | + | - | 10 | - | + | ND | ND |
| 5 | mixed blood meal | 10 | + | - | 10 | + | +* | - | ND |
| 6 | plasma powder + 1 % collagen | 10 | + | - | 10 | + | + | - | ND |

**(Legend: ND = not tested, + = systematically detected, - = systematically not detected, +* = not systematically detected)**

The homogeneity was studied by light microscopy on 10 g of sample material for 10 replicates. Analyses of replicates were performed following strictly EC/152/2009 regulation [3]. For PCR analysis, a DNA extraction was performed on 2 test portions of 100 mg of sample material for each of the 10 replicates.

**Sample 1** (milk replacer) was microscopically free from any trace of animal origin on the exception of milk globules. The PCR analyses confirmed the presence of ruminant DNA.

**Sample 2** (tuna meal) showed systematically the presence of fish bones and muscle fibres (more than 5 bones on each slide). PCR tests revealed only the presence of fish DNA.

**Sample 3** (salmon meal + 1 % pig PAP) showed systematically the presence of both fishbones and terrestrial bones. Muscle fibres were also systematically observed. The sample was positive for the presence of fish DNA ; the presence of porcine DNA was not systematically detected probably due to inhibition issues.

**Sample 4** (pig feed + 1% haemoglobin powder) showed systematically the presence of blood particles, no bone was detected (fishbones or terrestrial bones). PCR analyses revealed the sample as negative for ruminant DNA and positive for pig DNA.

**Sample 5** (mixed blood meal) was systematically positive for the presence of blood particles. Some few hairs and terrestrial bones were observed. No presence of fish was detected. Ruminant DNA was detected and traces of porcine DNA were detected but not systematically using PCR.

**Sample 6** (plasma powder + 1% collagen) was, on the exception of plasma particles which were systematically observed, free from any identifiable animal particles. PCR analyses revealed the presence of ruminant and porcine DNA.

Results from the homogeneity study allowed declaring the samples as fit for their purpose.

## 3.6. Stability of the samples

Internal stability studies performed on similar samples from past studies have demonstrated that such samples were stable over time (years) for both light microscopic and PCR analyses. There are no reasonable elements which would indicate that present samples should be unstable.

# 4. Results

Gross results for microscopy and PCR from all participants are to be found in Annexes 4 and 5 respectively.

## 4.1. Microscopy results

4.1.1. Qualitative analyses from the NRLs

4.1.1.1. Results and performance of the network

Table 3 summarizes the results reported by the 26 NRLs for the sample types submitted to microscopic analysis.

The overall results, expressed in terms of global accuracy (AC) reveal the performance of the NRL network for the detection of PAPs from the present test. The percentage of total error accounted for 21 % of the total responses.

**Table 3: Global results expressed as accuracy (AC) – light microscopy**

| Sample | Material | n | AC | |
|---|---|---|---|---|
| | | | Terrestrial | Fish |
| 1 | milk replacer | 26 | 1.000 | 0.923 (2) |
| 2 | tuna meal | 26 | 0.885 (3) | 1.000 |
| 3 | salmon meal + 1 % pig PAP | 26 | 0.885 (3) | 1.000 |
| 4 | pig feed + 1 % haemoglobin powder | 26 | 0.615 (10) | 0.962 (1) |
| 5 | mixed blood meal | 26 | 0.962 (1) | 1.000 |
| 6 | plasma powder + 1 % collagen | 26 | 0.500 (13) | 1.000 |

**Accuracy means sensitivity in case of ND and specificity in case of PD.**
**In brackets the absolute number of ND or PD. (Legend: n = number of results).**

Regarding the detection of fish constituents, sensitivity scores are perfect while still some few specificity issues are observed: two cases of false positive results in sample 1 and one case in sample 4.

Regarding the detection of terrestrial vertebrates' constituents, the situation is different. Some specificity issues are noted due to false positive findings of terrestrial material in sample 2 (tuna meal), but the majority of the problems are related to sensitivity issues. The absence of terrestrial vertebrates' findings leads to false negative results. Such situation is found in sample 3 where the presence of bones is overlooked (3 cases). More interestingly for the present proficiency test is the apparent difficulty of detecting particles from blood meals or blood derived products. For sample 5 (mixed blood meal), only one case of false negative results is observed. This number of false negative results increases spectacularly in samples 4 (pig feed adulterated at 1 % with haemoglobin powder) to reach 39 % and in sample 6 (plasma powder with the addition of 1 % of collagen) to reach 50 % of incorrect results.

The origin of this considerable sensitivity issue related to blood meals and blood derivates is commented into the discussion.

4.1.1.2. Detailed review of results per sample

**Sample 1 : milk replacer**

Eleven NRLs (42 %) reported the presence of milk, milk powder, milk derivates, milk globules and lactose crystals. These references to dairy products were logically considered as correct.

PD for fish particles :

- Lab 10 reported the presence of "baby squid",
- Lab 21 misidentified fishbones, scales and muscles.

Lab 19 reported the sample as < LOD after having reported one fishbone (on two determinations).

**Sample 2: tuna meal**

PD for terrestrial vertebrates' particles :

- Labs 4 and 21 reported the presence of terrestrial bones and muscles,
- Lab 25 mentioned the finding of muscles and blood.

**Sample 3: salmon meal + 1 % pig PAP**

ND for terrestrial vertebrates' particles :

- Labs 4, 13 and 22 failed at detecting terrestrial particles.

**Sample 4: pig feed + 1 % haemoglobin powder**

ND for terrestrial vertebrates' particles :

- Labs 2, 3, 4, 8, 9, 11, 13, 15, 16, 17 failed at detecting haemoglobin particles.

PD for fish particles :

- Lab 10 reported the finding of oyster particles

Lab 2 reported the sample as < LOD after the finding of few fishbones (on two determinations).

**Sample 5: mixed blood meal**

ND for terrestrial vertebrates' particles :

- Lab 13 declared the sample as negative while mentioning in its comment blood particles.

Lab 2 reported the sample as < LOD for fish after the finding of few fishbones (on two determinations).

**Sample 6: plasma powder + 1% collagen**

ND for terrestrial vertebrates' particles :

- Labs 2, 3, 4, 6, 8, 9, 10, 15, 23, 24 and 25 failed at detecting any terrestrial particles,
- Lab 13 declared the sample as negative while mentioning in its comment plasma particles for the sample.

One <LOD case was reported for terrestrial material presence by lab 19. The particles reported were blood particles (on two determinations)

Lab 26 although reporting the sample as positive for terrestrial vertebrates, only mentioned bones and muscles which is not correct.

One <LOD case was reported for fish by lab 23 after having reported one "pearl-like" shell fragment and one fish scale.

4.1.1.3. Individual performances of NRLs in qualitative analysis

Individual performance parameters were assessed for each participant by calculating the accuracy, sensitivity and specificity over the blind sample set. This was calculated separately for both the detection of terrestrial vertebrates' material and of fish material. Results are to be found in Tables 4 and 5 of next page. A ranking of the labs was prepared based on the consolidated accuracy.

**Tables 4 (left) and 5 (right): NRL proficiencies regarding the detection of terrestrial and fish material respectively. Ranking follows AC values for primary key and SE for second key**

| Terrestrial | | | |
|---|---|---|---|
| **lab code** | **AC** | **SE** | **SP** |
| 1, 5, 7, 12, 14, 18, 19, 20 and 26 | 1.000 | 1.000 | 1.000 |
| 21 | 0.833 | 1.000 | 0.500 |
| 6, 10, 11, 16, 17, 22, 23 and 24 | 0.833 | 0.750 | 1.000 |
| 2, 3, 8, 9, 15 and 25 | 0.667 | 0.500 | 1.000 |
| 4 | 0.333 | 0.250 | 0.500 |
| 13 | 0.333 | 0.000 | 1.000 |

| Fish | | | |
|---|---|---|---|
| **lab code** | **AC** | **SE** | **SP** |
| 1, 2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 22, 23, 24, 25 and 26 | 1.000 | 1.000 | 1.000 |
| 21 | 0.833 | 1.000 | 0.750 |
| 10 | 0.667 | 1.000 | 0.500 |

A general ranking of the NRLs was also performed on a consolidated evaluation including their proficiency in detecting both terrestrial and fish materials through the set of blind samples (Table 6).

**Table 6: General NRL proficiency regarding the detection of terrestrial and fish material. Ranking follows AC values as primary key and SE as second key. Lines in black refer to excellent results, lines in blue to satisfying results and lines in red to underperforming results.**

| Consolidated | | | |
|---|---|---|---|
| **lab code** | **AC** | **SE** | **SP** |
| 1, 5, 7, 12, 14, 18, 19, 20 and 26 | 1.000 | 1.000 | 1.000 |
| 6, 11, 16, 17, 22, 23 and 24 | 0.917 | 0.833 | 1.000 |
| 21 | 0.833 | 1.000 | 0.667 |
| 2, 3, 8, 9, 15 and 25 | 0.833 | 0.667 | 1.000 |
| 10 | 0.750 | 0.833 | 0.667 |
| 4 | 0.667 | 0.500 | 0.833 |
| 13 | 0.667 | 0.333 | 1.000 |

From the 26 NRLs, 9 performed very well (34.5 %), 8 performed satisfyingly (31 %) and 9 were underperforming (34.5 %).

In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participants (labs 2, 3, 4, 8, 9, 10, 13, 15 and 25) are asked to report on the origin of their errors as well as on the actions they will undertake in order to solve the problems.

4.1.2. Qualitative analyses and individual performances the non-EU participants

Individual performances from the 9 participants outside the EU were assessed exactly as in the previous section (4.1.1.3). A ranking of those labs was prepared as well based on the consolidated accuracy. Results are to be found in Tables 7 and 8.

**Tables 7 (left) and 8 (right): non-EU lab proficiencies regarding the detection of terrestrial and fish material respectively. Ranking follows AC values for primary key and SE for second key. (Legend: n.a., not applicable)**

| Terrestrial | | | |
|---|---|---|---|
| **lab code** | **AC** | **SE** | **SP** |
| 40 | 0.833 | 0.750 | 1.000 |
| 35 | 0.667 | 1.000 | 0.000 |
| 30 and 38 | 0.667 | 0.750 | 0.500 |
| 27, 31 and 32 | 0.667 | 0.500 | 1.000 |
| 39 | 0.500 | 0.250 | 1.000 |
| 29 | 0.333 | 0.250 | 0.500 |

| Fish | | | |
|---|---|---|---|
| **lab code** | **AC** | **SE** | **SP** |
| 27, 30, 39 and 40 | 1.000 | 1.000 | 1.000 |
| 32 and 38 | 0.833 | 1.000 | 0.750 |
| 35 | 0.714 | 0.500 | 0.800 |
| 31 | 0.667 | 1.000 | 0.500 |
| 29 | n.a. | n.a. | n.a. |

The error details are described per sample:

**Sample 1 : milk replacer**

PD for terrestrial vertebrates' particles :

- Lab 38 reported the presence of feathers,
- Lab 35 reported the presence of plasma

**Sample 2: tuna meal**

PD for terrestrial vertebrates' particles :

- Lab 29 declared the sample as positive but without giving details of the found particles
- Lab 30 mentioned the finding of blood meal.
- Lab 35 reported the presence of bone fragments, muscles and "jelly"

Lab 39 reported the sample as <LOD due to the presence of a few bones (on a single determination).

ND for fish particles :

- Lab 35 failed at identifying fish particles.

**Sample 3: salmon meal + 1 % pig PAP**

ND for terrestrial vertebrates' particles :

- Lab 32 failed at detecting terrestrial particles.

**Sample 4: pig feed + 1 % haemoglobin powder**

ND for terrestrial vertebrates' particles :

- Labs 27, 29, 31 and 39 failed at detecting haemoglobin particles.

PD for fish particles :

- Lab 31 reported the finding of oyster particles, otoliths and squid meal.

Lab 39 reported the sample as < LOD after the finding of few fishbones (on a single determination).

**Sample 5: mixed blood meal**

ND for terrestrial vertebrates' particles :

- Lab 29 declared the sample as negative.

Lab 39 reported the sample as < LOD after the finding of few bones (on a single determination).

PD for fish particles :

- Lab 31 declared the sample as positive for fish without details on the found particles
- Labs 32 and 38 misidentified fishbones, scales and muscles

**Sample 6: plasma powder + 1% collagen**

ND for terrestrial vertebrates' particles :

- Labs 27, 29, 30, 31,38 and 39 failed at detecting any terrestrial particles,
- Lab 32 declared the sample as negative while mentioning in its comment plasma particles for the sample,
- Lab 40 commented on the presence of milk but declared it as negative.

A general ranking as for the NRL network was established (Table 9).

**Table 9: 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. Lines in black refer to excellent results, lines in blue refer to satisfying results and lines in red refer to underperforming results**

| Consolidated | | | |
|---|---|---|---|
| lab code | AC | SE | SP |
| 40 | 0.917 | 0.833 | 1.000 |
| 30 | 0.833 | 0.833 | 0.833 |
| 27 | 0.833 | 0.667 | 1.000 |
| 38 | 0.750 | 0.833 | 0.667 |
| 32 | 0.750 | 0.667 | 0.833 |
| 39 | 0.750 | 0.500 | 1.000 |
| 35 | 0.692 | 0.833 | 0.571 |
| 31 | 0.667 | 0.667 | 0.667 |
| 29 | 0.333 | 0.250 | 0.500 |

One participant performed satisfyingly (line in blue in Table 9). The other participants were classified as underperforming (lines in red in Table 9) according to the applied criteria.

## 4.2. PCR results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. On the respect of the instructions

The NRLs seem to stick generally to the SOPs. Nevertheless, very few labs do not use one of the EURL-AP recommended mastermixes. It was also noticed that the cut-off of some labs was not updated for more than one year.

4.2.1.2. Overview of results and global performance of the network

Table 10 summarizes the results provided by 26 NRLs for the six sample types submitted to qualitative PCR analysis.

**Table 10: Global results expressed as accuracy (AC) – PCR**

| Sample | Material | n | AC |
|---|---|---|---|
| 1 | milk replacer | 26 | 1.000 |
| 2 | tuna meal | 26 | 0.962 (1) |
| 3 | salmon meal + 1 % pig PAP | 26 | 1.000 |
| 4 | pig feed + 1 % haemoglobin powder | 26 | 1.000 |
| 5 | mixed blood meal | 26 | 1.000 |
| 6 | plasma powder + 1 % collagen | 26 | 0.846 (4) |

**Accuracy means sensitivity in case of ND and specificity in case of PD.**
**The absence of a PCR result is considered as a deviation (ND or PD).**
**In brackets the absolute number of false results. (Legend: n = number of results)**

On the overall, 5 deviations (3% of the 156 results) were recorded. Among these 5 deviations, one is due to an absence of result (Lab 16 – sample 2). The four remaining ones are negative deviations all obtained with sample 6.

**Sample 1** was a milk replacer. The PCR result expected for the presence of ruminant DNA was positive. No negative deviation was recorded for this sample.

**Sample 2** was a tuna meal. The PCR result expected for the presence of ruminant DNA was negative. The only one deviation recorded with this sample is due to a missing result (Lab 16).

**Sample 3** was a salmon meal containing 0.1 % of pig PAP. The PCR result expected for the presence of ruminant DNA was negative. No positive deviation was recorded for this sample.

**Sample 4** was a pig feed containing 1 % of haemoglobin powder. The PCR result expected for the presence of ruminant DNA was negative. No positive deviation was recorded for this sample.

**Sample 5** was a mixed blood meal containing ruminant DNA. No negative deviation was recorded for this sample.

**Sample 6** was a plasma powder containing 1 % of collagen. The PCR result expected for the presence of ruminant DNA was positive. Four negative deviations were recorded (Labs 7, 8, 21 and 25).

4.2.1.3. Individual performances of NRLs in qualitative analysis

Individual performances were assessed for each participant by calculating the accuracy, sensitivity and specificity over the samples. A ranking of the labs was prepared based on the accuracy. Results are to be found in Table 11 (next page) that summarizes the results obtained by the participants.

**Table 11: NRL proficiencies regarding the detection of ruminant material. Ranking follows AC values. Cells in black refers to excellent NRLs. Cells in blue refers to satisfying NRLs. Cells in red refer to underperforming NRLs**

| Lab code | AC | SE | SP |
|---|---|---|---|
| 1, 2, 3, 4, 5, 6, 9, 10, 11,12, 13, 14, 15, 17, 18, 19, 20, 22, 23, 24 and 26 | 1.000 | 1.000 | 1.000 |
| 16* | 0.800 | 1.000 | 0.500 |
| 7, 8, 21 and 25 | 0.800 | 0.667 | 1.000 |

* Absence of a PCR result is assimilated to a deviation

Excellent performances were recorded for 21 labs out of 26 NRLs (81 % of the NRLs) having no false result. The five remaining labs were satisfying: Labs 7, 8, 21 and 25 reported a ND for sample 6. The remaining deviation is a PD but due to an absence of result for sample 2 (Lab 16). No lab is underperforming.

4.2.1.4. Cut-off quality control

A quality control for the number of copies of the ruminant target reached with the Ct value of the cut-off, was developed to minimize the risk of false positive result. A minimum of 9.00 copies at the cut-off was required. Indeed, depending on the variability of the lab (PCR platform + operator), the cut-off value can correspond to a too low number of copies.

All participants reached the minimum criterion of 9.00 copies. The range of copies at the cut-off goes from 9.04 copies to 11.91 copies. The cut-off in cycles are comprised between 31.80 cycles and 38.05 cycles. The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 69.2 %.

The protocol used to set the cut-off was developed to obtain a robust and stable cut-off. The EURL-AP considers that an update of the cut-off have to be done once a year or after any breakdown of the thermocycler. Surprisingly, the cut-off of the platforms (in cycles and in number of copies) in some labs remains the same for more than one year (they were already in use during the PT 2018 or 2019). This situation is certainly not in line with ISO 17025 standard recommending a continuous control of the quality of the results even if the results for the EURL-AP PT are still satisfying or excellent.

4.2.2. Qualitative analyses from the non-EU participants

4.2.2.1. Individual performances

Individual performances were assessed for four non-EU participants who reported PCR results by calculating the accuracy, sensitivity and specificity over the samples. Their results are to be found in Table 12.

**Table 12 : Non-EU participant proficiencies regarding the detection of ruminant material. Ranking follows AC values. Cells in black refers to excellent labs. Cells in blue refers to satisfying labs.**

| Lab code | AC | SE | SP |
|---|---|---|---|
| 27 and 40 | 1.000 | 1.000 | 1.000 |
| 35 | 0.833 | 0.667 | 1.000 |
| 31 | 0.800 | 1.000 | 0.500 |

Labs 27 and 40 obtained excellent results (no deviation).

Lab 35, one negative deviation was recorded with sample 6 (pig plasma containing 0.1 % of bovine collagen).

Lab 31 had one positive deviation with the tuna meal (sample 2).

4.2.2.2. Assessment of the cut-off values

Labs 27, 31 and 40 have cut-off values that comply with the minimum criterion of 9 copies set by the EURL-AP.

No statement can be made for Lab 35 as they did not use the EURL-AP PCR test.

# 5. Discussion and conclusions

This 2021 edition of the EURL-AP proficiency test mainly focused on the detection of animal traces within feed materials such as milk products, blood meals, haemoglobin and plasma powders known to deliver insignificant amounts of sediments, if any.

The results obtained by light microscopy revealed some unexpected issues deserving comments.

If the ability to disclose and identify the presence of fish particles occurred without problems at the exception of few baseline specificity problems, the performance obtained by the participants for the detection of terrestrial vertebrates pointed some major sensitivity problems.

The identification of the sample containing the mixed blood meal was almost perfect with in the details of the observations a majority of NRLs mentioning the correct identification of blood particles (20/26), by or without the use of TMB + $H_2O_2$ test. Even the single negative deviation observed was inexplicably accompanied with a comment stating that blood was present. Nevertheless 6 NRLs declared the sample as positive for terrestrial vertebrates with the sole mention of bones, muscles or hairs but omitting to mention the presence of blood particles.

The situation worsened when haemoglobin powder was added to a compound feed for pig; only 62 % of the NRLs were then able to disclose the blood product. No clear or conclusive explanation for this sensitivity issue could be found. However, a possible reason may be linked to the fact that when a pure blood meal or haemoglobin powder is analysed, due to the absence of sediment, the analyst pay more attention on the observation of the slides made from the raw material or the flotate. On the contrary, when such materials are added to a matrix delivering a sediment (even at a low level of 0.8 % as for the pig feed used) attention is first paid on the slides prepared from the sediment, from where the majority of the blood particles are absent, then only on the slides prepared from the other fractions. The successive steps of the observation flowchart are effectively prioritising the slides from the sediment over the slides prepared from the flotate or the raw material. An element in favour of this hypothesis is that fact that the majority of negative deviations (8/10) was recorded after microscopic analyses based on one determination rather than two. One recommendation would certainly be of not underestimating the value of the flotate or raw material observation which should also deserve accurate observations, especially in such situations.

The second major sensitivity issue was related to the identification of the plasma powder. Half of the NRLs failed at identifying this feed material. Although it can be expected that this type of material does not frequently occurs in daily routine work, it is required being able to identify it. Simple tests, such as TMB + $H_2O_2$ or IKI, can help to discriminate plasma from milk products or starch flakes respectively.

From a more global perspective, through the reading of the comments delivered by the participants, it appears that the decision of declaring a sample as positive for terrestrial vertebrates' constituents based on the observations of milk or plasma detection is still somehow an hesitating one. It must be reminded that applicable Annex VI of Commission Regulation EC/152/2009 in its title refers to the determination of constituents of animal origin and does not strictly limit this determination to prohibited ones. Therefore, even the microscopic finding in authorised matrices of authorised ingredients (dairy products and plasma powders) has to be considered as positive for terrestrial vertebrates' presence. Next coming proficiency studies organised by the EURL-AP will systematically adhere to this position; thus, declared negative results

for terrestrial vertebrates with reference to milk in the details of the observations will no longer be accepted as it was for the present study.

To conclude on the microscopic results, the number of excellent and satisfactory scores obtained within the network of NRLs reached respectively 34.5 % and 31 %. The rate of underperforming NRL for the present study reached 34.5 %. Dedicated follow-up actions will be undertaken for each of them.

Concerning results from non-EU participants, encountered problems were similar. The detection of terrestrial vertebrates' elements in the pig feed fortified with haemoglobin powder and in the plasma powder was very problematic too, while the identification of blood particles from the mixed blood meal was all in all comparable to the situation of the NRLs. For these participants the percentage of satisfying results reached only 11 % while 89 % were, according to the applied performance criteria, categorised as underperforming.

Looking at the PCR results, the performances of the NRL network are very good with no underperforming lab. If the experience of the participants is probably an explanation, the instruction to analyse all the samples by PCR independently of the microscopic results has certainly helped to improve the results. Indeed the last proficiency tests during which no underperforming lab was recorded took place in 2015 and 2016 [8 ; 9]. Since 2017, the application of the SOP on the operational protocol for the combination of light microscopy and PCR was also in the assessment criteria and lets the choice to analyse the samples by PCR or not. The PCR skill of the NRLs network specifically with the ruminant PCR method is confirmed.

On the five deviations, four negative deviations were obtained with a sample of pig plasma containing bovine collagen. This sample was not considered by the organisers as the most difficult one. Before the PT, the sample of pig feed containing pig haemoglobin was considered as a challenging sample. Indeed this sample can give Ct values close to the cut-off and lead to positive deviations. Eventually, all the participants succeed to analyse correctly this sample.

A last point concerns the good laboratory practices. Few labs are using the same cut-off during a very long time (two or three years). This is certainly not in line with accreditation purposes which recommend a good follow-up of the results' quality. Until now, it had no impact on the proficiency of the participants. It is an indication of the robustness and the stability of the cut-off along the time. Nevertheless, the EURL-AP recommends to check regularly the cut-off of the PCR platforms. A frequency of once a year is realistic to prevent deviations.

## Acknowledgment

We are grateful to the EURL-AP technical staff for their preparation work and the efforts made to meet the ISO 17043 requirements: A. Cordonnier, L. Plasman and J. Maljean. We also thank the participants for their fruitful collaboration.

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# Annex 1

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

| Country | Institute Name |
|---|---|
| *Argentina* | *SENASA - DILAB - DLA - CIALOA - Departamento evaluacion y desarrollo* |
| *Australia* | *Biosecurity Sciences Laboratory* |
| Austria | Austrian Agency for Health and Food Safety |
| Belgium | Federal Agency for the Safety of the Food Chain |
| Bulgaria | National Diagnostic Research Veterinary Medical Institute |
| *China* | *China Agricultural University* |
| Croatia | Croatian Veterinary Institute |
| Cyprus | Cyprus Veterinary Services |
| Czech Republic | State Veterinary Institute Jihlava |
| Denmark | The Danish Plant Directorate |
| Estonia | Veterinary and Food Laboratory |
| Finland | Finnish Food Safety Authority |
| France | DG for Fair Trading, Consumer Affairs and Fraud Control-Laboratory Directorate Rennes |
| Germany | Federal Institute for Risk Assessment |
| Greece | Feedstuffs Control Laboratory |
| Hungary | Central Agricultural Office-Directorate Food and Feed Safety-Central Feed Investigation Lab. |
| Ireland | Department of Agriculture and Food Microscopy Laboratory - Seed Testing Station |
| Italy | National Reference Centre for the Surveillance and Monitoring of Animal Feed |
| Latvia | Institute of Food Safety, Animal Health and Environment "BIOR" |
| Lithuania | National Food and Veterinary Risk Assessment Institute |
| Luxemburg | Agroscope Liebefeld-Posieux Research Station (Switzerland) |
| Netherlands | Wageningen Food Safety Research |
| *Norway* | *Institute of Marine Research* |
| *Peru* | *Bureau Veritas* |
| Poland | National Veterinary Research Institute |
| Portugal | Instituto Nacional de Investigacao Agraria e Veterinaria |
| Romania | Hygiene Institute of Veterinary Health |
| *Serbia* | *Institute of Veterinary Medicine of Serbia* |
| Slovakia | State Veterinary and Food Institute |
| Slovenia | Veterinary faculty - National Veterinary Institute - Institute of Food Safety, Feed and Environment - Department of Environment, Animal Nutrition, Welfare and Hygiene |
| *South Africa* | *Stellenbosch University, Department of Animal Sciences* |
| Spain | Laboratorio Arbitral Agroalimentario |
| Sweden | National Veterinary Institute, Department of Animal Feed |
| *Thailand* | *Bureau of Quality Control of Livestock Products* |
| *United Kingdom* | *Animal and Plant Health Agency* |

# Annex 2

Announcement letter

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Knowledge & valorisation of Agricultural Products Department

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## Announcement of the EURL-AP proficiency test 2021/01 for the determination of Processed Animal Proteins (PAPs) in feed

### Introduction

The use of processed animal by-products as ingredient for animal feedingstuffs within the European Union is regulated by the TSE Regulation (Regulation EC N°999/2001), as amended. In particular, Article 7 imposes a prohibition to use processed animal proteins in the feeding of farmed animals (extended feed ban).

Commission implementing Regulation (EU) No 2020/1560 amending Annex VI of Regulation (EC) No 152/2009, imposes the methods of analysis for the determination of constituents of animal origin for the official control of feed.

### Objectives

The objective of the present proficiency test is to assess the performance of the NRLs to detect the presence of PAPs in feed by the reference methods using light microscopy and PCR as stated in Regulation EC 152/2009 as amended by Commission implementing Regulation (EU) No 2020/1560 and related SOPs.

### The organizer team

The test will be coordinated by the European Union Reference Laboratory for Animal Proteins in feedingstuffs (EURL-AP).

### Test material

Samples containing typical compound feed fortified with processed animal proteins (PAPs) will be prepared. The EURL-AP will endorse the homogeneity of the samples. Nevertheless, each laboratory participating to the test is sole responsible to reach appropriate homogeneity for the sample sub-portions taken for analysis.

Each participant will receive a maximum of 6 samples, each of about 40g. Each sample shall be analysed both by light microscopy and PCR.

### General outline of the exercise

- The light microscopic and PCR methods to use are described in Annex VI of Commission Regulation EC 152/2009 and related SOPs.
- The EURL-AP will provide participants with an Excel file for reporting the results of the proficiency test analyses.
- Each participating laboratory will be assigned a unique code and only the organizer of the study knows the key to this code. After completing the test each laboratory will get a report including its results and lab code. A final report of the study will be published with anonymised results.
- The participation in this proficiency study is mandatory and free of charge for national reference laboratories within Member States of the European Union.

European Union Reference Laboratory for Animal Proteins in feedingstuffs

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## Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : 10 September 2021
- Sending of the sample boxes and communication of the instructions : 15 October 2021

> *By default, samples will be sent to the NRL microscopy contact person referred on the intranet. You are asked to check if this person is still your contact and to inform the organizer from any change.*

- Deadline for returning of results to the organizer : 15 November 2021

## Further information

- Refer to the address and coordinates mentioned in the heading,

or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager
  ☎32 (0) 81 87 52 28
  32 (0) 81 87 40 19
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or

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# Annex 3

Excel result report form

## Proficiency Test Microscopy-PCR 2021/01

**Laboratory identification**

Laboratory code : 1

Responsibility agreement : No

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

**Report**

| | | | | | | |
|---|---|---|---|---|---|---|
| Lab code | 1 | 1 | 1 | 1 | 1 | 1 |
| Sample rank | 1st | 2nd | 3th | 4th | 5th | 6th |
| Sample N° | | | | | | |
| **Method of analysis** | **Light microscopy** | | | | | |
| Light microscopy analyses | | | | | | |
| Terrestrial animal particles | | | | | | |
| details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.* | | | | | | |
| Fish particles | | | | | | |
| details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.* | | | | | | |
| Analyses performed on | | | | | | |
| Number of determinations | | | | | | |
| Free comment *Example : presence of unusual fragments,...* | | | | | | |
| **Method of analysis** | **PCR** | | | | | |
| PCR analyses | | | | | | |
| Ruminant DNA | | | | | | |
| **Cut-off at 15 copies of the PCR platform used (in cycles)** | | | | | | |
| **Copy number at the cut-off of the PCR platform used (in copies)** | | | | | | |
| **Maxter mix used** | | | | | | |
| Dilution 1 (e.g. 1 fold) | | | | | | |
| Ct value replicate 1 | | | | | | |
| Ct value replicate 2 | | | | | | |
| Dilution 2 (e.g. 10 fold) | | | | | | |
| Ct value replicate 1 | | | | | | |
| Ct value replicate 2 | | | | | | |

# Annex 4

Gross results of participants for microscopy (in numerical order of lab ID)

Laboratory identification code : **1**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 9 | Present | Bones, blood, hairs | Absent | | Sed. + Flot. | 2 |
| 1 | 385 | Absent | | Absent | | Sed. + Flot. | 1 |
| 2 | 435 | Absent | | Present | Bones, gills | Sed. + Flot. | 2 |
| 3 | 485 | Present | Bones | Present | Bones, gills | Sed. + Flot. | 2 |
| 6 | 563 | Present | Only blood plasma | Absent | | Sed. + Flot. | 2 |
| 4 | 655 | Present | Only blood | Absent | | Sed. + Flot. | 2 |

*Tetramethylbenzidine – Hydrogen peroxide were used as a mounting medium for detecting blood and blood plasma in flotate.*

Laboratory identification code : **2**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 61 | Absent | | Absent | | Sed. + Flot. | 1 |
| 6 | 179 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 305 | Present | bones, muscle fibers, cartilages | Present | fishbones, muscle fibers, cartilages, scales | Sed. + Flot. | 2 |
| 5 | 417 | Present | bones | < LOD | fishbones | Sed. + Flot. | 2 |
| 2 | 483 | Absent | | Present | fishbones, gills, cartilages, muscle fibers | Sed. + Flot. | 2 |
| 4 | 631 | Absent | | < LOD | fishbones | Sed. + Flot. | 2 |

Laboratory identification code : **3**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 253 | Absent | | Absent | | Sed. + Flot. | 2 |
| 3 | 497 | Present | bones | Present | fishbones, scales, gills | Sed. + Flot. | 2 |
| 2 | 519 | Absent | | Present | fishbones, gills | Sed. + Flot. | 2 |
| 6 | 611 | Absent | | Absent | | Sed. + Flot. | 2 |
| 4 | 619 | Absent | | Absent | | Sed. + Flot. | 2 |
| 5 | 633 | Present | bones | Absent | | Sed. + Flot. | 2 |

Laboratory identification code : **4**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 2 | 51 | Present | bones, muscles | Present | Fishbones, muscles, gills, cartilages | Sed. + Raw | 1 |
| 4 | 307 | Absent | | Absent | | Sed. + Raw | 1 |
| 6 | 395 | Absent | | Absent | | Sed. + Raw | 1 |
| 5 | 453 | Present | bones, muscles | Absent | | Sed. + Raw | 1 |
| 1 | 481 | Absent | | Absent | | Sed. + Raw | 1 |
| 3 | 557 | Absent | | Present | Fishbones, muscles, gills, cartilages, otoliths | Sed. + Raw | 1 |

Laboratory identification code : **5**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 2 | 3 | Absent | | Present | bones | Sed. + Flot. | 1 |
| 6 | 95 | Present | blood plasma | Absent | | Sed. + Raw | 2 |
| 5 | 129 | Present | bones, blood meal of unknown origin | Absent | | Sed. + Flot. | 1 |
| 4 | 187 | Present | hemoglobin of unknown origin | Absent | | Sed. + Raw | 2 |
| 3 | 473 | Present | bones, muscles | Present | bones, muscles | Sed. + Flot. | 1 |
| 1 | 553 | Absent | | Absent | | Sed. + Flot. | 1 |

Laboratory identification code : **6**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 119 | Absent | | Absent | | Sed. + Flot. | 1 |
| 4 | 151 | Present | haemoglobin | Absent | | Sed. + Flot. | 2 |
| 5 | 153 | Present | bones, blood meal | Absent | | Sed. + Flot. | 1 |
| 2 | 171 | Absent | | Present | fishbones, scales, muscles | Sed. + Flot. | 1 |
| 3 | 221 | Present | bones | Present | fishbones, scales, muscles | Sed. + Flot. | 2 |
| 1 | 469 | Absent | milk powder | Absent | | Sed. + Flot. | 1 |

Laboratory identification code : **7**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 37 | Absent | | Absent | | Sed. + Raw | 1 |
| 4 | 43 | Present | Blood | Absent | | Sed. + Raw | 1 |
| 6 | 323 | Present | Plasma | Absent | | Sed. + Raw | 1 |
| 2 | 531 | Absent | | Present | fish bones, meat, cartilage, scales, gills | Sed. + Raw | 1 |
| 3 | 581 | Present | bone, meat | Present | fish bones, cartilage, scales, gills, otholits, meat | Sed. + Raw | 1 |
| 5 | 597 | Present | bone, blood, hair | Absent | | Sed. + Raw | 1 |

Laboratory identification code : **8**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 11 | Absent | | Absent | | Sed. + Flot. | 1 |
| 5 | 273 | Present | Bones, muscle, blood | Absent | | Sed. + Flot. | 1 |
| 1 | 349 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 425 | Present | Bone | Present | Bone, muscle | Sed. + Flot. | 1 |
| 4 | 487 | Absent | | Absent | | Sed. + Flot. | 1 |
| 2 | 591 | Absent | | Present | Bone, muscle | Sed. + Flot. | 1 |

Laboratory identification code : **9**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 97 | Present | milk | Absent | | Sed. + Raw | 1 |
| 6 | 143 | Absent | | Absent | | Sed. + Raw | 1 |
| 4 | 163 | Absent | | Absent | | Sed. + Flot. | 1 |
| 2 | 291 | Absent | | Present | bones, scales, gilles, muscle | Sed. + Flot. | 1 |
| 5 | 573 | Present | muscle, hear | Absent | | Sed. + Raw | 1 |
| 3 | 641 | Present | bones, muscle | Present | bones, scales, gilles, muscle | Sed. + Flot. | 1 |

Laboratory identification code : **10**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 5 | Present | bone | Present | scale, shark scale, cartilage, teeth, gill, fish bone | Sed. + Raw | 1 |
| 2 | 207 | Absent | | Present | scale, gill, cartilage, teeth | Sed. + Raw | 1 |
| 4 | 403 | Present | blood | Present | oyster | Sed. + Raw | 1 |
| 6 | 515 | Absent | | Absent | | Sed. + Raw | 1 |
| 1 | 529 | Present | milk | Present | baby squid | Sed. + Raw | 1 |
| 5 | 549 | Present | blood, muscle | Absent | | Sed. + Raw | 1 |

*515: blood test positive*

Laboratory identification code : **11**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 2 | 63 | Absent | | Present | Bone fragments, teeth, muscle fibres | Sed. + Flot. | 1 |
| 6 | 167 | Present | Plasma (ingredient) | Absent | | Sed. + Flot. | 1 |
| 3 | 209 | Present | Bone fragments | Present | Bone fragments, teeth, gills, scales, muscle fibres | Sed. + Flot. | 1 |
| 4 | 331 | Absent | | Absent | | Sed. + Flot. | 1 |
| 1 | 433 | Present | Milk (ingredient) | Absent | | Sed. + Flot. | 1 |
| 5 | 657 | Present | Blood meal, hairs | Absent | | Sed. + Flot. | 1 |

*Sample 209: the few terrestrial bone fragments detected are weakly coloured with Alizarin Red*

Laboratory identification code : **12**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 2 | 195 | Absent | | Present | Bones, gills, scales, cartilage, muscles | Sed. + Flot. | 1 |
| 6 | 215 | Present | Blood plasma | Absent | | Sed. + Flot. | 1 |
| 4 | 223 | Present | Blood | Absent | | Sed. + Flot. | 1 |
| 3 | 353 | Present | Bones | Present | Bones, gills, scales | Sed. + Flot. | 1 |
| 5 | 429 | Present | Blood, feathers | Absent | | Sed. + Flot. | 1 |
| 1 | 493 | Present | Milk powder | Absent | | Sed. + Flot. | 1 |

*The samples 215, 223, 429 and 493 gave negligible amount of sediment.*

Laboratory identification code : **13**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 203 | Absent | | Absent | | Sed. + Raw | 1 |
| 1 | 301 | Absent | | Absent | | Sed. + Raw | 1 |
| 4 | 439 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 449 | Absent | | Present | Bones, cartilage, gills, otholite, scales, muscles | Sed. + Flot. | 1 |
| 5 | 489 | Absent | | Absent | | Sed. + Raw | 1 |
| 2 | 639 | Absent | | Present | Teeth, 'Bones, cartilage, gills, muscles | Sed. + Raw | |

*Plasma detected in #203; Milk powder in #301; Blood in #489*

Laboratory identification code : **14**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 33 | Present | Bones,blood meal of unknown origin | Absent | | Sed. + Flot. | |
| 1 | 157 | Absent | | Absent | | Sed. + Flot. | |
| 4 | 175 | Present | hemoglobin of unknow origin | Absent | | Sed. + Raw | |
| 3 | 365 | Present | bones,muscle | Present | Bones,muscle | Sed. + Flot. | |
| 6 | 467 | Present | blood plasma | Absent | | Sed. + Flot. | |
| 2 | 567 | Absent | | Present | bones ,muscle | Sed. + Flot. | |

Laboratory identification code : **15**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 47 | Absent | | Absent | | Sed. + Flot. | 1 |
| 4 | 139 | Absent | | Absent | | Sed. + Flot. | 1 |
| 2 | 147 | Absent | | Present | fish bones, muscle fibers | Sed. + Flot. | 1 |
| 3 | 185 | Present | bones | Present | fish bones, muscle fibers | Sed. + Flot. | 1 |
| 5 | 285 | Present | muscle fibers, blood, bones | Absent | | Sed. + Flot. | 1 |
| 1 | 325 | Absent | | Absent | | Sed. + Flot. | 1 |

Laboratory identification code : **16**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 237 | Present | bones, blood, feathers | Absent | | Sed. + Flot. | 1 |
| 6 | 443 | Present | milk product (milk powder / derivates) | Absent | | Sed. + Flot. | 1 |
| 3 | 533 | Present | bones, blood | Present | bones, muscles, cartilages, scales, gill | Sed. + Flot. | 1 |
| 2 | 555 | Absent | | Present | bones, muscles, cartilages, scales, gill | Sed. + Flot. | 1 |
| 4 | 607 | Absent | | Absent | | Sed. + Flot. | 1 |
| 1 | 625 | Present | milk product (milk powder / derivates) | Absent | | Sed. + Flot. | 1 |

*Since we had limited time to perform the PT test (as we have already told you), we were still able to complete the analysis after the first determination. Nevertheless, the samples were unmarked, i.e., without declaration, and according to the new regulation we would have to make another determination because we found animal fragments in the samples (after the first determination). According to the regulation, we know that if there is no declaration and if animal particles are found, another determination is mandatory. Finally, the particles found (or terrestrial or fish) are added up – sum of particles of same nature (from D1+D2) and then the sample is declared according to point 2.1.5.2 or 2.1.5.3. However, we concluded after the first determination, because we had found more than 10 fragments of certain animal particles in the samples. As the instructions stated that SOP should not be followed for the combination of LM and PCR and that all samples had to be examined by both methods, we assumed that no second determination was necessary for LM as the results were clear after the first determination.*

Laboratory identification code : **17**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 59 | Present | blood plasm | Absent | | Sed. + Flot. | 1 |
| 3 | 77 | Present | bones, muscle fibres | Present | fish bones, scales, otholits, muscle fibres | Sed. + Flot. | 1 |
| 2 | 243 | Absent | | Present | fish bones, scales, otholits, muscle fibres<br><br>it can't be excluded, that the muscle fibres found only derive from fish | Sed. + Flot. | 1 |
| 4 | 343 | Absent | | Absent | | Sed. + Flot. | 1 |
| 5 | 357 | Present | bones, blood products, muscle fibres<br><br>it can't be excludet, that the muscle fibres found are only from terestrial origin | Absent | | Sed. + Flot. | 1 |
| 1 | 397 | Present | milk powder | Absent | | Sed. + Flot. | 1 |

Laboratory identification code : **18**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 25 | Present | milk globules | Absent | | Sed. + Flot. | 2 |
| 2 | 327 | Absent | | Present | fish bones, muscle fibers | Sed. + Flot. | 2 |
| 4 | 451 | Present | blood spray-dried globules, hemoglobine? | Absent | | Sed. + Flot. | 2 |
| 5 | 561 | Present | bone particles, blood meal, hair, muscle fiber | Absent | | Sed. + Flot. | 2 |
| 6 | 587 | Present | blood plasma? | Absent | | Sed. + Flot. | 2 |
| 3 | 593 | Present | bone particles, muscle fibers | Present | bone particles, muscle fibers | Sed. + Flot. | 2 |

*Samples 561 and 587 have delivered only very little sediment.*

*In samples 25, 451 and 587 we found spray-dried particles which could not be 100 % verified but ressemble: milk globules (sample 25), heamoglobine powder (sample 451), plasma (sample 587).*

*Sample 561 seems to be blood meal, but we did not verify it 100 %.*

*Each of 2 determinations was performed by one analyst with different sedimentation glassware and coloration.*

*In case of sample 561, with closed separation funnels and sediment collected on a filter paper, we found no sediment at all. Applying the sedimentation in a conical bottomed settling beaker and collecting the sediment (without filter paper) on a glass plate, we found a small sediment containing more than 15 bone particles.*

Laboratory identification code : **19**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 109 | Absent | | < LOD | 1 fish bone | Sed. + Flot. | 2 |
| 5 | 189 | Present | blood particles, bones, muscle fibres | Absent | | Sed. + Flot. | 2 |
| 3 | 401 | Present | bones, muscle fibres, cartilage | Present | fish bones, gill, fish scales, cartilage, muscle fibres, fish skin | Sed. + Flot. | 2 |
| 2 | 447 | Absent | | Present | fish bones, gill, fish scales, cartilage, muscle fibres, fish skin | Sed. + Flot. | 2 |
| 6 | 455 | < LOD | blood particles | Absent | | Sed. + Flot. | 2 |
| 4 | 559 | Present | blood particles | Absent | | Sed. + Flot. | 2 |

*sample 109: due to the color, the typical smell in combination with the microscopic findings it is a milk product ; sample 455: due to the color, the smell in combination with the microscopic findings it is a blood plasma product*

Laboratory identification code : **20**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 89 | Present | bones | Present | fishbone, gill, scale, muscle | Sed. + Raw | 2 |
| 5 | 93 | Present | bones, blood | Absent | | Sed. + Raw | 2 |
| 4 | 199 | Present | blood | Absent | | Sed. + Raw | 2 |
| 6 | 251 | Present | plasm | Absent | | Sed. + Raw | 2 |
| 1 | 409 | Present | milk | Absent | | Sed. + Raw | 2 |
| 2 | 459 | Absent | | Present | fishbone, gill, tooth, muscle | Sed. + Raw | 2 |

Laboratory identification code : **21**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 4 | 67 | Present | haemoglobine | Absent | | Sed. + Flot. | 2 |
| 1 | 169 | Absent | | Present | fish bones, scales,muscles | Sed. + Raw | 2 |
| 2 | 303 | Present | 6 bones and muscles | Present | fish bones,muscles,scales | Sed. + Flot. | 2 |
| 5 | 405 | Present | blood and feathers, muscle | Absent | | Sed. + Raw | 2 |
| 6 | 419 | Present | plasma | Absent | | Sed. + Raw | 2 |
| 3 | 605 | Present | bones an d muscles | Present | fish bones, scales and muscles | Sed. + Flot. | 2 |

Laboratory identification code : **22**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 53 | Absent | | Present | gill, scale, tooth, otolith, bone | Sed. + Raw | 2 |
| 4 | 79 | Present | blood | Absent | | Sed. + Raw | 2 |
| 2 | 183 | Absent | | Present | bone, gill, tooth | Sed. + Raw | 2 |
| 6 | 263 | Present | blood products (plasma) | Absent | | Sed. + Raw | 2 |
| 5 | 297 | Present | blood, terrestrial bone | Absent | | Sed. + Raw | 2 |
| 1 | 601 | Present | milk globules and lactose crystals | Absent | | Sed. + Raw | 2 |

*Sample 263 reacted with TMB+H2O2 stain. Blue colour development within seconds.*

Laboratory identification code : **23**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 4 | 55 | Present | blood particles | Absent | | Sed. + Raw | 1 |
| 1 | 361 | Absent | | Absent | | Sed. + Raw | 1 |
| 2 | 387 | Absent | | Present | fish bones | Sed. + Raw | 1 |
| 5 | 609 | Present | bone fragments, muscle fibres, hairs | Absent | | Sed. + Raw | 1 |
| 3 | 617 | Present | bone fragments (11) | Present | fish bone fragments, muscle fibres attributed to fish | Sed. + Raw | 1 |
| 6 | 659 | Absent | | < LOD | pearl-like shell fragment (1), fish scale fragment (1) | Sed. + Raw | 2 |

*sample 659: some very thin (10 um) translucent flakes have been observed.*

Laboratory identification code : **24**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 6 | 23 | Absent | | Absent | | Sed. + Raw | 1 |
| 4 | 91 | Present | blood | Absent | | Sed. + Raw | 1 |
| 3 | 245 | Present | bones, muscle fibers, cartilages | Present | scales, fishbones, cartilages, muscle fibers, otholithes, gills | Sed. + Raw | 2 |
| 2 | 267 | Absent | | Present | scales, fishbones, gills, cartilages, muscles fibers | Sed. + Raw | 2 |
| 5 | 369 | Present | blood | Absent | | Sed. + Raw | 1 |
| 1 | 541 | Absent | | Absent | | Sed. + Raw | 1 |

Laboratory identification code : **25**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 73 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 317 | Present | bones, muscles | Present | gills, fish bones, scales, cartilage, muscles | Sed. + Flot. | 2 |
| 2 | 351 | Present | muscles, blood | Present | 'gills, fish bones, scales, cartilage, muscles | Sed. + Flot. | 2 |
| 4 | 415 | Present | blood | Absent | | Sed. + Flot. | 2 |
| 6 | 539 | Absent | | Absent | | Sed. + Raw | 1 |
| 5 | 645 | Present | bone, blood, hair | Absent | | Sed. + Flot. | 2 |

*Sample No 73 contain milk powder particles. Sample No 539 contain blood plasma. TMB positive for samples No 351, 415, 539 and 645.*

Laboratory identification code : **26**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 85 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 149 | Present | bones, muscles | Present | bones, scales, otolith. | Sed. + Flot. | 1 |
| 5 | 345 | Present | bones | Absent | | Sed. + Flot. | 1 |
| 4 | 535 | Present | bones, muscles | Absent | | Sed. + Flot. | 1 |
| 2 | 579 | Absent | | Present | bones, scales, cartilage | Sed. + Flot. | 1 |
| 6 | 623 | Present | bones, muscles | Absent | | Sed. + Flot. | 1 |

*Have been found acaries in 85 sample.*

Laboratory identification code : **27**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 277 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 281 | Present | Bones | Present | Fishbones, scales, gills | Sed. + Flot. | 1 |
| 4 | 283 | Absent | | Absent | | Sed. + Flot. | 1 |
| 5 | 393 | Present | Bones (low presence) | Absent | | Sed. + Flot. | 1 |
| 6 | 491 | Absent | | Absent | | Sed. + Flot. | 1 |
| 2 | 507 | Absent | | Present | Fishbones, gills, muscles | Sed. + Flot. | 1 |

Laboratory identification code : **29**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 41 | Present | | | | | |
| 5 | 105 | Absent | | | | | |
| 2 | 111 | Present | | | | | |
| 1 | 145 | Absent | | | | | |
| 4 | 367 | Absent | | | | | |
| 6 | 575 | Absent | | | | | |

Laboratory identification code : **30**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 213 | Present | Blood meal and mammalian hair | Absent | | Sed. + Flot. | 1 |
| 2 | 219 | Present | Blood meal in flotate | Present | Fish bone, muscle and cartilage. | Sed. + Flot. | 1 |
| 4 | 247 | Present | Blood meal (spray dried). | Absent | | Sed. + Flot. | 1 |
| 3 | 329 | Present | Bone particles. | Present | Fish bone, muscle and cartilage. | Sed. + Flot. | 1 |
| 6 | 371 | Absent | | Absent | | Sed. + Flot. | 2 |
| 1 | 637 | Present | Milk powder. | Absent | | Sed. + Flot. | 1 |

*Sample 637 contains milk powder. Lactose crystals and other milk powder particles have been observed under polarized lighgt. Although milk powder is not a rendered product and per se not an animal particle, but the product of an animal, I have still chosen "present" in the "Terrestrial animal particles" block, to indicate that particles that originated from a terrestrial animal have been observed.*

Laboratory identification code : **31**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 81 | Present | | Present | | Sed. + Flot. | 1 |
| 6 | 131 | Absent | | Absent | | Sed. + Flot. | 2 |
| 1 | 181 | Absent | | Absent | | Sed. + Flot. | 1 |
| 4 | 211 | Absent | | Present | Otolit from fish. Also oyster shels, squid meal. | Sed. + Flot. | 1 |
| 3 | 389 | Present | bone particles | Present | cartilage and gills | Sed. + Flot. | 1 |
| 2 | 423 | Absent | | Present | Gills, scales, bone and otolit | Sed. + Flot. | 1 |

*Sample nr 131 did not contain any sediment following final washing step. This happend after two attempts to recover sediment.*

Laboratory identification code : **32**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 1 | 1 | Absent | | Absent | | Sed. + Raw | 2 |
| 3 | 17 | Absent | | Present | fishbones, scale, muscle fibres | Sed. + Flot. | 2 |
| 2 | 39 | Absent | | Present | fishbones, scale, muscle fibres | Sed. + Flot. | 2 |
| 5 | 141 | Present | blood particles (drum dried), hairs | Present | fishbones, scale, muscle fibres | Sed. + Flot. | 2 |
| 6 | 407 | Absent | | Absent | | Sed. + Raw | 2 |
| 4 | 523 | Present | blood particles (spray dried) | Absent | | Sed. + Flot. | 2 |

*sample N1 we found milk powder and vitamin B2 particles. sample N407 we found plasma powder*

Laboratory identification code : **35**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 101 | Present | bone fragments | Present | thorns and scales | Sed. + Flot. | 2 |
| 4 | 115 | Present | spray blood | Absent | | Sed. + Flot. | 2 |
| 2 | 123 | Present | bone fragments, muscle and jelly | Absent | | Sed. + Flot. | 2 |
| 5 | 309 | Present | bone fragments and hairs | Absent | | Sed. + Flot. | 2 |
| 1 | 373 | Present | plasma | Absent | | Sed. + Flot. | 2 |
| 6 | 635 | Present | plasma | Absent | | Sed. + Flot. | 2 |

*sample 101 contains unusual bone fragments*

Laboratory identification code : **38**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 4 | 19 | Present | spray blood | Absent | | Sed. + Flot. | 1 |
| 1 | 265 | Present | feathers | Absent | | Sed. + Flot. | 2 |
| 2 | 315 | Absent | | Present | bones, scales | Sed. + Flot. | 1 |
| 6 | 383 | Absent | | Absent | | Sed. + Flot. | 1 |
| 3 | 461 | Present | bones, feathers | Present | bones, scales, otoliths, gill | Sed. + Flot. | 1 |
| 5 | 537 | Present | feathers, hairs | Present | bones, scales | Sed. + Flot. | 1 |

Laboratory identification code : **39**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 3 | 341 | Present | Bones | Present | fishbones | Sed. + Flot. | 1 |
| 2 | 363 | < LOD | bones | Present | fishbones | Sed. + Flot. | 1 |
| 4 | 511 | Absent | | < LOD | fishbones | Sed. + Flot. | 1 |
| 5 | 249 | < LOD | bones | Absent | | Sed. + Flot. | 1 |
| 1 | 241 | Absent | | Absent | | Sed. + Flot. | 1 |
| 6 | 431 | Absent | | Absent | | Sed. + Flot. | 1 |

Laboratory identification code : **40**

| Sample type | Sample N° | Terrestrial animal part. | Details of terrestrial part. | Fish part. | Details of fish part. | Fractions used | Number of determinations |
|---|---|---|---|---|---|---|---|
| 5 | 69 | Present | Bone, blood | Absent | | Sed. + Raw | 1 |
| 6 | 275 | Absent | | Absent | | Sed. + Raw | 1 |
| 3 | 293 | Present | Bone, muscle fibres | Present | Bone, scale, otolith, cartilage, gill, muscle fibres. | Sed. + Raw | 1 |
| 4 | 391 | Present | Blood | Absent | | Sed. + Raw | 1 |
| 1 | 613 | Absent | | Absent | | Sed. + Raw | 1 |
| 2 | 615 | Absent | | Present | Bone, otolith, gill, cartilage, muscle fibres. | Sed. + Raw | 1 |

*Blood seen in sample no 391, PCR result indicates from non-ruminant source. Milk seen in sample 275 and in sample 613, PCR result indicates both from a ruminant sou*

# Annex 5

Gross results of participants for PCR (in numerical order of lab ID)

Laboratory identification code : **1**

| | |
|---|---|
| Cut-off at 15 copies : | 34,23 cycles |
| Copy number at the cut-off : | 11,08 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 5 | 9 | Present | |
| 1 | 385 | Present | |
| 2 | 435 | Absent | |
| 3 | 485 | Absent | PCR inhibition, more dilutions were needed |
| 6 | 563 | Present | |
| 4 | 655 | Absent | |

Laboratory identification code : **2**

| | |
|---|---|
| Cut-off at 15 copies : | 37,84 cycles |
| Copy number at the cut-off : | 9,37 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 61 | Present | |
| 6 | 179 | Present | |
| 3 | 305 | Absent | |
| 5 | 417 | Present | PCR inhibition when sample run without dilution |
| 2 | 483 | Absent | |
| 4 | 631 | Absent | |

Laboratory identification code : **3**

| | |
|---|---|
| Cut-off at 15 copies : | 34,26 cycles |
| Copy number at the cut-off : | 9,43 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 253 | Present | |
| 3 | 497 | Absent | |
| 2 | 519 | Absent | |
| 6 | 611 | Present | |
| 4 | 619 | Absent | |
| 5 | 633 | Present | |

Laboratory identification code : **4**

| | |
|---|---|
| Cut-off at 15 copies : | 33,76 cycles |
| Copy number at the cut-off : | 10,30 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 2 | 51 | Absent | |
| 4 | 307 | Absent | |
| 6 | 395 | Present | |
| 5 | 453 | Present | |
| 1 | 481 | Present | |
| 3 | 557 | Absent | |

Laboratory identification code : **5**

| | |
|---|---|
| Cut-off at 15 copies : | 35,76 cycles |
| Copy number at the cut-off : | 9,09 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 2 | 3 | Absent | |
| 6 | 95 | Present | |
| 5 | 129 | Present | |
| 4 | 187 | Absent | |
| 3 | 473 | Absent | |
| 1 | 553 | Present | |

Laboratory identification code : **6**

| | |
|---|---|
| Cut-off at 15 copies : | 34,20 cycles |
| Copy number at the cut-off : | 10,44 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 119 | Present | |
| 4 | 151 | Absent | inhibition |
| 5 | 153 | Present | |
| 2 | 171 | Absent | |
| 3 | 221 | Absent | inhibition |
| 1 | 469 | Present | |

Laboratory identification code : **7**

| | |
|---|---|
| Cut-off at 15 copies : | 34,30 cycles |
| Copy number at the cut-off : | 9,26 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 37 | Present | PCR inhibition, but very positive |
| 4 | 43 | Absent | |
| 5 | 323 | Absent | |
| 2 | 531 | Absent | |
| 3 | 581 | Absent | |
| 1 | 597 | Present | |

Laboratory identification code : **8**

| | |
|---|---|
| Cut-off at 15 copies : | 34,27 cycles |
| Copy number at the cut-off : | 11,91 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 11 | Absent | It should have crossreactivity with porcine DNA in ruminant test |
| 5 | 273 | Present | |
| 1 | 349 | Present | |
| 3 | 425 | Absent | |
| 4 | 487 | Absent | |
| 2 | 591 | Absent | |

Laboratory identification code : **9**

| | |
|---|---|
| Cut-off at 15 copies : | 31,91 cycles |
| Copy number at the cut-off : | 10,32 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 97 | Present | |
| 6 | 143 | Present | |
| 4 | 163 | Absent | |
| 2 | 291 | Absent | |
| 5 | 573 | Present | |
| 3 | 641 | Absent | No signal at all |

Laboratory identification code : **10**

| | |
|---|---|
| Cut-off at 15 copies : | 36,79 cycles |
| Copy number at the cut-off : | 9,77 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 3 | 5 | Absent | |
| 2 | 207 | Absent | |
| 4 | 403 | Absent | DNA extraction and PCR was repeated, because of inconsistent result on first determination |
| 6 | 515 | Present | |
| 1 | 529 | Present | |
| 5 | 549 | Present | |

Laboratory identification code : **11**

| | |
|---|---|
| Cut-off at 15 copies : | 35,30 cycles |
| Copy number at the cut-off : | 11,00 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 2 | 63 | Absent | |
| 6 | 167 | Present | |
| 3 | 209 | Absent | little PCR inhibition in replicate 2 |
| 4 | 331 | Absent | |
| 1 | 433 | Present | |
| 5 | 657 | Present | |

Laboratory identification code : **12**

| | |
|---|---|
| Cut-off at 15 copies : | 35,34 cycles |
| Copy number at the cut-off : | 11,00 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 2 | 195 | Absent | |
| 6 | 215 | Present | |
| 4 | 223 | Absent | |
| 3 | 353 | Absent | |
| 5 | 429 | Present | |
| 1 | 493 | Present | |

Laboratory identification code : **13**

| | |
|---|---|
| Cut-off at 15 copies : | 36,17 cycles |
| Copy number at the cut-off : | 9,04 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 2 | 203 | Present | |
| 6 | 301 | Present | |
| 4 | 439 | Absent | |
| 3 | 449 | Absent | |
| 5 | 489 | Present | |
| 1 | 639 | Absent | |

Laboratory identification code : **14**

| | |
|---|---|
| Cut-off at 15 copies : | 35,23 cycles |
| Copy number at the cut-off : | 9,10 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 5 | 33 | Present | |
| 1 | 157 | Present | |
| 4 | 175 | Absent | |
| 3 | 365 | Absent | |
| 6 | 467 | Present | |
| 2 | 567 | Absent | |

Laboratory identification code : **15**

| | |
|---|---|
| Cut-off at 15 copies : | 35,07 cycles |
| Copy number at the cut-off : | 9,43 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 47 | Present | |
| 4 | 139 | Absent | |
| 2 | 147 | Absent | |
| 3 | 185 | Absent | |
| 5 | 285 | Present | |
| 1 | 325 | Present | |

Laboratory identification code : **16**

| | |
|---|---|
| Cut-off at 15 copies : | 35,92 cycles |
| Copy number at the cut-off : | 10,76 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 5 | 237 | Present | |
| 6 | 443 | Present | |
| 3 | 533 | Absent | |
| 2 | 555 | | We apologize for sample 555, as we obtained an inconsistent result after the first isolation and RT-PCR reaction. As indicated in the SOP, RT-PCR was repeated first, but obtained again as in the first PCR reaction result for dilution 1 (replicate 1 - / replicate 2 +) and for dilution 2 (replicate 1 - / replicate 2 -). Therefore, we decided to repeat the DNA extraction, but we could not perform the PCR reaction because our device broke down last Thursday. To date, we have not been able to perform a PCR test. There is a high probability that the result is negative, but since we do not have a result, we leave this field blank. As soon as the device is repaired, we will perform a test and send you the result for this sample as well. |
| 4 | 607 | Absent | |
| 1 | 625 | Present | |

Laboratory identification code : **17**

| | |
|---|---|
| Cut-off at 15 copies : | 36,86 cycles |
| Copy number at the cut-off : | 10,76 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 59 | Present | |
| 3 | 77 | Absent | |
| 2 | 243 | Absent | |
| 4 | 343 | Absent | |
| 5 | 357 | Present | |
| 1 | 397 | Present | |

Laboratory identification code : **18**

| | |
|---|---|
| Cut-off at 15 copies : | 32,19 cycles |
| Copy number at the cut-off : | 11,45 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 25 | Present | |
| 3 | 327 | Absent | no pcr inhibition : Ct of sample+40cp =31.92 and 32.00 |
| 2 | 451 | Absent | no pcr inhibition : Ct of sample+40cp =31.13 and 31.37 |
| 4 | 561 | Present | |
| 5 | 587 | Present | |
| 1 | 593 | Absent | no pcr inhibition : Ct of 10X sample + 40cp = 30.97 and 31.25 |

Laboratory identification code : **19**

| | |
|---|---|
| Cut-off at 15 copies : | 37,32 cycles |
| Copy number at the cut-off : | 10,00 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 109 | Present | |
| 5 | 189 | Present | |
| 3 | 401 | Absent | significant inhibition; 10-fold dilution undetermined too |
| 2 | 447 | Absent | |
| 6 | 455 | Present | |
| 4 | 559 | Absent | |

Laboratory identification code : **20**

| | |
|---|---|
| Cut-off at 15 copies : | 35,98 cycles |
| Copy number at the cut-off : | 11,18 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 3 | 89 | Absent | PCR inhibition, positive signal inhibition control at 30x dilution, result sample 30x dilution: no signal |
| 5 | 93 | Present | |
| 4 | 199 | Absent | |
| 6 | 251 | Present | |
| 1 | 409 | Present | |
| 2 | 459 | Absent | |

Laboratory identification code : **21**

| | |
|---|---|
| Cut-off at 15 copies : | 36,13 cycles |
| Copy number at the cut-off : | 11,24 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 4 | 67 | Absent | low inhibition |
| 1 | 169 | Present | no inhibition |
| 2 | 303 | Absent | low inhibition |
| 5 | 405 | Present | no inhibition |
| 6 | 419 | Absent | low inhibition |
| 3 | 605 | Absent | at 1F: inhibition. At 10F: partial |

Laboratory identification code : **22**

| | |
|---|---|
| Cut-off at 15 copies : | 35,99 cycles |
| Copy number at the cut-off : | 11,72 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 3 | 53 | Absent | undiluted DNA caused inhibition. DNA tested at 10fold, 20fold and also 40fold and 80fold dilutions. |
| 4 | 79 | Absent | |
| 2 | 183 | Absent | |
| 6 | 263 | Present | |
| 5 | 297 | Present | |
| 1 | 601 | Present | |

Laboratory identification code : **23**

| | |
|---|---|
| Cut-off at 15 copies : | 38,05 cycles |
| Copy number at the cut-off : | 11,22 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 4 | 55 | Absent | |
| 1 | 361 | Present | |
| 2 | 387 | Absent | |
| 5 | 609 | Present | |
| 3 | 617 | Absent | |
| 6 | 659 | Present | |

Laboratory identification code : **24**

| | |
|---|---|
| Cut-off at 15 copies : | 36,90 cycles |
| Copy number at the cut-off : | 11,41 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 23 | Present | 2021-1905 |
| 4 | 91 | Absent | 2021-1906 |
| 3 | 245 | Absent | 2021-1907 PCR inhibition (new extract 15/11/21) |
| 2 | 267 | Absent | 2021-1908 |
| 5 | 369 | Present | 2021-1909 |
| 1 | 541 | Present | 2021-1910 |

Laboratory identification code : **25**

| | |
|---|---|
| Cut-off at 15 copies : | 34,49 cycles |
| Copy number at the cut-off : | 10,20 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 6 | 73 | Present | |
| 4 | 317 | Absent | replicate 1 and 2 have inhibition |
| 3 | 351 | Absent | |
| 2 | 415 | Absent | |
| 5 | 539 | Absent | |
| 1 | 645 | Present | |

Laboratory identification code : **26**

| | |
|---|---|
| Cut-off at 15 copies : | 31,80 cycles |
| Copy number at the cut-off : | 11,44 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 85 | Present | |
| 3 | 149 | Absent | |
| 5 | 345 | Present | |
| 4 | 535 | Absent | |
| 2 | 579 | Absent | |
| 6 | 623 | Present | |

Laboratory identification code : **27**

| | |
|---|---|
| Cut-off at 15 copies : | 37,59 cycles |
| Copy number at the cut-off : | 9,66 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 1 | 277 | Present | |
| 3 | 281 | Absent | PCR inhibition determined, and after several dilutions the presence of ruminant DNA was not detected. |
| 4 | 283 | Absent | |
| 5 | 393 | Present | |
| 6 | 491 | Present | |
| 2 | 507 | Absent | |

Laboratory identification code : **31**

| | |
|---|---|
| Cut-off at 15 copies : | 37,03 cycles |
| Copy number at the cut-off : | 11,30 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 5 | 81 | Present | |
| 6 | 131 | Present | |
| 1 | 181 | Present | |
| 4 | 211 | Absent | |
| 3 | 389 | Absent | |
| 2 | 423 | Present | |

| Laboratory identification code : | **35** |
|---|---|

| | |
|---|---|
| Cut-off at 15 copies : | 30,00 cycles |
| Copy number at the cut-off : | 35,00 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 3 | **101** | Absent | |
| 4 | **115** | Absent | porcine species presence |
| 2 | **123** | Absent | |
| 5 | **309** | Present | bovine, porcine and chicken species presence |
| 1 | **373** | Present | bovine species presence |
| 6 | **635** | Absent | porcine species presence |

| Laboratory identification code : | **40** |
|---|---|

| | |
|---|---|
| Cut-off at 15 copies : | 37,77 cycles |
| Copy number at the cut-off : | 9,22 copies |

| Sample type | Sample N° | Ruminant DNA | Comment |
|---|---|---|---|
| 5 | **69** | Present | No inbition observed |
| 6 | **275** | Present | No inbition observed |
| 3 | **293** | Absent | No inbition observed |
| 4 | **391** | Absent | No inbition observed |
| 1 | **613** | Present | No inbition observed |
| 2 | **615** | Absent | No inbition observed |