Combined microscopy-PCR EURL-AP Proficiency Test 2016

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Wallonie

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 Commission Regulation EU/51/2013. It is the second time that the proficiency of the NRLs is assessed for the two methods through a single study. It allows also evaluating the process of the sample management within the labs.

The total number of participants was 32 (27 NRLs and 5 labs outside the NRL network). The study was based on a set of eight blind samples (all to be analysed by light microscopy while five of them were to be analysed by PCR) consisting of blank feed matrices or feeds fortified with terrestrial processed animal proteins and/or fishmeal or contaminated feed sent to the participants.

All participants provided their results on due time. Each participant received after the closure of the results an individual table giving them an immediate feedback of their results.

Regarding the detection of PAP by light microscopy the overall results indicate an excellent level of global performance for 71 % of the NRLs, a satisfying level of global performance for 7 % of the NRLs and an underperforming level of global performance for 22 % of the NRLs. The sample set composition highlighted the difficulty of detecting feathers at low levels of adulteration as well as a general misidentification of shell grits from bivalves being confused with fish particles. The PCR results reflect also an excellent level of performance. 96.3 % of the 27 NRLs had no false result. The remaining lab (3.7 %) obtained a satisfactory level of performance by providing only one incorrect result.

Keywords :

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

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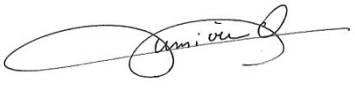
ISO 17043 coordinators signature for approval:

Olivier Fumière *Pascal Veys*



Wallonie

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 29th April 2004, the European Parliament and the Council adopted Regulation EC/882/2004 [1], improving the effectiveness of the official food and feed controls while redefining the obligations of the relevant authorities and their obligations in the organization of these controls.

On 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, http://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 since 2006 yearly proficiency tests for the assessment of the implementation of the reference methods for the detection of animal proteins in feed as described by Commission Regulation EU/51/2013 [3] amending Annex VI of Commission Regulation EC/152/2009 [4]. The present study report is part of this activity scope.

2. Introduction

According to modified Annex VI of Commission Regulation EC/152/2009 [4] official controls for the detection of animal proteins in feed inside the EU have been performed by light microscopy and/or PCR since June 2013.

The objective of the present proficiency test is strictly to evaluate the performance of the network of 27 NRLs to detect the presence of processed animal proteins in feed by light microscopy and PCR. Their participation 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/125/2009 was not imposed to them.

3. Material and methods

3.1. Study organisation

Twenty seven NRLs and five laboratories outside this EU network participated to the study. A detailed list of the 32 participating labs is included in Annex 1.

Official announcement (Annex 2) of the study was made on the 30th September 2016 to all participants.

On the 7th November 2016, 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 do not have access to this intranet.

The deadline for the delivery of the results was fixed in the announcement and in the instructions at the 9th December 2016.

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. Only when both the Excel file and a copy of the summarized results sheet were received by the EURL-AP were results taken into consideration.
- Participants were notified that results arriving later would not be accepted.

All participants delivered their results on time. On the exception of two non-EU participant who only sent microscopic results, all other participants returned results for both microscopic and PCR analyses. The proficiencies of NRLs and other participants were analysed separately in this report.

3.2. Material

3.2.1. Description of the samples

Eight different test materials were prepared for the proficiency test.

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

- Use of fishfeeds as matrices for assessing the detection capabilities of terrestrial PAPs because since the 1st June 2013 non-ruminant PAPS are authorized in aquafeed according to Commission Regulation EU/56/2013 [5].
- Use of feed matrices containing or adulterated with microscopically almost undetectable materials from terrestrial origin (ruminant plasma and milk powder) able to deliver positive responses for ruminant DNA by PCR.

Each participating lab received about 50g for each of the eight blind samples to which a unique random number was assigned. Details of the samples are indicated in Table 1.

Table 1: Composition of the blind sample set used.

Colour code	Sample	Material	Nr of replicates	Expected results * Microscopy: Terrestrial particles	Microscopy: Fish particles	PCR: Ruminant DNA
	1	Feed I	1	-	-	
	2	Feed I + 0.1% feather meal	1	+	-	
	3	Feed II	1	-	-	
	4	Fishfeed I	1	-	+	-
	5	Fishfeed I + 0.05 % ruminant PAP	1	+	+	+
	6	Fishfeed I + 1 % porcine PAP	1	+	+	-
	7	Fishfeed I + 3 % bovine plasma	1	-	+	+
	8	Fishfeed II + 0.1 % milk powder	1	-	+	+
	Total		**8**	**3**	**5**	**3**

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

The 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.

For avoiding ambiguities colour labels, stuck on the vials, were used to indicate which method or method combination had to be used. As mentioned in Table 1, the 3 first samples were intended to be analysed by light microscopy only (red code); the 5 following ones had to be analysed both by light microscopy and PCR (green code).

3.2.2. Materials used in the preparation of the samples

Four matrices were used:

- Feed I was a **compound feed for lay hens** bought from a local producer. It was composed of maize, wheat, roasted soy, peas, sorghum, lin seeds, barley, sunflower seeds, shell grit, spinach seeds, hulled oat, paddy rice and feed complements (salts, vitamins, minerals). Its sediment content was of 4.6 %. This feed was used for preparing samples 1 and 2.
- Feed II was a **compound feed for pigs**. It consisted of wheat, maize, barley, wheat gluten, wheat hull, rapeseed cake, palm cake, soybean cake, peas, sunflower cake, pig fat, calcium carbonate, vitamins, minerals, feed additives and antioxidants. The sediment content of the mixture was about 1.8 %. It was used for preparing sample 3.
- Fishfeed I was a **complete feed for fry**. It consisted of fishmeal, corn starch, fish oil, wheat gluten, protein concentrate from peas and feed additives (vitamins, salts, minerals and antioxidants). Its sediment was of 0.9 %. It was used for preparing samples 4, 5, 6 and 7.
- Fishfeed II was a **compound feed** made of fishmeal, soya meal, fish oil, wheat gluten, maize gluten, rapeseed, sunflower seeds, protein concentrate from soya, rapeseed oil, vitamins and minerals. Its percentage of sediment was of 2 %. This feed was used for sample 8 only.

Adulterant material used:

- A **non-hydrolysed feather meal** was used for preparing sample 2. This material was used after removing of the bones for a proficiency test in 2008 [6]. This feather meal had a low sediment content of about 0.1 %. It contained some few bone particles as well as muscle fibres, always more than 5 per analysis.
- A **pure bovine PAP** was used for preparing sample 5. This PAP, already used in 2015 [7], was produced by a pilot plant. Its bone content reached 52.4 %. Its purity was controlled by microscopy and PCR. By PCR the mean Ct values for ruminant PCR test was about 20 cycles corresponding to an estimated copy number of 850 000 copies. Some traces of porcine and poultry DNA were however detected.

- A **pure porcine PAP** was used for preparing sample 6. This PAP was used in 2015 [7]. Its bone content was of about 14.0 % and its purity was checked by microscopy and PCR.
- A **bovine plasma powder** was used for sample 7. It was free from sediment. PCR analyses revealed it from ruminant origin and free from porcine DNA.
- A **skimmed milk powder** was used for sample 8. PCR analyses proved it only to be from bovine origin.

3.2.3. Description of the mixing procedures

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 the vials.

All matrices were ground at 2 mm before any other treatment.

Adulteration of samples 5 and 6 was performed by successive dilutions.

Samples 2 and 7 were directly spiked with the adulterant.

For sample 8, six sub-portions of 500 g were prepared by direct spiking with the adulterant. All sub-portions were thoroughly homogenised by mixing during 1 hour prior to be ground. After grinding, the sub-portions were pooled and homogenised by mixing during 1 hour.

3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [8].

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/51/2013 [3] amending regulation EC/152/2009 [4]:

- 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. 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 the 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 [8].

3.4.1. Light microscopy

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

Samples 1 and 2 are based on a poultry feed matrix containing shell grits. Participants that would have identified these shell fragments and for this reason would interpret them as positive for fish should not be penalised. In such situation the results would have to be assimilated to a correct negative assignment. Indeed, according to Commission Regulation EC/1069/2009 [9] this type of ingredient is not subject to animal by-product regulation since soft tissues and flesh are removed. In case shellfish soft tissues are processed then they have to be included in the animal by-product regulation and categorised as processed animal proteins from aquatic origin, or fishmeal according to Commission Regulation EU/142/2011 [10]. This ambiguity is motivating this decision. On the other hand, participants that would have declared these samples as positive for fish due to erroneous identifications of fish bones, scales or gill fragments were delivering false positive results for fish.

Samples 7 and 8, respectively with the addition of bovine plasma and skimmed milk powders, are considered to be declared negative for terrestrial particles as these two ingredients are hardly visible by microscopic investigation. Nevertheless participants that would be able to disclose plasma particles and milk and would therefore declare the sample as positive for terrestrial material (as these types of product are not known to be obtained from fish) should logically not be penalised, therefore such results would have to be assimilated to a correct negative assignment.

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, i.e. having no more than 1 wrong result.
- **Satisfying** level of global performance = consolidated AC below 0.90 and having no more than 2 wrong results including a maximum of 1 ND for terrestrial material.
- **Underperforming** level of global performance = consolidated AC below 0.90 and having more than 2 wrong results –or 2 ND for terrestrial material.

3.4.2.PCR

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

Samples 4 and 6 are considered to be declared negative for the presence of ruminant DNA. Samples 5, 7 and 8 contain an ingredient from ruminant origin (ruminant PAP, bovine plasma and milk powder respectively) and must be declared positive for the presence of ruminant DNA. The ruminant PAP content of sample 6 (Fishfeed + 0.05 % ruminant PAP) is below 0.1 %. The method is usually sensitive enough to detect the presence of ruminant DNA in that sample but it can be considered as a more challenging sample for the participants.

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 2 wrong results and a maximum of 1 ND or 1 PD for the detection of ruminant DNA.
- **Underperforming** level of global performance = more than 2 wrong results or 2 ND or 2 PD 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			NIRM		PCR			
		Nr of replicates	Terrestrial	Fish	Nr of replicates	Animal	Nr of replicates	Ruminant	Porcine	Fish
1	Feed I	10	-	-	5	-	/	/	/	/
2	Feed I + 0.1% feather meal	10	+	-	5	-	/	/	/	/
3	Feed II	10	-	-	5	-	/	/	/	/
4	Fishfeed I	10	-	+	5	+	10	-	/	+
5	Fishfeed I + 0.05 % ruminant PAP	10	+	+	5	+	10	+	/	+
6	Fishfeed I + 1 % porcine PAP	10	+	+	5	+	10	-	+	+
7	Fishfeed I + 3% bovine plasma	10	-	+	5	+	10	+	/	+
8	Fishfeed II + 0.1 % milk powder	10	-	+	5	+	10	+	/	+

(Legend: blank cells = not tested, + = systematically detected, - = systematically not detected, NIRM = near infrared microscopy)

The homogeneity was studied by light microscopy on 10 g of sample material for each replicate. Analyses of replicates were performed following strictly EC/152/2009. For PCR analysis of each replicate a double extraction was performed on 100 mg of sample material. Near infrared microscopy has also been performed on sediments of the samples and materials used for this study in complement to the official methods. For each sample 400 spectra were recorded on a fraction of 5 different sediments.

Sample 1 (Feed I) was systematically negative for any vertebrate particle traces; no muscles were detected. All slides from the sediments contained shell fragments.

Sample 2 (Feed I + 0.1 % feather meal) revealed to be always positive for terrestrial particles and always negative for fish. In terms of terrestrial particles, feathers fragments were always identified in large numbers. Some few bone fragments were also observed (5 on a total of 60 slides). All slides from the sediments contained shell fragments.

Sample 3 (Feed II) was systematically negative for fish particles and terrestrial particles.

Sample 4 (Fishfeed I) was always negative for presence of terrestrial particles but always positive for fish particles. PCR revealed the sample as positive for fish DNA and negative for ruminant DNA.

Sample 5 (Fishfeed I + 0.05 % ruminant PAP) was always positive for fish presence and for terrestrial particles. PCR analyses revealed the sample as positive for both fish and ruminant DNA.

Sample 6 (Fishfeed I + 1 % porcine PAP) was always found positive for both terrestrial and fish particles. PCR analyses showed the sample as always negative for ruminant DNA and always positive for porcine and fish DNA.

Sample 7 (Fishfeed I + 3 % bovine plasma) was always positive for fish particle presence and negative for terrestrial particles. Plasma powder was not visualised. By PCR, results were always positive for ruminant and fish DNA.

Sample 8 (Fishfeed II + 0.1% milk powder) was found to be always positive for fish particles and negative for terrestrial particles by microscopy. PCR analyses led to positive results for ruminant and fish DNA.

Near infrared microscopy analyses performed on the sediment did not reveal inconsistencies in the materials used and the samples prepared this considering the adulteration levels.

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 Annex 4 and 5 respectively.

4.1. Microscopy results

4.1.1. Qualitative analyses from the NRLs

4.1.1.1. On the respect of the instructions

NRLs respected the instructions related to the proficiency test itself.

Regarding the respect of EU regulation, remarks made over the last 2 years on the respect of the number of determinations [7, 11] did not improve the fact that still some laboratories (6 in totals) do not respect the diagrams. Most of them concerned correctly identified negative results for both terrestrial and fish presence (Sample 1 and 3) but based on an unauthorised number of determinations, i.e. two instead of a single. This represents only 6 % of unnecessary repetitions over the total number of results (in 2015, 4 % and in 2014, 8 %).

In addition EU regulation was not followed by some NRLs as it imposes a minimum of two determinations before using the "<LOD" result expression: Lab 16 and 6 declared cases of "<LOD" based on a single determinations which fortunately did not lead to an erroneous conclusion.

4.1.1.2. Results and performance of the network

Table 3 summarizes the results submitted by the 27 NRLs for the eight sample types submitted to qualitative analysis.

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

Sample	Material	n	AC	
			Terrestrial	Fish
1	Feed I	27	0.963 (1)	0.704 (8)
2	Feed I + 0.1 % feather meal	27	0.519 (13)	0.741 (7)
3	Feed II	27	1.000	1.000
4	Fishfeed I	27	1.000	1.000
5	Fishfeed I + 0.05 % ruminant PAP	27	1.000	1.000
6	Fishfeed I + 1 % porcine PAP	27	1.000	1.000
7	Fishfeed I + 3 % bovine plasma	27	0.926 (2)	1.000
8	Fishfeed II + 0.1% milk powder	27	0.926 (2)	1.000

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

The overall results, expressed in terms of global accuracy (AC) reveal the excellence of the network of NRLs for PAP detection in fishfeed. Fish material is systematically detected when it must, illustrating a perfect sensitivity score. The sensitivity for the detection of terrestrial animal particles is faultless in these fishfeed matrices. Some few false positive results are however detected on sample 7 and 8. These cases are commented in the next section.

Specificity and sensitivity scores are also perfect for sample 3 consisting of a compound feed for pig.

Results obtained from the two samples based on the compound feed for laying hens (samples 1 and 2) are less optimal. Whether this matrix was adulterated or not it gave rise to a large number of false positive results for fish material. Explanations for this specificity issue are commented in the next section. Sample 2 also illustrates a problem of sensitivity related to the disclosure of terrestrial particles when feather meal is introduced in this matrix. For reminder this feather meal was not a hydrolysed product.

4.1.1.3. Detailed review of results per sample

Sample 1: Feed I

PD for terrestrial particles:

- Lab 23 : feathers detected*

Some <LOD cases were also reported. Lab 6 identified insect particles but less than 5 and only based on a single determination. Lab 15 declared to have seen less than 10 feather fragments (as from 2 determinations).

PD for fish particles

- Lab 1: otolith reported
- Lab 2: fishbones and scales
- Labs 3 and 16: otolith, fishbones
- Lab 10: otoliths, fishbones and muscle fibres
- Lab 11: scales, gills and muscles
- Labs 19 and 22: fishbones

This high number of false positive detection of fish particles is revealing the confusion that can be made by participants between fish material and mollusc material (shell grits in the present case). A detailed discussion on this topic is provided in the conclusion.

Some NRLs were able to identify properly shell fragments (labs 3, 14, 15 and 26). Two NRLs (labs 5 and 9) also mentioned having identified shell fragments in their accompanying email. They represent only 22 % of the NRLs. Based on this sole reason, two participants declared the sample as positive for fish. As referred in the performance criteria section this decision could not be penalized.

Sample 2: Feed I + 0.1 % feather meal

ND for terrestrial particles:

- Labs 4, 8, 11, 14, 16, 18, 19, 22 and 23*.

Some labs also reported terrestrial particles but at a level <LOD (labs 1, 21, 25 and 26). From these 4 participants 3 out of them detected feathers but in reduced numbers.

PD for fish particles

- Lab 1: otolith reported
- Lab 2: fishbones
- Lab 3: fishbones, otolith
- Lab 11: scales, gills and muscles
- Labs 16 and 19: fishbones and scales
- Lab 22: fishbones

The NRLs that were able to identify properly shell fragments in sample 1 also successfully identified them in sample 2. Once again, based on this sole reason, two participants declared the sample as positive for fish. As referred in the performance criteria section this decision could not be penalized.

A correlation was observed, four laboratories that misidentified fish particles were also ineffective in finding feather material (labs 11, 16, 19 and 22).

Sample 3: Feed II

No errors were noted.

Sample 4: Fishfeed I

* Lab 23 showed evidence for a reporting error, referring to sample 2 instead of sample 1.

No errors were noted. It has to be noted that Lab 19 identified fragments from mollusc origin.

Sample 5: Fishfeed I + 0.05 % ruminant PAP

No errors were noted.

Sample 6: Fishfeed I + 1 % porcine PAP

No errors were noted.

Sample 7: Fishfeed I + 3 % bovine plasma

PD for terrestrial particles:

- Lab 1: muscles and hairs
- Lab 3: bones

Two <LOD cases were reported. Lab 16 identified less than 5 bones but only based on a single determination. Lab 21 indicated the sample was possibly containing milk powder but did not mention plasma powder.

The presence of bovine plasma is hardly detectable by light microscopy, even at a level of 3 %, for this reason according the performance criteria the sample had to be considered as negative for terrestrial particles. No participant was able to detect it.

Sample 8: Fishfeed II + 0.1 % milk powder

PD for terrestrial particles:

- Lab 1: muscles and hairs
- Lab 3: bones

Five <LOD cases were reported. Lab 4 identified 3 bones but did not mention the number of determinations. Labs 8 and 15 declared less than 10 bones as based on two determinations. Lab 21 indicated the sample was possibly containing milk powder after three determinations. Lab 24 observed less than 10 particles identified as bones, muscle fibres and cartilage fragments from two determinations.

The presence of milk powder is difficult to determine by light microscopy, therefore the sample had to be considered as negative for terrestrial. A single participant, lab 21, suspected it could contain milk powder.

Interestingly labs 1 and 3 reported exactly the same observations made on sample 7 although the compound feeds of the matrices were not the same.

4.1.1.4. 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 material and of fish material. Results are to be found in Tables 4 and 5. 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
2	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
9	1.000	1.000	1.000
10	1.000	1.000	1.000
12	1.000	1.000	1.000
15	1.000	1.000	1.000
17	1.000	1.000	1.000
20	1.000	1.000	1.000
24	1.000	1.000	1.000
27	1.000	1.000	1.000
28	1.000	1.000	1.000
4	0.875	0.667	1.000
8	0.875	0.667	1.000
11	0.875	0.667	1.000
14	0.875	0.667	1.000
16	0.875	0.667	1.000
18	0.875	0.667	1.000
19	0.875	0.667	1.000
21	0.875	0.667	1.000
22	0.875	0.667	1.000
25	0.875	0.667	1.000
26	0.875	0.667	1.000
3	0.750	1.000	0.600
23	0.750	0.667	0.800
1	0.625	0.667	0.600

Fish			
lab code	AC	SE	SP
4	1.000	1.000	1.000
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
8	1.000	1.000	1.000
9	1.000	1.000	1.000
12	1.000	1.000	1.000
14	1.000	1.000	1.000
15	1.000	1.000	1.000
17	1.000	1.000	1.000
18	1.000	1.000	1.000
20	1.000	1.000	1.000
21	1.000	1.000	1.000
23	1.000	1.000	1.000
24	1.000	1.000	1.000
25	1.000	1.000	1.000
26	1.000	1.000	1.000
27	1.000	1.000	1.000
28	1.000	1.000	1.000
10	0.875	1.000	0.667
1	0.750	1.000	0.333
2	0.750	1.000	0.333
3	0.750	1.000	0.333
11	0.750	1.000	0.333
16	0.750	1.000	0.333
19	0.750	1.000	0.333
22	0.750	1.000	0.333

Details of the results were commented in section 4.1.1.3.

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

19 labs out of 27 NRLs or in other words 70 % of the NRLs performed very well (2015 : 93 % [7], 2014 : 78 % [11] and 2013 : 63 % [12]). This is quite less than the two last years. Two NRLs performed satisfyingly.

Six NRLs were classified according the ranking criteria as underperforming for the present proficiency test. These labs require improvement of proficiency. In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), these underperforming participants are asked to report on the origin of their multiple errors as well as on the actions they will undertake in order to solve the problems.

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 blue refer to satisfying NRLs, lines in red refer to underperforming NRLs.

Consolidated			
lab code	AC	SE	SP
5	1.000	1.000	1.000
6	1.000	1.000	1.000
7	1.000	1.000	1.000
9	1.000	1.000	1.000
12	1.000	1.000	1.000
15	1.000	1.000	1.000
17	1.000	1.000	1.000
20	1.000	1.000	1.000
24	1.000	1.000	1.000
27	1.000	1.000	1.000
28	1.000	1.000	1.000
10	0.938	1.000	0.875
4	0.938	0.875	1.000
8	0.938	0.875	1.000
14	0.938	0.875	1.000
18	0.938	0.875	1.000
21	0.938	0.875	1.000
25	0.938	0.875	1.000
26	0.938	0.875	1.000
2	0.875	1.000	0.750
23	0.875	0.875	0.875
11	0.813	0.875	0.750
16	0.813	0.875	0.750
19	0.813	0.875	0.750
22	0.813	0.875	0.750
3	0.750	1.000	0.500
1	0.688	0.875	0.500

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

Individual performances from the 5 participants outside the EU were assessed exactly as in previous section (4.1.1.4). 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.

Terrestrial			
lab code	AC	SE	SP
30	0.875	0.667	1.000
32	0.875	0.667	1.000
31	0.625	0.667	0.600
33	0.500	0.333	0.600
34	0.500	0.333	0.600

Fish			
lab code	AC	SE	SP
32	1.000	1.000	1.000
33	1.000	1.000	1.000
34	0.875	1.000	0.667
30	0.750	0.600	1.000
31	0.625	0.600	0.667

The error details are described per sample:

Sample 1: Feed I

Two <LOD cases were reported for fish material (labs 33 and 34).

Sample 2: Feed I + 0.1 % feather meal

ND for terrestrial particles:

- Labs 30, 31, 32, 34

PD for fish particles

- Lab 31: fishbones

A <LOD case was recorded by lab 33.

Sample 3: Feed II

PD for fish particles

- Lab 34: fishbones

Sample 4: Fishfeed I

PD for terrestrial particles:

- Lab 31: bone fragments

A <LOD case was reported by lab 34.

ND for fish particles

- Lab 30

Sample 5: Fishfeed I + 0.05 % ruminant PAP

ND for terrestrial particles:

- Labs 33

A <LOD case was also reported by lab 34.

ND for fish particles

- Lab 31

Sample 6: Fishfeed I + 1 % porcine PAP

Lab 33 declared having detected terrestrial bones but <LOD.

ND for fish particles

- Labs 30 and 31. However lab 30 reported one particle of fish tooth.

Sample 7: Fishfeed I + 3 % bovine plasma

PD for terrestrial particles:

- Labs 31 and 34: bone fragments

- Lab 34: bones and muscles

Sample 8: Fishfeed II + 0.1 % milk powder

PD for terrestrial particles:

- Labs 33 and 34: bones

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.

Consolidated			
lab code	AC	SE	SP
32	0.938	0.875	1.000
30	0.813	0.625	1.000
33	0.750	0.750	0.750
34	0.688	0.750	0.625
31	0.625	0.625	0.625

One participant performed excellently and another one performed satisfyingly (line in blue in Table 9).

The three 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. Overview of results and global performance of the test

Table 10 summarizes the results provided by the 27 NRLs for the five sample types submitted to qualitative analysis.

Table 10: Global results expressed as accuracy (AC) for the five sample types

Sample	Material	n	AC
4	Fishfeed I	27	0.963 (1)
5	Fishfeed I + 0.05 % ruminant PAP	27	1.000
6	Fishfeed I + 1 % porcine PAP	27	1.000
7	Fishfeed I + 3 % bovine plasma	27	1.000
8	Fishfeed II + 0.1% milk powder	27	1.000

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

For all the samples, the overall results, expressed in terms of global accuracy (AC), are quite good. The occurrence of false positive results is improved (1.9 %) compared to the previous PT whereas the presence of 0.05 % of ruminant PAP is again always detected underlining the correct implementation of the method through the network.

4.2.1.2. Individual performances of NRLs in qualitative analysis

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

Table 11: NRL proficiencies regarding the detection of ruminant material starting from the five samples. Ranking follows AC values. Cells in blue refer to satisfying NRLs.

Ranking	Lab code	AC	SE	SP
1	1	1.000	1.000	1.000
	2	1.000	1.000	1.000
	3	1.000	1.000	1.000
	4	1.000	1.000	1.000
	5	1.000	1.000	1.000
	6	1.000	1.000	1.000
	7	1.000	1.000	1.000
	8	1.000	1.000	1.000
	9	1.000	1.000	1.000
	10	1.000	1.000	1.000
	11	1.000	1.000	1.000
	12	1.000	1.000	1.000
	14	1.000	1.000	1.000
	15	1.000	1.000	1.000
	16	1.000	1.000	1.000
	17	1.000	1.000	1.000
	18	1.000	1.000	1.000
	19	1.000	1.000	1.000
	20	1.000	1.000	1.000
	21	1.000	1.000	1.000
	22	1.000	1.000	1.000
	23	1.000	1.000	1.000
	25	1.000	1.000	1.000
	26	1.000	1.000	1.000
	27	1.000	1.000	1.000
	28	1.000	1.000	1.000
27	24	0.800	1.000	0.500

Table 11 illustrates the excellent level of global performance for 26 labs out of 27 NRLs (96.3 % of the NRLs) having no false result. Only one lab (line in blue in Table 11) out of the 27 (3.7 %) obtained satisfactory level of performance by providing only one incorrect result (1 false positive deviation). No lab was underperforming.

4.2.1.3. 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 the participants reached the minimum criterion of 9.00 copies. The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test is 59.3 % (65.4 % in 2015 [7] ; 70.4 % in 2014 [13] ; 55.6 % in 2013 [14]). The only one positive deviation observed is not due to a cut-off problem.

4.2.2. Qualitative analyses from the non-EU participants

4.2.2.1. Overview of results

There were only three non-EU labs providing results for the five sample types submitted to qualitative PCR analysis. Table 12 summarizes the results provided by the 3 labs for the five sample types submitted to qualitative analysis.

Table 12: Global results of non NRL participants expressed as accuracy (AC) for the five sample types

Sample	Material	n	AC
4	Fishfeed I	3	1.000
5	Fishfeed I + 0.05 % ruminant PAP	3	0.667 (1)
6	Fishfeed I + 1 % porcine PAP	3	1.000
7	Fishfeed I + 3 % bovine plasma	3	1.000
8	Fishfeed II + 0.1 % milk powder	3	1.000

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

4.2.2.2. Individual performances

Individual performances were assessed for each of these participants by calculating the accuracy, sensitivity and specificity over the blind samples. Their results are to be found in Table 13.

Table 13 : Non NRL participant proficiencies regarding the detection of ruminant material starting from the five samples. Ranking follows AC values.

Ranking	Lab code	AC	SE	SP
1	30	1.000	1.000	1.000
	32	1.000	1.000	1.000
3	33	0.800	0.667	1.000

Labs 30 and 32 obtained excellent results (no deviation). Concerning Lab 33, one negative deviation is recorded with the sample 5 (Fishfeed I + 0.05 % ruminant PAP). This participant probably uses another method than the one described in the EURL-AP SOP as no cut-off value nor Ct values were reported. Nevertheless, none of the three participants was underperforming.

4.2.2.3. Assessment of the cut-off values

Lab 33 gave no information about the cut-off value and the Ct values probably indicating the use of another PCR method than the official one in EU. Labs 30 and 32 have cut-off values that comply with the minimum criterion of 9 copies set by the EURL-AP.

5. Discussion and conclusions

Results of the present proficiency test for the detection of PAP by light microscopy show a satisfying level of global performance of the NRL network. Nevertheless the rate of 71 % of NRLs performing excellently is not comparable to the scores obtained for the last two years. The number of underperforming NRLs, representing 22 %, is also the highest since years.

Considering the sample set composition of the study this situation is not alarming since explanation for this can easily be found. The numerous errors originate from two samples only: the first sample, a classical compound feed for laying hens, and the second sample, the same compound feed but this time adulterated with a feather meal at a level of 0.1% w/w.

A first major source of error is linked to the presence of feather meal. Almost half of the NRL participants revealed to be unable to detect the 0.1 % level of terrestrial material fragments with this type of PAP. Only 14 out 27 NLRs (52 %) succeeded in identifying feather particles. However from these 14 NRLs, three did not obtain a number of fragments allowing declaring the sample as positive for terrestrial. Interestingly in 2008 the same feather meal was used as an adulterant at a level of 0.5 % within a compound feed for bovine. At that time 98 % of correct detection for terrestrial material was obtained; nevertheless only 60 % of the participants were able to characterise feather fragments as such, thus shedding light on a hidden problem because of the apparent high number of correct answers for terrestrial fragments. It has also to be reminded that in 2008 no limitation in the number of slides from the flotate or raw material was imposed as is now the case by the operational schemes of with the current legal reference. Whether in 2008 more slides made from the flotate of the raw material were prepared allowing a better identification is impossible to know. When considering the 5 fold adulteration level that was used in 2008 compared to the present adulteration level, an improvement of the feather identification capabilities of the NRL cannot be proven – neither a worsening of identification capabilities. Therefore training is still required and special attention to the imposed slide made from the flotate is recommended. Among the possibilities to improve the detection skills the use of cystine reagens is advocated. However this staining for keratinic structures is usually performed as a complement to the basic observations made from the flotate. So far the current legal method does not deal with additional slides using more specific stainings. Considerations for an improvement in this respect would certainly bring added value to this lack of sensitivity.

The second source of error is bound to a specific feed matrix that was used: a compound feed for laying hens containing shell grits. The latter material being composed of crushed seashells is commonly used as a source of calcium for egg shell production. With regard to Commission Regulation EC/1069/2009 [9] this authorised material is not a source of protein and is neither a PAP or a fishmeal since it is only prepared from calcareous remains of molluscs. However it appears from this study that some fragments of shell grits are erroneously interpreted as from fish origin and described as fishbones, scales or otoliths. This misinterpretation occurred in one third of the NRLs. These errors of interpretation were made by the same participants (7 on 8) on the two blind samples using this compound feed for laying hens, so there is a consistency in the interpretation. A reduced number of participants (6) perfectly identified this type of material – whether or not classifying them as positive or negative for fish as explained in point 3.4.1.

A correct identification of shell fragments from seashell may correctly be achieved provided following data and considerations are followed:

1. The production of shell is a biomineralization of calcium and bicarbonate ions which are concentrated up to supersaturation into the extrapallial space of seashells. The process is thus an extracellular one and as a result the shell “in se” is a dead acellular tissue [15]. So the presence of osteocytes is excluded. Since the ultimate microscopic characteristic of bones is the proper presence of osteocytes the confusion is not possible. Some structural details, such as cracks, spaces between mineral crystals, tubular networks of pore duct combined with the elongated shape of fragments should not be confused with fish osteocytes (fig. 1A, 1B and 4 from Annex 4).

2. The chemical composition of shell from seashells is $CaCO_3$ in the form of calcite and aragonite. The inner layer of a shell is consisting of nacre (mother-of-pearl) exclusively made of aragonite. The organisation of aragonite crystals or microstructures into dense packs is responsible for an iridescent aspect. Their organisation is only visible by microscopy (fig. 2A from Annex 4). The outer layer is also made of crystals not exclusively made of aragonite but also, and in some cases exclusively, made of calcite. On the contrary to bones, hydroxyapatite is absent from the composition of shells. Therefore in case of hesitation, for shell fragments that would resemble for instance fish scales, a simple Alizarin Red staining will allow the distinction (figs. 1A, 1B, 2A and 2B from Annex 4). Shells fragments are not coloured by the staining while fish scales are. This last test was successfully experienced by lab 4 for which absence of coloration of the suspicious fragments by the Alizarin Red staining helped to come to the correct conclusion regarding the absence of fish material. The structural birefringence of some nacre fragments is also a characteristic that supports the identification (such as illustrated in the EURL-AP micrographs collection).

3. Some shell fragments show structural growing strips and might be interpreted as fragments of otoliths. In this case the distinction between the two types of fragments is difficult. First of all there is no difference in chemical nature; both are composed principally of aragonite. Whereas otoliths shows daily growth strips more regular in distance between strips than that which can be observed from shell fragments (figs. 3A and 3B from Annex 4). The almost perfectly perpendicular organisation of the growth strips to the main direction of the aragonite crystal in otoliths [16] is also a feature allowing the distinction with mollusc shells. In addition mollusc shell fragment resembling otoliths also present sometimes crossing structures which are absent from fish otoliths (fig. 3A from Annex 4). Finally in case of hesitation, it should be stated that so far the only presence of otoliths without any other structures from fish origin, such as fishbones, gills, and scales has never been reported. Therefore the sole presence of “otolith” resembling fragments should not lead to the conclusion of fish material presence.

The official microscopic method as described in Annex VI of Commission Regulation EC/152/2009 [4] enables to make the distinction between terrestrial and fish material; mollusc particles are excluded from these two categories. With regard to the recent proposal of authorising insect PAP in aquafeed, it is important to create an additional category of microscopic detectable particles corresponding to the presence of invertebrates. This third category, or nature when referring to the wording of Annex VI of Commission Regulation EU/152/2009, would allow an improved reporting precision in line with legal recommendations. For the present, in case mussel meal (prepared from entire mollusc including soft tissues and therefore legally categorised as fishmeal since prepared from) is included in a feed as both a source of protein and calcium, the microscopic method is unable to state on the presence of fish, since no scales, gills or fishbones will be detected leading to declare the sample as free from fish while it contains a “fishmeal”.

Concerning the non-EU participants, only one out of five performed excellently. It seems that these participants were equally affected by problem of specificity and sensitivity for terrestrial animal particles, while for fish particles specificity issues are more frequent. However absence of details about the methods used by these participants does not allow in depth comparison with results of the NRL network.

The PCR results of this second combined microscopy-PCR proficiency test are excellent. All the participants submitted their results in time and 96.3 % of the NRLs (26 out 27 NRLs) obtained perfect results without any deviation. Only one lab provided an incorrect result (1 false positive deviation) but was nevertheless considered as having a satisfactory level of performance.

Concerning the non-EU participants, three labs out of the 5 delivered results for the PCR. Two of them submitted perfect results. They used the PCR method described in the SOPs. The last lab obtained a satisfactory level of performance by providing only one incorrect result (1 false negative deviation).

The level of excellence achieved through this second combined proficiency test is demonstrating the maturity of the NRL network for both methods.

Acknowledgment

We are grateful to the EURL-AP technical staff that succeeded at addressing the challenge of a combined proficiency test. 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
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	Central Institute of sampling and testing in Agriculture
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
Japan	*Food and Agricultural Materials Inspection Center*
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	RIKILT Institute of Food Safety, Wageningen UR
Norway	*LabNett AS and National Institute of Nutrition and Seafood Research*
Poland	National Veterinary Research Institute
Portugal	Laboratorio Nacional de Investigaçao 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-Unit for pathology of animal nutrition and environmental hygiene
Spain	Laboratorio Arbitral Agroalimentario
Sweden	National Veterinary Institute, Department of Animal Feed
United Kingdom	Animal and Plant Health Agency

Annex 2

Announcement letter

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval building
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e-mail: secretary@eurl.cra.w.eu Internet : http://eurl.cra.w.eu

Announcement of the EURL-AP proficiency test 2016/01 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 Regulation (EU) No 51/2013 of 16 January 2013, 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.

Objective

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 Regulation EU 51/2013 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 8 samples, each of about 50g. According to the instructions and colour codes, some samples will have to be analysed by microscopy only, by PCR only and by combining both 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. These methods, alone or combined, shall be applied for the analyses.
- 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.
- The participation in this proficiency study is mandatory and free of charge for national reference laboratories within Member States of the European Union.

Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : 30 September 2016
- Sending of the sample boxes and communication of the instructions : 7 November 2016

> ***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 : 9 December 2016

Further information

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

  or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager

  ☎32 (0) 81 62 03 75

  32 (0) 81 62 03 88
  E-mail: p.veys@cra.wallonie.be

  or

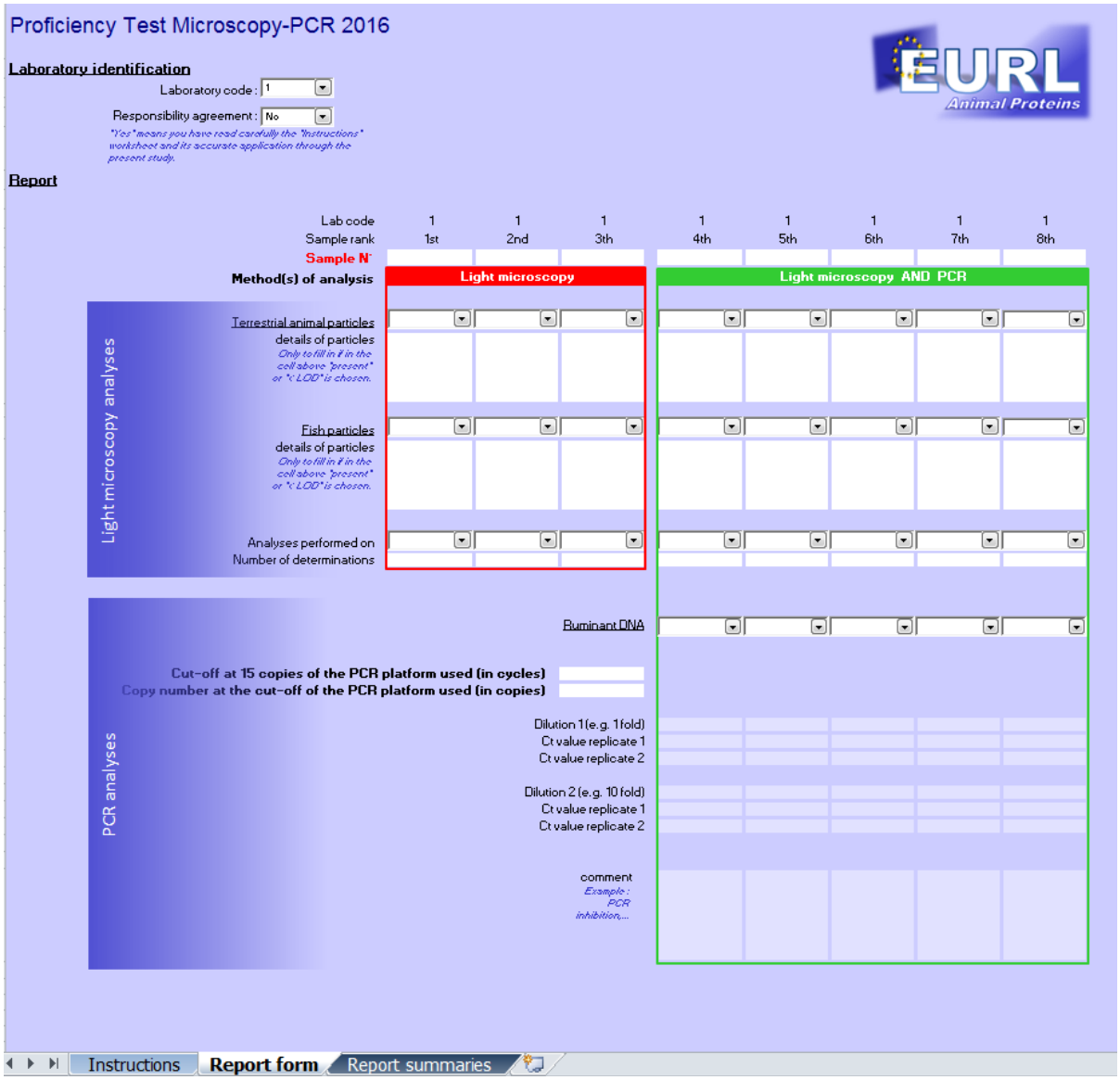
- Dr Olivier FUMIERE
  Head of EURL-AP Molecular biology team

  ☎32 (0) 81 62 03 51

  32 (0) 81 62 03 88
  E-mail: o.fumiere@cra.wallonie.be

Annex 3

Excel result report form



Wallonie

Annex 4

On the confusion of shell from seashells with fish fragments.

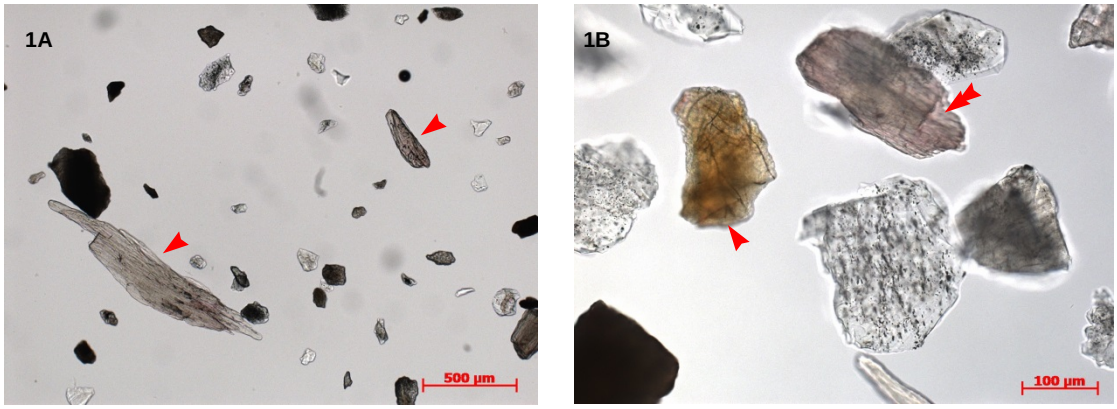


Fig 1: Overview of sediment from the compound feed for laying hens. A) some elongated shell fragments are visible (arrowheads), B) a shell fragment with textured cracks (arrowhead) and another shell fragment showing a pale colour (double arrowhead) are visible between other undetermined mineral structures. *Bright field. Alizarin Red staining*.

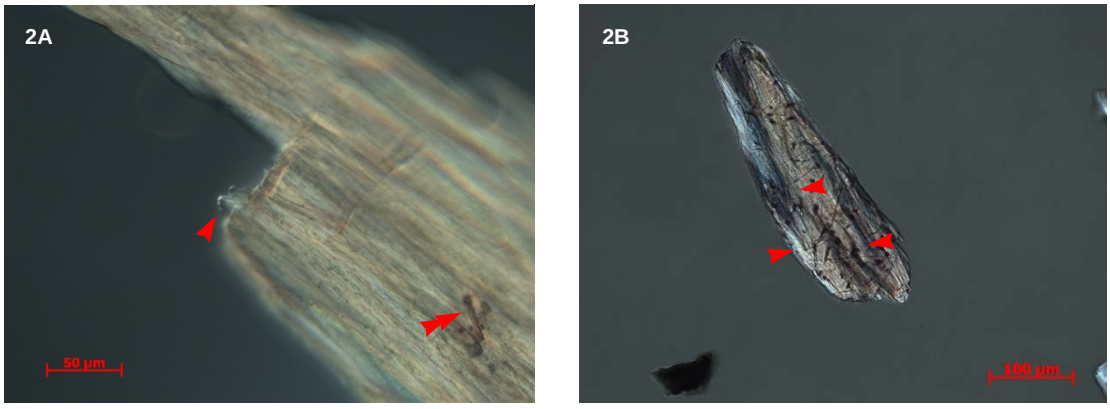


Fig 2: Details the shell fragments from Fig 1A. A) Crystals are at the edge of the fragment (arrowhead). A tubular, channel like, structure is also illustrated (double arrowhead). *Differential interference contrast. Alizarin Red staining*. B) Strong birefringence of the shell fragment is noted as well as the numerous tubular structures (arrowheads). *Polarized light. Alizarin Red staining*.

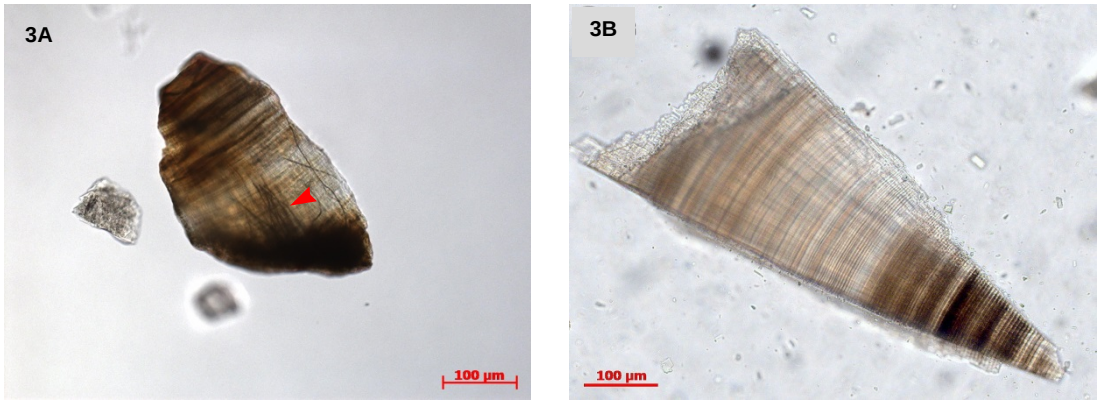


Fig 3: A) Bivalve shell fragment presenting growth strips more irregular and with some crossing structures (arrowhead). *Bright field.* B) Fish otolith fragment with more regular succession of growth strips and perfectly perpendicular to the aragonite crystals. *Bright field*.

Wallonie

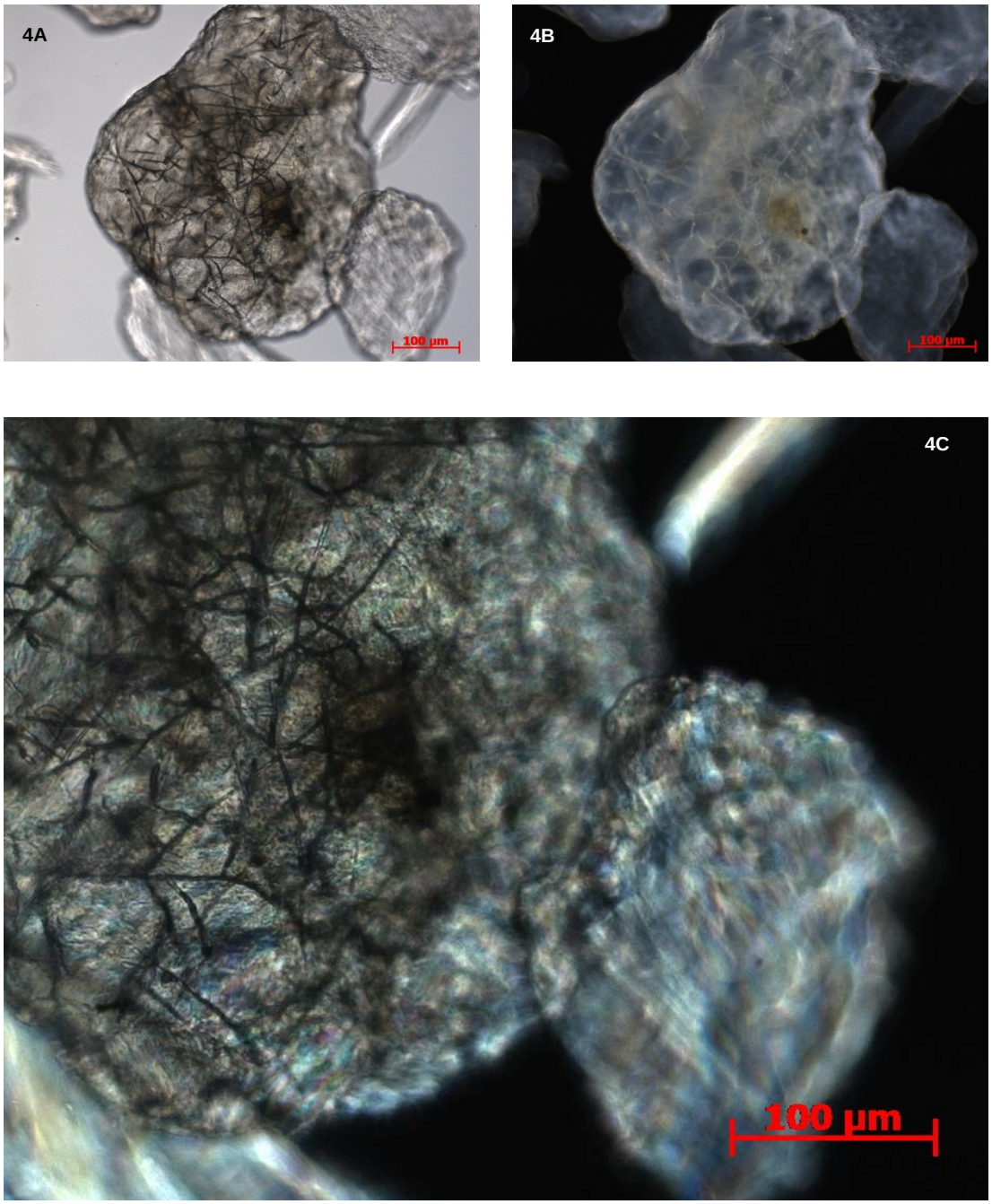


Fig 4: Calcitic oyster shell fragment presenting elongate pore ducts crossing each other in mixed ways. A) *Bright field.* B) *Dark field.* C) Detail of the same fragment by *Polarized light.*

Annex 5

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
3	169	Absent		Absent		Sed. + Raw	2
1	831	Absent		Present	otolith	Sed. + Raw	3
2	1121	< LOD	bones, muscle, feather	Present	otolith	Sed. + Raw	3
6	85	Present	bones, muscles, hair	Present	fishbones, muscles	Sed. + Raw	2
7	357	Present	muscles, hair	Present	fishbones, cartilage, gills, muscles	Sed. + Raw	3
4	747	Absent		Present	fishbones, cartilage, muscles	Sed. + Raw	2
5	983	Present	bones, muscles, feathers	Present	fishbones, cartilage, gills, muscles	Sed. + Raw	2
8	1277	Present	muscles, hairs	Present	fishbones, gills, muscles, scales	Sed. + Raw	3

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	111	Absent	N/A	Present	Fishbone, Scales	Sed. + Raw	1
2	1067	Present	Bone	Present	Fishbone	Sed. + Raw	1
3	1141	Absent	N/A	Absent	N/A	Sed. + Raw	1
6	121	Present	Bone, Muscle, Cartilage	Present	Fishbone, Muscle, Cartilage, Gills, Otolith	Sed. + Raw	1
7	429	Absent	N/A	Present	Fishbone, Muscle, Cartilage, Gills, Otolith	Sed. + Raw	1
5	533	Present	Bone, Muscle, Cartilage	Present	Fishbone, Muscle, Cartilage, Otolith	Sed. + Raw	1
4	873	Absent	N/A	Present	Fishbone, Cartilage, Gills, Muscle	Sed. + Raw	1
8	1079	Absent	N/A	Present	Fishbone, Muscle, Cartilage, Gills, Otolith, Scales	Sed. + Raw	1

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
3	475	Absent		Absent		Sed. + Flot.	1
2	617	Present	feathers	Present	bones, otolith,shell, squid	Sed. + Flot.	1
1	1155	Absent		Present	bones, otolith, shell	Sed. + Flot.	1
5	29	Present	bones	Present	bones, scale, cartilage, muscles	Sed. + Flot.	1
4	153	Absent		Present	bones, scale, cartilage, muscles	Sed. + Flot.	1
6	337	Present	bones, blood	Present	bones, scale, cartilage, muscles	Sed. + Flot.	1
7	1113	Present	bones	Present	bones, scale, cartilage, muscles	Sed. + Flot.	1
8	1133	Present	bones	Present	bones, scale, cartilage, muscles	Sed. + Flot.	1

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
3	745	Absent		Absent		Sed. + Flot.	2
2	923	Absent		Absent	we have stained with alizarin red the sediment but the particles didn´t stained	Sed. + Flot.	2
1	1011	Absent		Absent	we have stained with alizarin red the sediment but the particles didn´t stained	Sed. + Flot.	2
5	317	Present	Bones > 5, muscles	Present	fish bones, scales , otholits, muscles	Sed. + Flot.	2
6	535	Present	Bones >5, muscles	Present	Fish bones, scales, otholits, muscles	Sed. + Flot.	2
7	627	Absent		Present	fish bones, scales , otholits, muscles	Sed. + Flot.	2
4	801	Absent		Present	fish bones, scales , otholits, muscles	Sed. + Flot.	
8	863	< LOD	< 5 bones, we have found 3 bones, muscles	Present	fish bones, scales , otholits, muscles	Sed. + Flot.	

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	59	Present	hydrolysed feathers, 2 bones	Absent		Sed. + Flot.	1
1	597	Absent		Absent		Sed. + Flot.	1
3	817	Absent		Absent		Sed. + Flot.	1
6	49	Present	bones	Present	bones, cartilages, gills, scales, otoliths, muscles	Sed. + Flot.	1
8	161	Absent		Present	bones, cartilages, gills, scales, otoliths, muscles	Sed. + Flot.	1
5	389	Present	bones	Present	bones, cartilages, gills, scales, otoliths, muscles	Sed. + Flot.	1
7	537	Absent		Present	bones, cartilages, gills, scales, otoliths, muscles	Sed. + Flot.	1
4	1071	Absent		Present	bones, cartilages, gills, scales, otoliths, muscles	Sed. + Flot.	1

This participant explained in its accompanying email to have identified in sample types 1 and 2 “the presence of particles derived from sea shells (oysters, mussels, cockles, clams, etc)”

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
2	905	Present	muscles (possible from	Absent		Sed. + Raw	2
3	979	Absent		Absent		Sed. + Raw	1
1	1173	< LOD	parts of insects	Absent		Sed. + Raw	1
5	461	Present	bones	Present	muscles, fishbones	Sed. + Raw	3
8	485	Absent		Present	muscles, fishbones, cartilage, gills, scales	Sed. + Raw	1
6	607	Present	bones	Present	muscles, fishbones	Sed. + Raw	1
7	681	Absent		Present	muscles, fishbones, otolite, cartilage	Sed. + Raw	1
4	1017	Absent		Present	muscles, fishbones, cartilage, otolite	Sed. + Raw	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
2	203	Present	Bones	Absent		Sed. + Flot.	2
3	673	Absent		Absent		Sed. + Flot.	1
1	1101	Absent		Absent		Sed. + Flot.	1
7	195	Absent		Present	Bones	Sed. + Flot.	1
5	497	Present	Bones	Present	Bones	Sed. + Flot.	1
8	503	Absent		Present	Bones, gills, cartilage	Sed. + Flot.	1
6	1021	Present	Bones	Present	Bones	Sed. + Flot.	1
4	1179	Absent		Present	Bones	Sed. + Flot.	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
1	561	Absent		Absent		Sed. + Flot.	2
3	583	Absent		Absent		Sed. + Flot.	2
2	743	Absent		Absent		Sed. + Flot.	2
4	9	Absent		Present	fish bones, muscle fibers	Sed. + Flot.	2
6	103	Present	bones, muscle fibers	Present	fish bones, muscle fibers	Sed. + Flot.	2
7	321	Absent		Present	fish bones, muscle fibers	Sed. + Flot.	2
5	587	Present	bones, muscle fibers	Present	fish bones, muscle fibers	Sed. + Flot.	2
8	773	< LOD	bones	Present	fish bones, muscle fibers, scales, cartridges, gills	Sed. + Flot.	2

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
2	77	Present	feathers, hydrolysed feathers	Absent		Sed. + Flot.	1
1	669	Absent		Absent		Sed. + Flot.	1
3	871	Absent		Absent		Sed. + Flot.	1
6	157	Present	bones	Present	fishbones, scales, muscles	Sed. + Flot.	1
8	539	Absent		Present	fishbones, scales, cartilage, muscles	Sed. + Flot.	1
5	623	Present	bones	Present	fishbones, scales, muscles	Sed. + Flot.	1
7	1167	Absent		Present	fishbones, scales, muscles	Sed. + Flot.	1
4	1251	Absent		Present	fishbones, scales, muscles	Sed. + Flot.	1

This participant explained in its accompanying email to have identified in sample types 1 and 2 “more than 5 particles of shellfish meal (oyster, mussels, etc)”

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	637	Absent		Absent		Sed. + Raw	1
1	705	Absent		Present	fishbones, otholites, muscle fibres	Sed. + Raw	1
2	851	Present	feathers	Absent		Sed. + Raw	1
4	171	Absent		Present	fishbones, scales, otholites, muscles fibres	Sed. + Raw	1
6	625	Present	bones, blood, muscle fibres	Present	fishbones, scales, otholites, muscles fibres	Sed. + Raw	1
5	1145	Present	bones, muscle fibres	Present	fishbones, scales, otholites, muscles fibres	Sed. + Raw	1
7	1221	Absent		Present	fishbones, scales, otholites, muscles fibres	Sed. + Raw	1
8	1241	Absent		Present	otholithes, fishbones, scales, muscle fibres	Sed. + Raw	1

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
3	241	Absent		Absent		Sed. + Flot.	1
2	491	Absent		Present	scalles,gills,muscle	Sed. + Flot.	1
1	273	Absent		Present	scalles,gills,muscle	Sed. + Flot.	1
6	175	Present	bons	Present	scalles gills, muscle	Sed. + Flot.	1
4	639	Absent		Present	scalles,gills,muscle	Sed. + Flot.	1
5	659	Present	bons	Present	scalles,gills,muscle	Sed. + Flot.	1
7	843	Absent		Present	scalles,gills,muscle	Sed. + Flot.	2
8	971	Absent		Present	scalles,gills,muscle	Sed. + Flot.	2

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
1	777	Absent		Absent		Sed. + Flot.	1
3	1033	Absent		Absent		Sed. + Flot.	1
2	1175	Present	hydrolized feathers, muscle fibres no diff. of the origin of the found muscle fibres possible	Absent		Sed. + Flot.	1
6	31	Present	bones, feathers no diff. between MBM- and FM muscle fibres possible	Present	fishbones, scales, muscle fibres no diff. between MBM- and FM muscle fibres possible	Sed. + Flot.	1
7	339	Absent		Present	fishbones, scales, muscle fibres it can't be excludet, that the muscle fibres found only derive from fish meal	Sed. + Flot.	1
8	647	Absent		Present	fishbones, scales, muscle fibres it can't be excludet, that the muscle fibres found only derive from fish meal	Sed. + Flot.	1
4	729	Absent		Present	fishbones, scales, muscle fibres it can't be excludet, that the muscle fibres found only derive from fish meal	Sed. + Flot.	1
5	1253	Present	bones no diff. between MBM- and FM fibres possible	Present	fishbones, scales, muscle fibres no diff. between MBM- and FM fibres possible	Sed. + Flot.	1

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
3	25	Absent		Absent		Sed. + Raw	1
2	455	Absent		Present	Shell fish scales. These are included in the definition of fish meal according to R (EC) 142/2011, Annex I. The competent authority should make the final desicion. Here only the analytical result is given.	Sed. + Raw	1
1	579	Absent		Present	Shell fish scales. These are included in the definition of fish meal according to R (EC) 142/2011, Annex I. The competent authority should make the final desicion. Here only the analytical result is given.	Sed. + Raw	1
4	207	Absent		Present	Fish bones and muscle fibres.	Sed. + Raw	1
7	411	Absent		Present	Fish bones and muscle fibres.	Sed. + Raw	1
5	425	Present	Land animal bones and muscle fibres.	Present	Fish bones and muscle fibres.	Sed. + Raw	1
8	611	Absent		Present	Fish bones and muscle fibres.	Sed. + Raw	1
6	805	Present	Land animal bones and muscle fibres.	Present	Fish bones and muscle fibres.	Sed. + Raw	1

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
3	709	Absent		Absent		Sed. + Flot.	2
1	993	< LOD	feather	Absent	shell present	Sed. + Flot.	2
2	1013	Present	feather	Absent	shell present	Sed. + Flot.	2
5	227	Present	bones	Present	fishbones, muscle, splinters	Sed. + Flot.	2
6	373	Present	bones, blood, pig hair	Present	fishbones, muscle, splinters	Sed. + Flot.	2
7	735	Absent		Present	fishbones, muscle, splinters	Sed. + Flot.	2
8	917	< LOD	bones	Present	fishbones, muscle, splinters, scale	Sed. + Flot.	2
4	963	Absent		Present	fishbones, muscle, splinters	Sed. + Flot.	2

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
2	473	Absent		Present	bone, scale	Sed. + Flot.	1
1	1047	Absent		Present	bone, scale, otolith	Sed. + Flot.	1
3	1195	Absent		Absent		Sed. + Flot.	1
6	499	Present	bone	Present	bone, cartilage, muscle	Sed. + Flot.	1
5	605	Present	bone	Present	bone, scale, gill, otolith, cartilage, muscle	Sed. + Flot.	1
7	699	< LOD	bone	Present	bone, scale, otolith, cartilage, muscle	Sed. + Flot.	1
8	1025	Absent		Present	bone, scale, gill, otolith, cartilage, muscle	Sed. + Flot.	1
4	1233	Absent		Present	bone, cartilage, muscle	Sed. + Flot.	1

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
3	205	Absent		Absent		Sed. + Flot.	3
2	221	Present	feathers	Absent		Sed. + Flot.	3
1	723	Absent		Absent		Sed. + Flot.	3
5	191	Present	bones	Present	fish bones	Sed. + Flot.	3
4	351	Absent		Present	fish bones	Sed. + Flot.	3
8	809	Absent		Present	fish bones, scales, gills	Sed. + Flot.	3
6	913	Present	bones	Present	fish bones, gills	Sed. + Flot.	3
7	1239	Absent		Present	fish bones	Sed. + Flot.	3

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
2	131	Absent		Absent		Sed. + Raw	2
1	345	Absent		Absent		Sed. + Raw	2
3	1087	Absent		Absent		Sed. + Flot.	1
8	251	Absent		Present	bones, cartilages	Sed. + Raw	2
7	375	Absent		Present	bones, cartilages	Sed. + Raw	2
6	391	Present	bones	Present	bones	Sed. + Flot.	1
5	551	Present	bones	Present	bones	Sed. + Flot.	1
4	1125	Absent		Present	bones	Sed. + Raw	2

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
2	347	Absent		Present	bone, scale	Sed. + Raw	2
3	691	Absent		Absent		Sed. + Raw	2
1	849	Absent		Present	bone	Sed. + Raw	2
7	285	Absent		Present	Bone, muscle fibres, scale, gill.	Sed. + Raw	2
8	323	Absent		Present	Bone, muscle fibres, scale,	Sed. + Raw	2
5	335	Present	Bone	Present	Bone, muscle fibres, otolith, tooth	Sed. + Raw	2
6	409	Present	Bone	Present	Bone, muscle fibres, skin,	Sed. + Raw	2
4	855	Absent		Present	Bone, muscle fibres, gill, mollusc	Sed. + Raw	2

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
2	239	Present	1bone particle + 5 feather particles	Absent		Sed. + Flot.	1
3	511	Absent		Absent		Sed. + Flot.	1
1	939	Absent		Absent		Sed. + Flot.	1
7	159	Absent		Present	bones, gills, muscle	Sed. + Flot.	1
4	333	Absent		Present	bones, gills muscle	Sed. + Flot.	1
8	413	Absent		Present	bones, gills muscle	Sed. + Flot.	1
6	571	Present	bones, teeth? (muscle and blood)	Present	bones, otolith, gills, muscle	Sed. + Flot.	1
5	1199	Present	bones, (muscle)	Present	bones, muscle	Sed. + Flot.	1

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
3	79	Absent		Absent		Sed. + Raw	3
2	113	< LOD	Bone	Absent		Sed. + Raw	3
1	147	Absent		Absent		Sed. + Raw	3
5	173	Present	Bone, catilage, meatfiber, feather	Present	bone, meatfiber, gills, cartilage, scales	Sed. + Raw	1
8	197	< LOD	possibly milk powder	Present	bone, meatfiber, gills, cartilage, scales	Sed. + Flot.	3
7	213	< LOD	possibly milk powder	Present	bone, meatfiber, gills, cartilage, scales	Sed. + Raw	3
6	355	Present	Bone, catilage, meatfiber, hair, blood	Present	bone, meatfiber, gills, cartilage, scales	Sed. + Raw	1
4	477	Absent		Present	bone, meatfiber, gills, cartilage, scales	Sed. + Raw	3

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
1	3	Absent		Present	fishbones	Sed. + Flot.	1
3	7	Absent		Absent		Sed. + Flot.	1
2	1211	Absent		Present	fishbones	Sed. + Flot.	1
5	47	Present	bones	Present	fishbones, muscle fibres	Sed. + Flot.	2
4	315	Absent		Present	fishbones, muscle fibres	Sed. + Flot.	1
6	463	Present	bones	Present	fishbones, otolites, muscle fibres	Sed. + Flot.	1
8	593	Absent		Present	fishbones, scales, cartilage, muscle fibres	Sed. + Flot.	1
7	897	Absent		Present	fishbones, muscle fibres	Sed. + Flot.	1

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
3	223	Absent		Absent		Sed. + Flot.	1
1	525	Present	feathers	Absent		Sed. + Flot.	2
2	635	Absent		Absent		Sed. + Flot.	1
4	45	Absent		Present	muscles, fish bones,	Sed. + Flot.	1
8	53	Absent		Present	muscles, fish bones, scales, cartilages, gills	Sed. + Flot.	1
7	249	Absent		Present	muscles, fish bones,	Sed. + Flot.	1
5	263	Present	bones	Present	muscles, fish bones, scales, cartilages, gills	Sed. + Flot.	1
6	553	Present	bones	Present	muscles, fish bones, scales, cartilages, gills	Sed. + Flot.	1

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
1	21	Absent		Absent		Sed. + Flot.	1
2	167	Present	feathermeal	Absent		Sed. + Flot.	1
3	601	Absent		Absent		Sed. + Flot.	1
8	71	< LOD	animal bones, muscle fibres, cartilage	Present	fish bones, fish scales, cartilage, gill, muscle fibres, fish skin	Sed. + Flot.	2
6	247	Present	'animal bones, muscle fibres, cartilage	Present	'fish bones, fish scales, cartilage, gill, muscle fibres, fish skin	Sed. + Flot.	1
5	299	Present	"animal bones, muscle fibres, cartilage	Present	'fish bones, fish scales, cartilage, gill, muscle fibres, fish skin	Sed. + Flot.	1
4	549	Absent		Present	'fish bones, cartilage, muscle fibres, fish skin	Sed. + Flot.	1
7	555	Absent		Present	'fish bones, fish scales, cartilage, gill, muscle fibres, fish skin	Sed. + Flot.	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	183	Absent		Absent		Sed. + Flot.	1
2	329	< LOD	feathers	Absent		Sed. + Flot.	2
3	565	Absent		Absent		Sed. + Flot.	1
6	229	Present	bones	Present	gills, bones	Sed. + Flot.	1
8	233	Absent		Present	gills, bones, cartilage, scales	Sed. + Flot.	1
4	297	Absent		Present	bones, gills, scales	Sed. + Flot.	1
7	573	Absent		Present	bones, gills, scales	Sed. + Flot.	1
5	821	Present	bones	Present	bones, gills, scales	Sed. + Flot.	1

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
2	5	< LOD	Feathers	Present	shell fragments	Sed. + Flot.	2
1	57	Absent		Present	Shell fragments	Sed. + Flot.	2
3	367	Absent		Absent		Sed. + Flot.	1
7	105	Absent		Present	bones scales gills cartilage muscle	Sed. + Flot.	2
6	265	Present	bones	Present	bones, scales cartilage, muscle	Sed. + Flot.	1
8	359	Absent		Present	bones scales gills, cartilage, muscle	Sed. + Flot.	1
4	531	Absent		Present	bones scales muscle	Sed. + Flot.	2
5	767	Present	bones	Present	bones scales cartilage muscle	Sed. + Flot.	2

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
3	43	Absent		Absent		Sed. + Flot.	1
1	255	Absent		Absent		Sed. + Flot.	1
2	311	Present	12 feathers, 4 bone particles	Absent		Sed. + Flot.	1
4	405	Absent		Present	fish bone particles	Sed. + Flot.	1
6	445	Present	terrestrial bone particles, blood meal particles	Present	fish bone particles	Sed. + Flot.	1
8	467	Absent		Present	fish bone particles	Sed. + Flot.	1
7	987	Absent		Present	fish bone particles	Sed. + Flot.	1
5	1037	Present	terrestrial bone particles	Present	fish bone particles	Sed. + Flot.	1

Laboratory identification code : **28**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	399	Absent		Absent		Sed. + Flot.	1
2	581	Present	Feathers	Absent		Sed. + Flot.	1
3	763	Absent		Absent		Sed. + Flot.	1
6	211	Present	Bones	Present	Fishbones, scales	Sed. + Flot.	1
4	279	Absent		Present	Fishbones, scales	Sed. + Flot.	1
8	305	Absent		Present	Fishbones, scales, gills	Sed. + Flot.	1
7	447	Absent		Present	Fishbones, scales	Sed. + Flot.	1
5	1091	Present	Bones	Present	Fishbones, scales	Sed. + Flot.	1

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
3	349	Absent		Absent		Sed. + Raw	2
1	507	Absent		Absent		Sed. + Raw	2
2	959	Absent		Absent		Sed. + Raw	2
7	33	Absent		Present		Sed. + Raw	2
8	89	Absent		Present		Sed. + Raw	2
5	281	Present		Present		Sed. + Raw	2
4	513	Absent		Absent		Sed. + Raw	2
6	715	Present		Absent	One particle of tooth detected	Sed. + Raw	2

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
8	107	Absent		Present		Sed. + Flot.	2
3	133	Absent		Absent		Sed. + Flot.	2
2	275	Absent		Present		Sed. + Flot.	2
1	381	Absent		Absent		Sed. + Flot.	2
6	427	Present		Absent		Sed. + Flot.	2
4	459	Present		Present		Sed. + Flot.	2
7	483	Present		Present		Sed. + Flot.	2
5	677	Present		Absent		Sed. + Flot.	2

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
2	23	Absent		Absent		Sed. + Flot.	1
1	291	Absent		Absent		Sed. + Flot.	1
3	295	Absent		Absent		Sed. + Flot.	1
4	27	Absent		Present	fishbones, (muscles of unknown origin in flotate)	Sed. + Flot.	1
6	283	Present	bones	Present	fishbones, gills (muscles of unknown origin in flotate)	Sed. + Flot.	1
7	501	Absent		Present	fishbones, gills (a lot of muscles of unknown origin in flotate)	Sed. + Flot.	1
8	755	Absent		Present	fishbones, gills, cartilage, scale (less muscles of unknown origin in	Sed. + Flot.	1
5	929	Present	bones	Present	fishbones, gills (muscles of unknown origin in flotate)	Sed. + Flot.	1

Laboratory identification code : **33**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
3	385	Absent		Absent		Sed. + Raw	1
2	437	Present	bones, feathers, muscles	< LOD	fishbones	Sed. + Raw	1
1	453	Absent		< LOD	scales, fishbones	Sed. + Raw	1
6	13	< LOD	bones	Present	scales, fishbones	Sed. + Raw	1
8	125	Present	bones	Present	scales, fishbones	Sed. + Raw	1
7	231	Present	bones, muscles	Present	fishbones	Sed. + Raw	1
4	243	Absent		Present	fishbones, muscles	Sed. + Raw	1
5	443	Absent		Present	fishbones, muscles	Sed. + Raw	1

Laboratory identification code : **34**

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
2	293	Absent		Absent		Sed. + Flot.	1
1	417	Absent		< LOD	Fishbones	Sed. + Flot.	2
3	547	Absent		Present	Fishbones	Sed. + Flot.	1
8	17	Present	Bones	Present	Fishbones	Sed. + Flot.	1
4	81	< LOD	Bones	Present	Fishbones	Sed. + Flot.	2
5	119	< LOD	Bones	Present	Fishbones	Sed. + Flot.	2
7	267	Present	Bones	Present	Fishbones	Sed. + Flot.	1
6	301	Present	Bones	Present	Fishbones	Sed. + Flot.	1

Annex 6

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

Laboratory identification code :	1

Cut-off at 15 copies :	34.30	cycles
Copy number at the cut-off :	11.17	copies

Sample type	Sample N°	Ruminant DNA
6	85	Absent
7	357	Present
4	747	Absent
5	983	Present
8	1277	Present

Laboratory identification code :	2

Cut-off at 15 copies :	36.02	cycles
Copy number at the cut-off :	10.77	copies

Sample type	Sample N°	Ruminant DNA
6	121	Absent
7	429	Present
5	533	Present
4	873	Absent
8	1079	Present

Laboratory identification code :	3

Cut-off at 15 copies :	35.92	cycles
Copy number at the cut-off :	10.76	copies

Sample type	Sample N°	Ruminant DNA
5	29	Present
4	153	Absent
6	337	Absent
7	1113	Present
8	1133	Present

Laboratory identification code :	4

Cut-off at 15 copies :	37.16	cycles
Copy number at the cut-off :	10.90	copies

Sample type	Sample N°	Ruminant DNA
5	317	Present
6	535	Absent
7	627	Present
4	801	Absent
8	863	Present

Laboratory identification code :	5

Cut-off at 15 copies :	35.34	cycles
Copy number at the cut-off :	9.58	copies

Sample type	Sample N°	Ruminant DNA
6	49	Absent
8	161	Present
5	389	Present
7	537	Present
4	1071	Absent

Laboratory identification code :	6

Cut-off at 15 copies :	36.43	cycles
Copy number at the cut-off :	11.25	copies

Sample type	Sample N°	Ruminant DNA
5	461	Present
8	485	Present
6	607	Absent
7	681	Present
4	1017	Absent

Laboratory identification code :	7

Cut-off at 15 copies :	34.23	cycles
Copy number at the cut-off :	11.08	copies

Sample type	Sample N°	Ruminant DNA
7	195	Present
5	497	Present
8	503	Present
6	1021	Absent
4	1179	Absent

Laboratory identification code :	8

Cut-off at 15 copies :	37.00	cycles
Copy number at the cut-off :	9.74	copies

Sample type	Sample N°	Ruminant DNA
4	9	Absent
6	103	Absent
7	321	Present
5	587	Present
8	773	Present

Laboratory identification code :	9

Cut-off at 15 copies :	34.96	cycles
Copy number at the cut-off :	10.90	copies

Sample type	Sample N°	Ruminant DNA
6	157	Absent
8	539	Present
5	623	Present
7	1167	Present
4	1251	Absent

Laboratory identification code :	10

Cut-off at 15 copies :	36.97	cycles
Copy number at the cut-off :	9.27	copies

Sample type	Sample N°	Ruminant DNA
4	171	Absent
6	625	Absent
5	1145	Present
7	1221	Present
8	1241	Present

Laboratory identification code :	11

Cut-off at 15 copies :	35.61	cycles
Copy number at the cut-off :	10.74	copies

Sample type	Sample N°	Ruminant DNA
6	175	Absent
4	639	Absent
5	659	Present
7	843	Present
8	971	Present

Laboratory identification code :	12

Cut-off at 15 copies :	35.72	cycles
Copy number at the cut-off :	11.68	copies

Sample type	Sample N°	Ruminant DNA
6	31	Absent
7	339	Present
8	647	Present
4	729	Absent
5	1253	Present

Laboratory identification code :	14

Cut-off at 15 copies :	34.12	cycles
Copy number at the cut-off :	11.87	copies

Sample type	Sample N°	Ruminant DNA
4	207	Absent
7	411	Present
5	425	Present
8	611	Present
6	805	Absent

Laboratory identification code :	15

Cut-off at 15 copies :	34.53	cycles
Copy number at the cut-off :	9.53	copies

Sample type	Sample N°	Ruminant DNA
5	227	Present
6	373	Absent
7	735	Present
8	917	Present
4	963	Absent

Laboratory identification code :	16

Cut-off at 15 copies :	37.56	cycles
Copy number at the cut-off :	9.09	copies

Sample type	Sample N°	Ruminant DNA
6	499	Absent
5	605	Present
7	699	Present
8	1025	Present
4	1233	Absent

Laboratory identification code :	17

Cut-off at 15 copies :	37.20	cycles
Copy number at the cut-off :	10.15	copies

Sample type	Sample N°	Ruminant DNA
5	191	Present
4	351	Absent
8	809	Present
6	913	Absent
7	1239	Present

Laboratory identification code :	18

Cut-off at 15 copies :	35.49	cycles
Copy number at the cut-off :	9.95	copies

Sample type	Sample N°	Ruminant DNA
8	251	Present
7	375	Present
6	391	Absent
5	551	Present
4	1125	Absent

Laboratory identification code :	19

Cut-off at 15 copies :	36.95	cycles
Copy number at the cut-off :	10.96	copies

Sample type	Sample N°	Ruminant DNA
7	285	Present
8	323	Present
5	335	Present
6	409	Absent
4	855	Absent

Laboratory identification code :	20

Cut-off at 15 copies :	35.85	cycles
Copy number at the cut-off :	9.65	copies

Sample type	Sample N°	Ruminant DNA
7	159	Present
4	333	Absent
8	413	Present
6	571	Absent
5	1199	Present

Laboratory identification code :	21

Cut-off at 15 copies :	34.90	cycles
Copy number at the cut-off :	10.13	copies

Sample type	Sample N°	Ruminant DNA
5	173	Present
8	197	Present
7	213	Present
6	355	Absent
4	477	Absent

Laboratory identification code :	22

Cut-off at 15 copies :	34.23	cycles
Copy number at the cut-off :	11.08	copies

Sample type	Sample N°	Ruminant DNA
7	195	Present
5	497	Present
8	503	Present
6	1021	Absent
4	1179	Absent

Laboratory identification code :	23

Cut-off at 15 copies :	35.44	cycles
Copy number at the cut-off :	10.12	copies

Sample type	Sample N°	Ruminant DNA
4	45	Absent
8	53	Present
7	249	Present
5	263	Present
6	553	Absent

Laboratory identification code :	24

Cut-off at 15 copies :	34.83	cycles
Copy number at the cut-off :	9.50	copies

Sample type	Sample N°	Ruminant DNA
8	71	Present
6	247	Absent
5	299	Present
4	549	Present
7	555	Present

Laboratory identification code :	25

Cut-off at 15 copies :	31.91	cycles
Copy number at the cut-off :	10.32	copies

Sample type	Sample N°	Ruminant DNA
6	229	Absent
8	233	Present
4	297	Absent
7	573	Present
5	821	Present

Laboratory identification code :	26

Cut-off at 15 copies :	36.56	cycles
Copy number at the cut-off :	9.04	copies

Sample type	Sample N°	Ruminant DNA
7	105	Present
6	265	Absent
8	359	Present
4	531	Absent
5	767	Present

Laboratory identification code :	27

Cut-off at 15 copies :	37.15	cycles
Copy number at the cut-off :	9.06	copies

Sample type	Sample N°	Ruminant DNA
4	405	Absent
6	445	Absent
8	467	Present
7	987	Present
5	1037	Present

Laboratory identification code :	28

Cut-off at 15 copies :	31.91	cycles
Copy number at the cut-off :	10.32	copies

Sample type	Sample N°	Ruminant DNA
6	211	Absent
4	279	Absent
8	305	Present
7	447	Present
5	1091	Present

Laboratory identification code :	30

Cut-off at 15 copies :	35.62	cycles
Copy number at the cut-off :	12.17	copies

Sample type	Sample N°	Ruminant DNA
7	33	Present
8	89	Present
5	281	Present
4	513	Absent
6	715	Absent

Laboratory identification code :	32

Cut-off at 15 copies :	37.95	cycles
Copy number at the cut-off :	9.86	copies

Sample type	Sample N°	Ruminant DNA
4	27	Absent
6	283	Absent
7	501	Present
8	755	Present
5	929	Present

Laboratory identification code :	33

Cut-off at 15 copies :	-	cycles
Copy number at the cut-off :	-	copies

Sample type	Sample N°	Ruminant DNA
6	13	Absent
8	125	Present
7	231	Present
4	243	Absent
5	443	Absent