Combined microscopy-PCR EURL-AP Proficiency Test 2017

Final version

Authors:

P. Veys, O. Fumière, A. Marien, V. Baeten and G. Berben

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European Union Reference Laboratory for Animal Proteins in feedingstuffs
Walloon Agricultural Research Centre
Agricultural Product Valorisation Department , Henseval Building, Chaussée de Namur 24, B-5030 GEMBLOUX (BELGIUM)
Phone: +32 (0) 81 62 03 75 - Fax : +32 (0) 81 62 03 88
secretary@eurl.craw.eu – website: eurl.craw.eu

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Editor :

Centre wallon de Recherches agronomiques
Service Communication
Rue de Liroux, 9
5030 Gembloux (Belgique)

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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 Commission Regulation EU/51/2013. For the third time, the study combined the NRLs assessment for the two official methods. Whereas the methods to apply were indicated in the last two proficiency tests, a new evaluation criterion of the NRLs was introduced this year; it concerned the correct application of the SOP (Standard Operating Procedures) on operational protocols for the combination of light microscopy and PCR with respect to the type of feed and the composition of the samples. For the first time, the participants had to decide, based on the labels linked to each of the feed samples, which method(s) they had to apply.

The total number of participating laboratories was 30 (26 NRLs and 4 labs outside the NRL network). The study was based on a set of eight samples (to be analysed by light microscopy and/or by PCR) consisting of blank feed matrices or feed fortified with terrestrial processed animal proteins and/or fishmeal. One of the samples was fortified with insect meal and was not considered in the evaluation of the participants.

Almost all participants provided their results in due time. One laboratory was only able to provide results by light microscopy. For a second laboratory, a server problem intervened during the transmission of the results. It was not possible to trace back that the results were effectively transmitted before the deadline and the participant was therefore excluded from this report. Each participant received after the closure of the results an individual table giving them a feedback of their results.

Testing the understanding of the SOP on operational protocols for the combination of light microscopy and PCR revealed particularly challenging. Results effectively demonstrated that some NRLs faced difficulties to apply the operational schemes. The number of non-compliant NRLs as regards the respect of the operational protocols is 36 %.

Regarding the detection of PAPs by light microscopy the overall results indicate an excellent level of global performance for 96 % of the NRLs (80 % of the NRLs having no wrong result), a satisfying level of global performance for 4 % of the NRLs and no underperforming NRL.

Concerning the PCR results, the global performance of the NRL network appeared to be lower than the two previous proficiency tests combining light microscopy and PCR. Four underperforming NRLs were pointed by the present study. However, the tendency reflects more the additional difficulty introduced by the choice of the method(s) to apply than a decrease of the proficiency per se. Indeed, the wide majority of the deviations recorded are missing results due to incorrect microscopic results and/or misapplication of the SOP on operational protocols for the combination of light microscopy and PCR. In the case of two NRLs, the cause of the underperformance is clearly an organizational problem that must be solved internally.

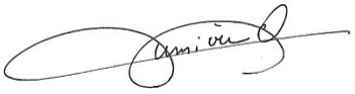
Keywords :

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 8th February 2018. After reception of the comments on the 1st March 2018, 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, 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 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 SOP on operational protocols for the combination of light microscopy and PCR [5] defines which of the methods shall be used alone or in combination according to the type of feed and its composition.

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. Moreover, the compliance with the legal requirements imposed by the SOP on operational protocols for the combination of light microscopy and PCR was evaluated. 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 four laboratories outside this EU network participated to the study. A detailed list of the 30 participating labs is included in Annex 1.

Official announcement (Annex 2) of the study was made on the 6th September 2017 to all participants.

On the 17th October 2017, 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 17th November 2017.

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.

On the exception of one participating NRL, which was excluded, all results were delivered on time to the organiser.

Twenty eight participants returned results for both microscopic and PCR analyses; one NRL was unable to perform PCR analyses. The proficiencies of NRLs and other participants were evaluated separately in this report.

3.2. Material

3.2.1. Description of the samples

Six different test materials were prepared for the study. Five were used for proficiency assessment and one for scientific purpose only.

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

- Use of feed matrices intended to different farmed animals (with the indication on the vial label) for assessing the correct interpretation and implementation of the operational schemes as described in the SOP on operational protocols for the combination of light microscopy and PCR [5].
- 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 [6].
- Use of insect PAP in order to determine if the current reference method is fit for the purpose of insect detection. The sample fortified with this PAP was not used for proficiency assessment of the participants.

Each participating lab received eight samples of about 50g each. Samples were not blind. Each sample was labelled with the type of feed matrix. Each sample was assigned with a unique random number. Details of the sample set are indicated in Table 1.

Table 1: Composition of the sample set.

				Expected results *		
				Microscopy		PCR
Sample	Label	Material	Nr of replicates	Terrestrial particles	Fish particles	Ruminant DNA
1	Poultry feed	Poultry feed	2	-	-	NA
2	Fish feed	Fishfeed I + 0.1 % porcine PAP	1	+	+	+
3	Horse feed	Horse feed	1	-	-	NA
4	Fish feed + haemoglobin meal	Fishfeed II with haemoglobin meal	2	NA	NA	-
5	Trout juveniles feed	Fishfeed III + 0.05 % ruminant PAP	1	+	+	+
6	Trout juveniles feed	Fishfeed IV + 0.5 % insect PAP	1	NA	NA	NA
Total			**8**			

(* Explanations on expected results are described in section 3.4, NA = not applicable)

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.

The labels were aimed at defining which analyses had to be performed for each sample in agreement with the binding SOP on operational protocol. No other information was delivered to the participants for determining the correct analytical choice.

3.2.2. Materials used in the preparation of the samples

Six commercial matrices were used:

- Poultry feed was a **compound feed for lay hens**. It was used in a previous proficiency test [7] in 2016. 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 sample 1.
- Fishfeed I was a **complete feed for sturgeon**. It consisted of fishmeal, wheat, soy, guar meal, sunflower, haemoglobin, fish oil, soy oil, rapeseed oil, lin seed oil, dried distillers grains with soluble, vitamins and minerals. The sediment content of the mixture was about 1.6 %. It was used for preparing sample 2. This fishfeed was positive for ruminant DNA presence.
- Horse feed was a **compound feed for poneys**. It contained wheat hulls, soy hulls, wheat gluten, maize gluten, molasses, maize, calcium carbonate and other minerals. Its sediment was of 0.8 %. Its was used for preparing sample 3.
- Fishfeed II was a **complete feed**. It consisted of rapeseed oil, soy, wheat, fishmeal, haemoglobin meal, fish oil, horsebean, wheat gluten, soybean toasted and feed additives, minerals and antioxidants. Its sediment was of 1.8 %. It was used for preparing samples 4.
- Fishfeed III was a **compound feed for fry** made of fishmeal, maize starch, fish oil, wheat gluten, protein concentrate from pea, vitamins and minerals. It was used in previous study in 2016 [7]. Its percentage of sediment was of 0.9 %. This feed was used for sample 5.
- Fishfeed IV was a **compound feed for fry**. Its composition was unknown but is analytically free of any terrestrial PAP (see section 3.5). Its sediment was of 3.7 %. It was used only for sample 6.

Adulterant material used:

- A **pure porcine PAP** was used for preparing sample 2. This PAP was used in 2015 and 2016 [7, 8]. Its bone content was of about 14.0 % and its purity was checked by microscopy and PCR.
- A **pure ruminant PAP** was used for preparing sample 5. Its purity was controlled by PCR.
- A **pure insect PAP** from *Tenebrio molitor* was used for sample 6. It was industrially produced and had a sediment of 0.1 %. Microscopic analysis showed it as free from both terrestrial and fish remains. PCR analyses showed it negative for DNA from ruminant, bovine, ovine, porcine, chicken, turkey and fish origin. It was only positive for insect DNA.

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 of the vials.

Blank matrices were conditioned first in order to avoid contamination.

On the exception of the matrices for samples 1 and 3, all other matrices were ground at 2 mm before any other treatment.

Samples 2 and 6 were directly spiked with the adulterant.

Adulteration of samples 5 was processed as follows to avoid problems of homogeneity due to the fat content the matrix. Several portions of 25 g of the matrix were degreased and pooled together to obtain a starting amount of defatted matrix for the initial step of a serial dilution. The amount of adulterant was added to this defatted matrix fraction. After homogenisation of this first step, the serial dilution was continued with the original matrix.

3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [9].

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, in the sense that both Ct values should be close 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 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 [9].

The performance was assessed on two different aspects: the correct implementation of the legislation (i.e. choice of method to apply in accordance with the SOP on operational protocol) and the analytical results.

Results from analyses which should not have been performed according to the legislation were not considered for the analytical proficiency assessment but well for the evaluation of the implementation of the legislation. This is notably justified by the fact that if doing so the number of analyses would have been different among participant thus excluding any ranking of them.

The absence of an analytical result while legally imposed was considered as incorrect for both legal and analytical performance assessment. On the other hand, an analytical error leading to a logical stop choice in the operational protocol was not considered as an error in the implementation of the legislation.

3.4.1. Legislation

The first performance evaluation concerned the correct implementation of the operational protocols that have to be followed, depending on the type of feed being analysed, in order to control the application of the prohibitions laid down in Article 7 and Annex IV to Regulation (EC) N°999/2001 (feed ban). The final destination of the compound feed or feed material determines which of the two operational protocols has to be followed: the one for feed or feed material intended for farmed animals others than aquaculture and fur animals, and the second one for feed or feed material intended for aquaculture animals. For the present test the only information conditioning the protocol to follow was the mention on the label referring to the type of feed.

The performance criteria for the legal implementation were decided as:

- **Complying** if no error was recorded concerning the operational scheme applied.
- **Non complying** if one or more errors were recorded concerning the operational scheme applied.

A ranking of legal non-compliance based on the number of errors was not considered for the present proficiency test. The reason for this choice is that it is the very first time that the correct implementation of the operational protocols is assessed.

Regarding the sample set labelling, the expected operational protocols were:

Table 2: Expected operational protocols and analyses to perform.

Sample	Label	Operational protocol for the analysis of feed or feed material intended for
1	Poultry feed	Farmed animals other than aquaculture and fur animals (microscopy)
2	Fish feed	Aquaculture animals (microscopy and PCR)
3	Horse feed	Farmed animals other than aquaculture and fur animals (microscopy)
4	Fish feed + haemoglobin meal	Aquaculture animals (PCR only)*
5	Trout juveniles feed	Aquaculture animals (microscopy and PCR)
6**	Trout juveniles feed	Aquaculture animals (microscopy only)

(* except if the last paragraph of point 7.3 of the SOP was argued, ** out of proficiency assessment)

3.4.2. Light microscopy

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

Samples 2 and 5 are to be declared positive for both terrestrial and fish material presence.

All other samples are to be declared negative for both parameters.

In addition special attention was put on the description of the type of identified particles. For instance sample 2 which would have been declared as positive for terrestrial PAP because of the sole identification of blood particles (linked to the presence of haemoglobin) but whitout any mention of terrestrial bones was considered as erroneous.

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.

3.4.3. PCR

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

Samples 2 and 5 are considered to be declared positive for the presence of ruminant DNA. Fishfeed I present in sample 2 contained a source of ruminant DNA (probably from the declared haemoglobin) and must be declared positive for the presence of ruminant DNA. The ruminant PAP content of sample 5 (Fishfeed III + 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 could 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 1 wrong results 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 3 summarizes the results.

Table 3: 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	Poultry feed	10	-	-	5	-	3	-	ND	ND
2	Fishfeed I + 0.1 % porcine PAP	10	+	+	5	+	10	+	+	+
3	Horse feed	10	-	-	5	-	3	-	ND	ND
4	Fishfeed II with haemoglobin meal	3	-*	+	3	+	10	-	+	+
5	Fishfeed III + 0.05 % ruminant PAP	10	+	+	5	+	10	+	ND	+
6	Fishfeed IV + 0.5 % insect PAP	10	-	+	5	+	3	-	ND	+

(Legend: ND = not tested, + = systematically detected, - = systematically not detected, NIRM = near infrared microscopy, *only blood particles)

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 (3 for sample 3).

Sample 1 (Poultry feed) was systematically negative for any vertebrate particle traces.

Sample 2 (Fishfeed I + 0.1 % porcine PAP) revealed to be always positive for terrestrial particles and always positive for fish. Slides prepared from the flotate always presented few blood particles. PCR revealed it as positive for ruminant, porcine and fish DNA.

Sample 3 (Horse feed) was free from any particle from animal origin.

Sample 4 (Fishfeed II with haemoglobin meal) was always positive for fish particles. About the detection of terrestrial particles, only blood particles were observed. PCR revealed the sample as positive for fish and porcine DNA and negative for ruminant DNA.

Sample 5 (Fishfeed III + 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 IV + 0.5 % insect PAP) was always found positive for fish particles and, on the exclusion of insect fragments which were always detected, negative for terrestrial particles. PCR analyses showed the sample as negative for ruminant DNA and positive for fish DNA.

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

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. Legal compliance

Many NRLs did not apply properly the SOP on operational protocols and were not complying (Labs 1, 2, 10, 13, 15, 18, 19, 20 and 26). Only 64 % of the NRL applied correctly the SOP related to operational protocols.

Microscopic analyses were performed on sample 4 whereas it was not authorised since it was a fishfeed knowing to contain blood product as labelled. NRLs in such situation were labs 1, 10, 13, 15, 18, 19, 20 and 26. In addition this led to erroneous results:

- Labs 15 and 20 declared sample 4 as negative for terrestrial animals (without mentioning the presence of blood particles).
- Lab 18 declared it as positive for terrestrial animals but based on the presence of feathers.
- Lab 19 declared one replicate of sample 4 as negative for terrestrial animals (without mentioning the presence of blood particles) and the second one as positive due to records of terrestrial bones.

Eventually, lab 24 mentioned at the delivery of the results to have chosen for microscopic observation first in reference to the last paragraph of point 7.3 of the SOP which specifies that “PCR method shall only be applied in the first instance at a frequency of one in ten samples (1/10) chosen on a random basis”. Although the intent of this paragraph is that of limiting the number of alerts to be posted on the RASFF platform and is not in line with the spirit of the proficiency test, this decision had to be considered as a correct interpretation of the SOP. For this reason lab 24 was strictly but paradoxally compliant with the SOP.

About correct appliance of PCR analyses:

- Lab 2 was not complying; it is the lab that did not deliver any PCR result.
- Lab 15 and 20 did not analyse the two replicates of sample 4 (probably as a consequence of having declared them as negative by microscopy).
- Lab 19 performed PCR analyses on the whole sample set.

From this synthesis it appears that lab 19 does not follow the mandatory SOP on the operational protocols. In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), this NRL is asked to deliver explanation on this legal non-compliance.

4.2. Microscopy results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. Results and performance of the network

Table 4 summarizes the results submitted by the 25 NRLs for the sample types submitted to microscopic analysis.

Table 4: Global results expressed as accuracy (AC) – light microscopy

Sample	Material	n	AC	
			Terrestrial	Fish
1	Poultry feed	50	1.000	0.960 (2)
2	Fishfeed I + 0.1% porcine PAP	25	0.920 (2)	1.000
3	Horse feed	25	1.000	1.000
4	Fishfeed II with haemoglobin meal		NA	NA
5	Fishfeed III + 0.05 % ruminant PAP	25	0.962 (1)	0.962 (1)
6	Fishfeed IV + 0.5 % insect PAP		NA	NA

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; NA = not applicable).

The overall results, expressed in terms of global accuracy (AC) reveal the excellence of the NRL network for the detection of PAPs. The percentage of total error only accounted for 5 % of the total responses. On one exception, fish material is systematically detected when it must. There are two cases of specificity issues for fish material in the poultry feed. The sensitivity for the detection of terrestrial animal particles is perfectible for sample 2 where two ND were recorded and for sample 5 where one ND was reported.

4.2.1.2. Detailed review of results per sample

Sample 1: Poultry feed

PD for fish particles:

- Lab 11 reported sea shells (in each replicate)

This participant erroneously considered shell grits as fish meal and therefore declared the sample as positive for the presence of fish, despite he mentioned in the details of the observation that no fish was detected. This misunderstanding situation was clearly explained in the report of the EURL-AP proficiency test of 2016 [8] and confirmed by DG Sante. A good example regarding such situation is delivered by labs 4, 22 and 24. Indeed these latter participants applied correctly the legislation: they declared in the details of their observations (or on the summary PDF) the presence of shell fragments but declared the sample as free from fish material.

Sample 2: Fishfeed I + 0.1 % porcine PAP

ND for terrestrial particles:

- Labs 1 and 7 failed at detecting the porcine PAP

The present situation deserves some explanation. Actually, these two participants mentioned the presence of terrestrial particles but only referred to blood and haemoglobin while being unable to detect the presence of the PAP and its bone fragments. This type of situation is unusual and requires investigation on the reason why the presence of bones was overlooked, especially when 2 repetitions of the analyses were performed as indicated by these labs.

The presence of blood or haemoglobin was mentioned by 11 NRLs out of the 25. From the fishfeed declaration, haemoglobin effectively represented 10 % of the feed composition. Although it accounts for a large mass fraction of the feed, its identification from the raw fraction (3 on 5) or from the flotate (8 on 20) still remains difficult.

Sample 3: Horse feed

No error was noted.

Sample 4: Fishfeed II with haemoglobin meal

No microscopic analysis had to be realised.

Non-compliant labs that nevertheless analysed this sample made several errors which are detailed under point 4.1.

Sample 5: Fishfeed III + 0.05 % ruminant PAP

ND for terrestrial particles:

Lab 19 recorded a <LOD case for terrestrial material. It detected not enough bones; the most likely explanation for this situation could be a low recovery rate from the sedimentation process.

ND for fish particles:

- Lab 27 failed at detecting any fish particle.

Sample 6: Fishfeed IV + 0.5 % insect PAP

As explained under point 3.2.1., this sample was not considered for proficiency assessment.

Results obtained for this sample were as regards the detection of:

- Fish particles : AC = 1.000
- Terrestrial particles : AC = 0.920
- Insect particles : AC = 0.160

If the detection of fish particles and terrestrial particles occurred without any difficulties, on the exception of two laboratories that declared it as positive for terrestrial due to the detection of bones and blood particles, the detection of insect particles failed. Only 4 laboratories out of 25 were able to identify correctly the presence of the insect PAP. From these 4 laboratories, only one mentioned this presence in the details of the microscopic observations, while the 3 others referred to this presence either as a comment in the PDF summarizing the results or in the accompanying email.

4.2.1.3. 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 material and of fish material. Results are to be found in Tables 5 and 6. A ranking of the labs was prepared based on the consolidated accuracy.

Tables 5 (left) and 6 (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
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
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
13	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
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
24	1.000	1.000	1.000
26	1.000	1.000	1.000
27	1.000	1.000	1.000
1	0.800	0.500	1.000
7	0.800	0.500	1.000
19	0.800	0.500	1.000

Fish

lab code	AC	SE	SP
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
12	1.000	1.000	1.000
13	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
24	1.000	1.000	1.000
26	1.000	1.000	1.000
27	0.800	0.500	1.000
11	0.600	1.000	0.333

Details of the results were commented in section 4.2.1.2.

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 7).

24 labs out of 25 NRLs or in other words 96 % of the NRLs performed very well. One NRL performed satisfyingly and no NRL was underperforming for microscopic analyses.

Table 7: 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.

Consolidated			
lab code	**AC**	**SE**	**SP**
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
8	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
13	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
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
24	1.000	1.000	1.000
26	1.000	1.000	1.000
1	0.900	0.750	1.000
7	0.900	0.750	1.000
19	0.900	0.750	1.000
27	0.900	0.750	1.000
11	0.800	1.000	0.667

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

Individual performances from the 4 participants outside the EU were assessed exactly as in previous section (4.2.1.3). A ranking of those labs was prepared as well based on the consolidated accuracy.

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

Tables 8 (left) and 9 (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	1.000	1.000	1.000
34	0.800	0.500	1.000
32	0.600	0.000	1.000
31	0.400	0.000	0.667

Fish			
lab code	AC	SE	SP
30	1.000	1.000	1.000
31	0.800	1.000	0.667
34	0.800	0.500	1.000
32	0.600	1.000	0.333

The error details are described per sample:

Sample 1: Poultry feed

PD for terrestrial particles:

- Lab 31 reported bones

PD for fish particles:

- Lab 31 reported fishbones
- Lab 32 (without details)

Sample 2: Fishfeed I + 0.1 % porcine PAP

ND for terrestrial particles:

- Labs 31 and 32 failed at detecting the porcine PAP

Lab 34 presented a <LOD case while reporting the presence of bones.

None of the non-EU lab reported on the presence of blood particles.

Sample 3: Horse feed

No error was noted. However lab 34 presented a <LOD case and mentioned the presence of bones.

Sample 4: Fishfeed II with haemoglobin meal

No microscopic analysis had to be realised. Lab 30 did not perform light microscopic analyses, indicating that the SOP on the operational schemes was followed.

Labs that nevertheless analysed this sample made errors: labs 31 and 32 recorded the presence of terrestrial bones.

Sample 5: Fishfeed III + 0.05 % ruminant PAP

ND for terrestrial particles:

Labs 19 and 32 recorded <LOD cases.

ND for fish particles:

Lab 34 recorded a <LOD case.

Sample 6: Fishfeed IV + 0.5 % insect PAP

As explained under point 3.2.1., this sample was not considered for proficiency assessment.

No insect particles were mentioned by the non-EU participants

A general ranking as for the NRL network was established (Table 10).

One participant performed excellently and another one performed satisfyingly (line in blue in Table 10). The two other participants were classified as underperforming (lines in red in Table 10) according to the applied criteria.

Table 10: 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 blue refer to satisfying results. Lines in red refer to underperforming results.

Consolidated			
lab code	**AC**	**SE**	**SP**
30	1.000	1.000	1.000
34	0.800	0.500	1.000
31	0.600	0.500	0.667
32	0.600	0.500	0.667

4.3. PCR results

4.3.1. Qualitative analyses from the NRLs

4.3.1.1 On the respect of the instructions

The NRLs seem to stick generally to the SOPs. Only one deviation is to notice: lab 26 deviated from the mandatory DNA extraction method ("Wizard® Magnetic DNA purification system for Food" kit, Promega, Madison, WI, USA). The participant extracted the DNA from a larger test portion (2 g) and performed the extraction according to a procedure with chloroform commonly in use in the laboratory. This deviation explains most probably the 2 PD obtained by lab 26 and its underperforming status.

4.3.1.2 Overview of results and global performance of the network

Table 11 summarizes the results provided by 25 NRLs* for the three sample types submitted to qualitative PCR analysis.

Table 11: Global results expressed as accuracy (AC) –PCR

Sample	Material	n	AC
2	Fishfeed I + 0.1 % porcine PAP	25	0.960 (1)
4	Fishfeed II with haemoglobin meal	50	0.840 (8)§
5	Fishfeed III + 0.05 % ruminant PAP	25	0.960 (1)

Accuracy means sensitivity in case of ND and specificity in case of PD. The absence of a PCR result when expected is considered as a deviation (ND or PD). In brackets the number of false results. (Legend: n = number of results, § one absent PCR result was not considered as a deviation based on the justification of the participant)

* One NRL provided results for light microscopy only and was not able to do it for PCR. All expected results were considered as erroneous.

For the two samples containing PAP (sample types 2 and 5), the overall results, expressed in terms of global accuracy (AC), are quite good. The presence of terrestrial PAP detected by all the participants performing a microscopic analysis was systematically followed by a PCR analysis to determine the origin of the PAP. All PCR analyses performed led to a correct detection of ruminant DNA in the samples. The deviations recorded (one per sample type) were only due to the absence of PCR results from lab 2.

Sample type 4 was a fishfeed containing haemoglobin meal. This sample type was present in duplicate in the sample set. According to the SOP about the operational protocols for combination of the methods (light microscopy and PCR) and due to the presence of blood product in the composition of the sample, the detection of ruminant DNA by PCR was requested. The absence of ruminant DNA was correctly concluded by a majority of the participants (80 % or 20 labs out of 25). Lab 26 returned two positive results (2 PD). Based on the last paragraph of point 7.3 of the SOP on operational protocol, lab 24 analysed correctly (NA) only one of the 2 samples and considered the PCR analysis of the second sample as superfluous. Two labs (labs 15 and 20) stopped the analyses of the 2 samples of this type after the microscopy. Lab 2 did not submit any PCR result.

4.3.1.3 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 12 that summarizes the results obtained by the participants for the analyses of the three sample types (sample type 2, 4 and 5) representing a total of 4 samples.

Table 12: NRL proficiencies regarding the detection of ruminant material. Ranking follows AC values. Cell in blue refers to a satisfying NRL. Cells in red refer to underperforming NRLs.

Lab code	AC	SE	SP
1	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
13	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
21	1.000	1.000	1.000
22	1.000	1.000	1.000
23	1.000	1.000	1.000
27	1.000	1.000	1.000
24*	1.000*	1.000*	1.000*
15	0.500	1.000	0.000
20	0.500	1.000	0.000
26	0.500	1.000	0.000
2	0.000	0.000	0.000

*** Lab 24 provided only 3 of the 4 expected PCR results but justified the absence of the last one on the basis of an objective interpretation of the SOP on operational protocol. Lab 24 is therefore considered as perfectly performing but its accuracy, specificity and sensitivity were calculated on 3 results instead of 4.**

Table 12 illustrates the excellent level of global performance for 21 labs out of 25 NRLs (84 % of the NRLs) having no false result. Four labs were underperforming: labs 15 and 20 did not deliver 2 PCR results whereas lab 26 obtained 2 PD. Lab 2 did not deliver any PCR result.

4.3.1.4 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.

Only one participant (lab 3) did not reach the minimum criterion of 9.00 copies (8.84 copies). The percentage of the labs with a cut-off corresponding to a number of copies > 10 for this proficiency test was 64.0 % (59.3 % in 2016 [8]; 65.4 % in 2015 [7] ; 70.4 % in 2014 [10] ; 55.6 % in 2013 [11]). In the case of lab 3, the too low copy number at the cut-off did not have any influence on the results (no deviation). The 2 positive deviations recorded (lab 26) are not due to a cut-off problem.

4.3.2. Qualitative analyses from the non-EU participants

4.3.2.1. Individual performances

Individual performances were assessed for each of these participants by calculating the accuracy, sensitivity and specificity over the samples. Their results are to be found in Table 14.

Table 14 : Non-EU participant proficiencies regarding the detection of ruminant material. Ranking follows AC values.

Lab code	AC	SE	SP
30	1.000	1.000	1.000
34	1.000	1.000	1.000
32	0.750	0.500	1.000
31	0.250	0.000	0.500

Labs 30 and 34 obtained excellent results (no deviation).

Concerning Lab 32, one deviation is recorded with the sample 2 (Fishfeed I + 0.1 % porcine PAP). The sample was not analysed by PCR. No terrestrial particle was identified by light microscopy. Under the assumption that this participant is following the SOP related to the operational protocols, it is justifying stopping the analyses.

Lab 31 obtained 3 false results (2 ND with sample types 2 and 5 as well as 1 PD out of 2 analyses with sample type 4). This participant probably uses another method as no cut-off value nor Ct values were reported.

4.3.2.2. Assessment of the cut-off values

Lab 31 gave no information about the cut-off value and the Ct values. Lab 34 commented the results with the mention “Final point PCR”.

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

This proficiency test is the first one proposed to the NRL network without any indication on the method to be used. The selection of the analytical method, light microscopy and/or PCR, was entirely under the responsibility of the operator. The only leading indication was the label on the vial referring to the type of feed. The choice of the method relied on the understanding and interpretation of the SOP on operational protocols for the combination of light microscopy and PCR [5] which is a mandatory complement to regulation EC/51/2013 [3]. Testing the understanding of this SOP revealed particularly challenging. Results effectively demonstrated that some NRLs have difficulties to apply the operational schemes. The number of non-compliant NRLs as regards the respect of the operational protocols is 36 %. Different interpretations of the SOP, especially of the last paragraph of point 7.3 mentioning that "PCR method shall only be applied in the first instance at a frequency of one in ten samples (1/10) chosen on a random basis", were highlighted by this study. This was not expected by the organiser. On the other hand, making non authorized analyses may lead to errors in the results and discrepancies between official control laboratories. Therefore following strictly the SOP on the operational schemes is a legal requirement. If it were not the case it would jeopardise the harmonisation of the method selection. It cannot be ruled out in the future that such errors would lead to an underperforming status (instead of the current "non-complying" mention) of the NRLs with consequences similar to underperformances in analytical errors, thus submitted to explanation and/or corrective actions. For this reason clear explanation is already requested for lab 19. For now the difference of interpretation of point 7.3 is revealing a weakness of the third version of the SOP. Two options can be envisaged to avoid this situation. The first one would be to mention in the instructions of future proficiency tests that participants should not consider the last paragraph of that point. This type of mention, or exception to the official method, is already used for the issue of the minimal amount of material. The reason is strictly for practical and organisational reasons and is therefore accepted. However in the present case, this option would only resolve the problem for the organisers of proficiency tests but not the one of lack of harmonisation in the method selection. A second option is therefore recommended, namely a revision work of the SOP consisting of a modification or a deletion of this confusing section.Results obtained from light microscopic analyses were very good. No major problems were observed. The only NRL that did not performed excellently was lab 11 which made an erroneous interpretation of legal text with regards to shell grids, still considering this material as fish material. This led to analytical errors. Nevertheless, this very good performance must be prudently interpreted. The organisers made a clear separation between legal non-compliance related to the combination of methods on one side and analytical errors on the other side. If both evaluations would have been merged the outcome would have been less optimal.

Furthermore, the challenge of the fishfeed fortified with insect PAP was also excluded from any proficiency assessment. This is justified by the current state of the art in insect PAP isolation and identification, but also by the absence of categorisation of this new type of material. Indeed Annex VI of EC/152/2009 only proposes two categories of origin (i.e. nature according to the wording of the text): terrestrial and fish. At present, since insect PAPs are authorised ingredients, according to present legal framework they should be identified as from terrestrial origin. Such situation would generate a lot of confusions if not alerts. Therefore a revision of the Annex VI is needed with both the introduction of the double PE/TCE sedimentation step, when insect PAPs are needed to be detected, and a third category allowing to sort insect particles as opposed to terrestrial and fish. This was also discussed and recommended in the preceding EURL-AP proficiency test [8]. The term that should be used to refer to this third category has to refer to invertebrates. Further specification in terrestrial invertebrates might be the most adequate since other invertebrates, from marine origin will fall in the fish category.

Coming back on the insect detection issue, this study evidenced that the current sample preparation and/or observation protocol as per Annex VI is not adapted for this purpose. The calculated sensitivity score for proper insect detection was of only 0.160 which is very poor. The dedicated sample preparation by the double PE/TCE sedimentation before microscopic observations is necessary to better concentrate insect fragments. Education through training and image libraries constitutes the second pillar for insect fragment identification and is certainly to promote.

Concerning non-EU participants, only one out of four performed excellently for microscopy. Two out of them performed unsatisfyingly when compared to the same performance criteria as those applied for the NRL network. The absence of insect detection by non-EU participants was generalised. This is not a surprise since this type of product is new and their analytical operators, like these from NRLs, are not trained for this.

In a first lecture, the PCR results recorded during this proficiency test could be interpreted as showing a lower performance of the NRL network. The occurrence of deviations (ND or PD) is higher than in the two previous combined microscopy-PCR proficiency tests and the number of underperforming NRLs (4 labs) is also higher [7, 8]. However, this observation must be put into perspectives.

The underperformances of 2 participants explaining 6 out of the 10 deviations are due to organizational and resources problems that must be addressed and solved :

- Lab 2 was not able to return any PCR result in time with the consequence that the four missing expected results were considered as deviations ;
- Lab 26 did not use the mandatory DNA extraction method. The 2 PD of this NRL can be easily explained by this deviation to the protocol and underlines once again the importance to stick to the mandatory SOPs.

Two other NRLs (lab 15 and lab 20) were declared as underperforming for the PCR as two PCR expected results were missing. The decision to not perform the PCR analyses on two samples of their set was probably taken on the basis of negative deviating results obtained with the light microscopy for the detection of terrestrial animal particles. Nevertheless, the indication on the label of the presence of haemoglobin meal in these samples should had incited to check the non-ruminant origin of this blood product. All the other expected PCR analyses lead to correct results.

In the case of lab 24, one expected PCR analysis was not performed based on an interpretation of point 7.3 of the SOP on operational protocols.

As a positive conclusion, it must be emphasized that a wide majority of the NRLs (80 %) performed the PCR analyses excellently. Moreover, except for lab 26, all other results delivered by the NRLs were correct. The apparent decrease of the PCR proficiency can therefore be attributed to the additional difficulty inherent in the choice of the method(s) to apply.

Concerning the non-EU participants, the four participating labs delivered results for the PCR. Two of them (lab 30 and lab 34) submitted perfect results. One negative deviation corresponding to a missing result was recorded for lab 32. Lab 31 is underperforming with only 1 correct result out of the four expected ones.

Acknowledgment

We are grateful to the EURL-AP technical staff for their preparation work and the efforts made to meet the ISO 17043 requirements: M. Collard, J. Hulin, J. Maljean and B. Scaut. 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
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
Croatia	Croatian Veterinary Institute
Cyprus	Cyprus Veterinary Services
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)
Mexico	*Centro Nacional de Servicios de Constatacion en Salud Animal*
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 - Institute of Food Safety, Feed and Environment - Department of Environment, Animal Nutrition, Welfare and 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
Chaussée de Namur 24, B – 5030 GEMBLOUX

☎ 32 (0) 81 62 03 75 ℻ 32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Announcement of the EURL-AP proficiency test 2017/01 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.

Objectives

The first 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 second objective is to assess the correct application of the legal conditions for the choice of the analytical method with respect to the SOP Operational protocols for the combination of light microscopy and PCR (Version 3.0)

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 legislation 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.

European Union Reference Laboratory for Animal Proteins in feedingstuffs

Walloon Agricultural Research Centre, Valorisation of Agricultural Products Department
Henseval building
Chaussée de Namur 24, B – 5030 GEMBLOUX

☎32 (0) 81 62 03 75 32 (0) 81 62 03 88
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : **6 September 2017**
- Sending of the sample boxes and communication of the instructions : **17 October 2017**

> ***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 : **17 November 2017**

Further information

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

or

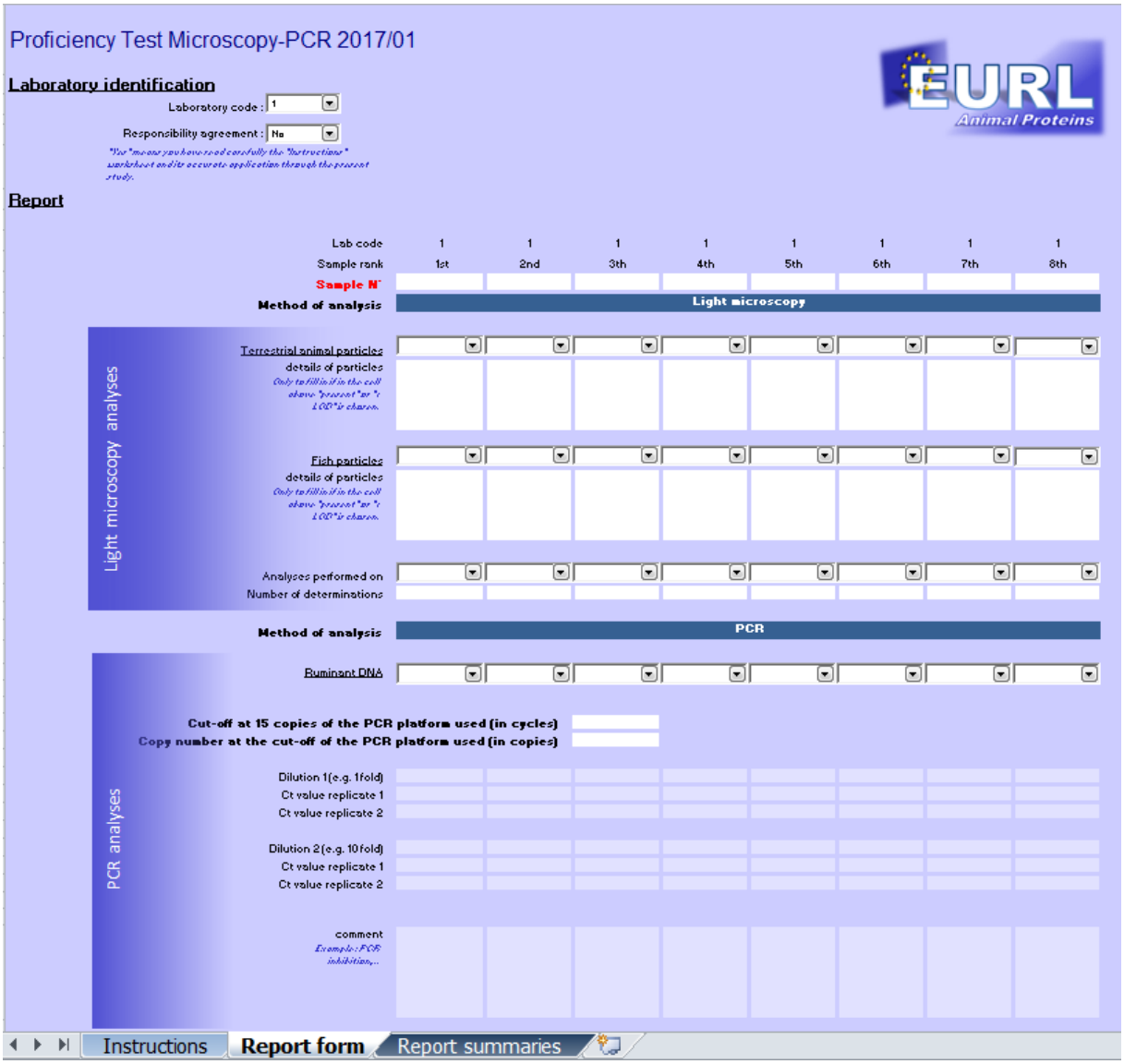
- Dr Pascal VEYS
  EURL-AP NRL Network Manager
  ☎32 (0) 81 62 03 75
  32 (0) 81 62 03 88
  E-mail: p.veys@cra.wallonie.be

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



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
2	682	Present	blood	Present	Muscle fibers, fishbones, scales, gills, cartilage	Sed. + Raw	2
4	1370	Present	blood	Present	Muscle fibers, fishbones, cartilage, gills	Sed. + Raw	1
4	1650	Present	blood	Present	Muscle fibers, fishbones, scales, cartilage	Sed. + Raw	1
5	1938	Present	bones	Present	Muscle fibers, fishbones	Sed. + Raw	1
3	2482	Absent		Absent		Sed. + Raw	1
6	2562	Absent		Present	Muscle fibers, fishbones, scales, cartilage	Sed. + Raw	1
1	3026	Absent		Absent		Sed. + Raw	1
1	3082	Absent		Absent		Sed. + Raw	1

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
3	578	Absent		Absent		Sed. + Flot.	1
2	2250	Present	bones, muscle fibers	Present	bones, gills, scales, muscle fibers	Sed. + Flot.	1
1	2690	Absent		Absent		Sed. + Flot.	1
5	2778	Present	bones, muscle fibers	Present	bones, cartilages, muscle fibers	Sed. + Flot.	1
1	3138	Absent		Absent		Sed. + Flot.	1
6	4242	Present	bones, muscle fibers	Present	bones, gills, scales, cartilages, muscle fibers	Sed. + Flot.	1
4	2042						
4	1818						

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	394	Absent		Absent		Sed. + Flot.	1
2	794	Present	bones cartilage	Present	bones, cartilage, muscle, tooth.gills, scales, otolith	Sed. + Flot.	1
5	1042	Present	bones cartilage	Present	bones, cartilage, muscle, tooth.gills, scales, otolith	Sed. + Flot.	1
6	4298	Absent		Present	bones, cartilage, muscle, tooth.gills, scales, otolith	Sed. + Flot.	1
4	1538						1
3	1922	Absent		Absent		Sed. + Flot.	1
1	2130	Absent		Absent		Sed. + Flot.	1
4	2490						

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	**186**	Absent		Absent		Sed. + Flot.	1
1	**562**	Absent		Absent		Sed. + Flot.	1
6	**938**	Present	blood particles	Present	bones, cartilage, gill, muscles	Sed. + Flot.	1
4	**1426**						
1	**1514**	Absent		Absent		Sed. + Flot.	1
5	**2442**	Present	bones	Present	bones, cartilage, muscles	Sed. + Flot.	1
2	**2642**	Present	bones, blood particles	Present	bones, cartilage, scale, muscles	Sed. + Flot.	1
4	**4114**						

This lab reported in the summary PDF the presence of insect in sample type 6 and shell particles in sample type 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
4	**418**						
5	**1714**	Present	bones, muscles	Present	bones, gills, scales, muscles	Sed. + Flot.	1
3	**2370**	Absent		Absent		Sed. + Flot.	1
2	**2530**	Present	bones, muscles, blood	Present	bones, gills, scales, muscles	Sed. + Flot.	1
4	**3274**						
6	**3346**	Absent		Present	bones, gills, scales, muscles	Sed. + Flot.	1
1	**3642**	Absent		Absent		Sed. + Flot.	1
1	**4314**	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	**1050**	Absent		Present	muscles, fishbones	Sed. + Flot.	2
4	**1202**						
5	**1994**	Present	bones	Present	muscles, fishbones	Sed. + Flot.	2
2	**2026**	Present	bones	Present	muscles, fishbones	Sed. + Flot.	2
3	**2202**	Absent		Absent		Sed. + Flot.	2
4	**3778**						
1	**4370**	Absent		Absent		Sed. + Flot.	2
1	**4426**	Absent		Absent		Sed. + Flot.	2

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
3	**1698**	Absent		Absent		Sed. + Flot.	2
1	**2018**	Absent		Absent		Sed. + Flot.	2
6	**2394**	Absent		Present	Fish bones, muscles, otholites, scales	Sed. + Flot.	2
4	**2658**						
2	**2698**	Present	blood and haemoglobine dried	Present	Fish bones, muscles, otholites, scales	Sed. + Flot.	2
5	**2722**	Present	bones and muscles	Present	Fish bones, muscles, otholites, scales	Sed. + Flot.	2
1	**2914**	Absent		Absent		Sed. + Flot.	2
4	**4450**						

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	226	Absent		Absent		Sed. + Flot.	1
3	1418	Absent		Absent		Sed. + Flot.	1
4	1762						
2	1970	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
5	2050	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
6	2226	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	3610						
1	4818	Absent		Absent		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
4	698						
6	994	Absent		Present	fish bone, scale, gill, cartilage, otolith, muscle	Sed. + Flot.	1
2	1410	Present	bone	Present	fish bone, scale, cartilage, muscle	Sed. + Flot.	1
5	1434	Present	bone	Present	fish bone, scale, muscle	Sed. + Flot.	1
3	2090	Absent		Absent		Sed. + Flot.	1
1	3922	Absent		Absent		Sed. + Flot.	1
4	3946						
1	4034	Absent		Absent		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	1474	Absent		Absent		Sed. + Raw	1
5	1826	Present	bones	Present	fish bones, scales, cartilage, otolithes, muscle fibers	Sed. + Raw	1
1	2298	Absent		Absent		Sed. + Raw	1
2	2418	Present	bones blood	Present	cartilage, scales, fishbones, muscle fibers, gills	Sed. + Raw	1
4	2770	Present	blood	Present	gills, fishbones, scales, cartilage	Sed. + Raw	1
1	3530	Absent		Absent		Sed. + Raw	1
4	3554	Present	blood	Present	gills, fishbones, scales, cartilage, otolithes	Sed. + Raw	1
6	4130	Absent		Present	gills, fishbones, scales, cartilage, otolithes	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
1	**58**	Absent		Present	Sea shells were detected. No fish was detected. According to Comm. Reg. 2017/786 mollusc belonging to the Phylum Mollusca are included in the definition of fish meal.	Sed. + Flot.	1
1	**1346**	Absent		Present	Sea shells were detected. No fish particles were detected. According to Comm. Reg. 2017/786 mollusc belonging to the Phylum Mollusca are included in the definition of fish meal.	Sed. + Flot.	1
5	**2106**	Present	terrestrial bone particles >10, meat fibers	Present	> 50 fish bone particles, scales, otoliths	Sed. + Flot.	1
3	**2146**	Absent		Absent		Sed. + Flot.	1
2	**2754**	Present	terrestrial bone particles >10, meat fibers, blood	Present	> 50 fish bone particles, scales, otoliths	Sed. + Flot.	1
4	**2882**						
6	**3626**	Absent		Present	> 50 fish bone particles, scales, otoliths, Notice! many particles of insects were detected	Sed. + Flot.	1
4	**4786**						

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
4	**250**						
4	**362**						
5	**650**	Present	bone fragments (cartilage fragments have been attributed to fish)	Present	fish bones, muscle fibres, cartilage	Sed. + Flot.	2
6	**1386**	Absent		Present	fish bones, muscle fibres,	Sed. + Flot.	1
2	**1522**	Present	bone fragments (cartilage fragments have been attributed to fish)	Present	fish bones, muscle fibres, cartilage	Sed. + Flot.	1
3	**1530**	Absent		Absent		Sed. + Flot.	1
1	**2802**	Absent		Absent			1
1	**4090**	Absent		Absent			1

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
4	138	Present	blood particles	Present	bones, cartilage, gills, muscle fibers	Sed. + Raw	1
1	1122	Absent		Absent		Sed. + Raw	1
2	1186	Present	blood particles, bones	Present	bones, cartilage, gills, muscle fibers	Sed. + Raw	1
5	2218	Present	bones	Present	bones, muscle fibers	Sed. + Raw	1
3	2538	Absent		Absent		Sed. + Raw	1
4	4002	Present	blood particles	Present	bones, cartilage, gills, muscle fibers	Sed. + Raw	1
6	4634	Absent		Present	bones, cartilage, gills, muscle fibers	Sed. + Raw	1
1	4874	Absent		Absent		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
1	618	Absent		Absent		Sed. + Flot.	1
2	1130	Present	bones	Present	bones,cartilage	Sed. + Flot.	2
5	1154	Present	bones	Present	bones,cartilage	Sed. + Flot.	1
3	1194	Absent		Absent		Sed. + Flot.	1
4	2434	Absent		Present	bones,cartilage,muscle.	Sed. + Flot.	1
1	2578	Absent		Absent		Sed. + Flot.	1
6	4354	Absent		Present	bones,cartilage,	Sed. + Flot.	1
4	4562	Absent		Present	bones,cartilage,muscle	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
3	746	Absent		Absent		Sed. + Raw	1
2	962	Present	Bone,Cartilage,Muscle	Present	Fishbone, Cartilage, Gills, Scales,Muscle	Sed. + Raw	1
4	1034						
4	1930						
5	2498	Present	Bone,Muscle,Cartilage	Present	Fishbone, Muscle, Cartilage	Sed. + Raw	1
6	3682	Absent		Present	Fishbone, Gills, Scales, Muscle	Sed. + Raw	1
1	3866	Absent		Absent		Sed. + Raw	1
1	4706	Absent		Absent		Sed. + Raw	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
5	**426**	Present	bones, muscle fibre, cartilage	Present	fish bone, scale,gill, fish skin	Sed. + Flot.	3
4	**866**						
1	**1010**	Absent		Absent		Sed. + Flot.	1
1	**1458**	Absent		Absent		Sed. + Flot.	1
2	**1690**	Present	bones, blood, muscle fibre, cartilage	Present	fish bone, scale,gill, fish skin, muscle fibre, cartilage	Sed. + Flot.	1
6	**2058**	< LOD	bones, muscle fibre, cartilage	Present	fish bone, scale,gill, fish skin, muscle fibre, cartilage	Sed. + Flot.	2
3	**2594**	Absent		Absent		Sed. + Flot.	1
4	**4226**						

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
6	**42**	Absent		Present	bones, cartilages, gills, scales, muscles	Sed. + Flot.	1
3	**354**	Absent		Absent		Sed. + Flot.	1
1	**506**	Absent		Absent		Sed. + Flot.	1
2	**1018**	Present	bones, blood	Present	bones, cartilages, gills, scales, muscles	Sed. + Flot.	1
5	**1266**	Present	bones	Present	bones, cartilages, gills, scales, muscles	Sed. + Flot.	1
1	**1682**	Absent		Absent		Sed. + Flot.	1
4	**2210**	Present	feathers	Present	bones, cartilages, gills, scales, muscles	Sed. + Flot.	1
4	**3386**	Present	feathers	Present	bones, cartilages, gills, scales, muscles	Sed. + Flot.	1

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	**2**	Absent		Absent		Sed. + Flot.	1
5	**258**	< LOD	Terrestrial bone	Present	Fish bone, scale, muscle, gill, cartilage, otolith.	Sed. + Flot.	2
4	**306**	Present	Terrestrial bone	Present	Fish bone, scale, muscle, gill, cartilage, otolith, blood.	Sed. + Flot.	1
6	**378**	Absent		Present	Fish bone, scale, muscle, gill, cartilage, otolith.	Sed. + Flot.	1
3	**1306**	Absent		Absent		Sed. + Flot.	1
1	**1794**	Absent		Absent		Sed. + Flot.	1
2	**2138**	Present	Terrestrial bone	Present	Fish bone, scale, muscle, gill, blood.	Sed. + Flot.	1
4	**4898**	Absent		Present	Fish bone, scale, muscle, gill, cartilage, blood.	Sed. + Flot.	1

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	**178**	Present	Bones	Present	Fishbones, cartilages	Sed. + Flot.	2
3	**970**	Absent		Absent		Sed. + Flot.	1
5	**1098**	Present	Bones	Present	Fishbones, cartilages	Sed. + Flot.	2
1	**2410**	Absent		Absent		Sed. + Flot.	2
4	**2994**	Absent		Present	Fishbones, cartilages	Sed. + Flot.	2
1	**3250**	Absent		Absent		Sed. + Flot.	2
6	**3290**	Absent		Present	Fishbones, cartilages	Sed. + Flot.	2
4	**3666**	Absent		Present	Fishbones, cartilages	Sed. + Flot.	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
1	**170**	Absent		Absent		Sed. + Flot.	1
2	**234**	Present	bones, blood	Present	bones, cartilage, gills,scales, meatfibers	Sed. + Flot.	1
6	**546**	Absent		Present	bones, cartilage, gills,scales, meatfibers	Sed. + Flot.	1
5	**818**	Present	bones	Present	bones, cartilage, gills,scales, meatfibers	Sed. + Flot.	1
3	**914**	Absent		Absent		Sed. + Flot.	1
1	**1850**	Absent		Absent		Sed. + Flot.	1
4	**2322**						
4	**3442**						

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
6	**770**	Absent		Present	Bones, muscle	Sed. + Flot.	1
5	**1378**	Present	Bones, muscle	Present	Bones, muscle	Sed. + Flot.	1
2	**1466**	Present	Bones, muscle, blood, hair	Present	Bones, muscle	Sed. + Flot.	1
3	**1754**	Absent		Absent		Sed. + Flot.	1
1	**2354**	Absent		Absent		Sed. + Flot.	1
1	**2522**	Absent		Absent		Sed. + Flot.	1
4	**2826**						
4	**4730**						

This lab reported in the summary PDF the presence of "insect meal" in sample type 6

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	**410**	Absent		Absent		Sed. + Raw	2
4	**586**						
2	**906**	Present	bones, muscle fibers	Present	fish bones, muscle fibers, scales, cartilage	Sed. + Raw	2
1	**1178**	Absent		Absent		Sed. + Raw	2
5	**2554**	Present	bones, muscle fibers	Present	fishbones, muscle fibers, scales	Sed. + Raw	2
6	**2618**	Absent		Present	fishbones, muscle fibers, scles, cartilage	Sed. + Raw	2
1	**2858**	Absent		Absent		Sed. + Raw	2
4	**4170**						

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
4	**810**	Present	blood	Present	fishbones,scales,cartilage,muscles	Sed. + Flot.	1
2	**1242**	Present	terrestrial bones + blood	Present	fishbones,scales,cartilage	Sed. + Flot.	1
3	**1250**	Absent		Absent		Sed. + Flot.	1
5	**1658**	Present	terrestrial bones	Present	fishbones,scales,cartilage,muscles	Sed. + Flot.	1
6	**3234**	Absent		Present	fishbones,scales,cartilage,muscles	Sed. + Flot.	1
1	**3362**	Absent		Absent	shells present	Sed. + Flot.	1
1	**4650**	Absent		Absent	shells present	Sed. + Flot.	1
4	**4954**	Present	blood	Present	fishbones,scales,cartilage,muscles	Sed. + Flot.	1

This lab reported in the email the presence of insect in sample type 6 (and delivered pictures)

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
5	**314**	Present	bones,	Present	fish bones, cartillages, muscle fibers, gills	Sed. + Flot.	1
4	**642**						
1	**786**	Absent		Absent		Sed. + Flot.	1
6	**1946**	Absent		Present	fish bones, cartillages, muscle fibers, gills, scales	Sed. + Flot.	1
2	**2194**	Present	bones,	Present	fish bones, cartillages, muscle fibers, gills, scales	Sed. + Flot.	2
4	**2378**						
3	**2650**	Present	feather,	Absent		Sed. + Flot.	2
1	**3698**	Absent		Absent		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
1	1402	Absent		Absent		Sed. + Flot.	1
5	1546	Present	Bones	Present	Bones, cartilage, gills	Sed. + Flot.	1
4	1986	Present	Blood	Present	Bones, cartilage, gills	Sed. + Flot.	1
3	2034	Absent		Absent		Sed. + Flot.	1
2	2474	Present	Bones	Present	Bones, cartilage, gills	Sed. + Flot.	1
4	2938	Present	Blood	Present	Bones, cartilage, gills	Sed. + Flot.	1
6	3178	Absent		Present	Bones, cartilage, gills	Sed. + Flot.	1
1	4538	Absent		Absent		Sed. + Flot.	1

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
5	90	Present	bones	Absent		Sed. + Flot.	3
3	690	Absent		Absent		Sed. + Flot.	3
2	738	Present	bones	Present	fish bones, gills, scales	Sed. + Flot.	3
6	882	Absent		Present	fish bones, gills, scales	Sed. + Flot.	3
4	1258						
1	1626	Absent		Absent		Sed. + Flot.	3
4	3722						
1	4146	Absent		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
3	466	Absent		Absent		Sed. + Flot.	1
5	762	Present	bones	Present	fishbones, gills, muscles	Sed. + Flot.	1
2	850	Present	bones	Present	fishbones, gills, scales, cartilage, muscles	Sed. + Flot.	1
1	1738	Absent		Absent		Sed. + Flot.	1
4	1874						
1	4258	Absent		Absent		Sed. + Flot.	1
4	4506						
6	4578	Present	bones	Present	fishbones, scales, gills, cartilage, muscles	Sed. + Flot.	1

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
1	282	Present	bones	Absent		Sed. + Raw	1
2	1914	Absent		Present	fishbones,scale	Sed. + Raw	1
5	2610	< LOD	bones	Present	fishbones,scale	Sed. + Raw	1
3	2762	Absent		Absent		Sed. + Raw	1
4	3218	Absent		Present	fishbones	Sed. + Raw	1
6	3738	Absent		Present	fishbones,scale	Sed. + Raw	1
4	4282	Present	bones	Present	fishbones	Sed. + Raw	1
1	4986	Absent		Present	fishbones	Sed. + Raw	1

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
3	**130**	Absent		Absent		Sed. + Raw	3
1	**674**	Absent		Present		Sed. + Raw	3
5	**1210**	< LOD		Present		Sed. + Raw	3
2	**1298**	Absent		Present		Sed. + Raw	3
1	**3306**	Absent		Present		Sed. + Raw	3
6	**3458**	Absent		Present		Sed. + Raw	3
4	**978**						
4	**3050**						

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	**402**	< LOD	bones, muscle	Present	bones, muscle	Sed. + Flot.	2
3	**522**	< LOD	bones, muscle	Absent		Sed. + Flot.	2
4	**922**	Present	bones, blood, muscle	Present	bones, blood, muscle	Sed. + Flot.	2
5	**986**	Present	bones, muscle	< LOD	bones, muscle	Sed. + Flot.	2
6	**1834**	Absent		Present	bones, muscle	Sed. + Flot.	2
1	**2186**	Absent		Absent		Sed. + Flot.	2
1	**4762**	Absent		Absent		Sed. + Flot.	2
4	**5010**	Present	bones, blood, muscle	Present	bones, blood, muscle	Sed. + Flot.	2

Annex 5

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

Laboratory identification code :	1

Cut-off at 15 copies of the PCR platform used (in cycles)	35.57
Copy number at the cut-off of the PCR platform used (in copies)	11.25

Sample type	Sample N°	Ruminant DNA
1	3026	
1	3082	
2	682	Present
3	2482	
4	1370	Absent
4	1650	Absent
5	1938	Present
6	2562	

Laboratory identification code :	2

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)	

Sample type	Sample N°	Ruminant DNA
1	2690	
1	3138	
2	2250	
3	578	
4	2042	
4	1818	
5	2778	
6	4242	

Laboratory identification code :	3

Cut-off at 15 copies of the PCR platform used (in cycles)	36.09
Copy number at the cut-off of the PCR platform used (in copies)	8.84

Sample type	Sample N°	Ruminant DNA
1	394	
1	2130	
2	794	Present
3	1922	
4	1538	Absent
4	2490	Absent
5	1042	Present
6	4298	Absent

Laboratory identification code :	4

Cut-off at 15 copies of the PCR platform used (in cycles)	35.92
Copy number at the cut-off of the PCR platform used (in copies)	10.76

Sample type	Sample N°	Ruminant DNA
1	562	
1	1514	
2	2642	Present
3	186	
4	1426	Absent
4	4114	Absent
5	2442	Present
6	938	Absent

Laboratory identification code :	5

Cut-off at 15 copies of the PCR platform used (in cycles)	31.91
Copy number at the cut-off of the PCR platform used (in copies)	10.32

Sample type	Sample N°	Ruminant DNA
1	3642	
1	4314	
2	2530	Present
3	2370	
4	418	Absent
4	3274	Absent
5	1714	Present
6	3346	

Laboratory identification code :	6

Cut-off at 15 copies of the PCR platform used (in cycles)	34.50
Copy number at the cut-off of the PCR platform used (in copies)	9.73

Sample type	Sample N°	Ruminant DNA
1	4426	
1	4370	
2	2026	Present
3	2202	
4	1202	Absent
4	3778	Absent
5	1994	Present
6	1050	

Laboratory identification code :	7

Cut-off at 15 copies of the PCR platform used (in cycles)	37.16
Copy number at the cut-off of the PCR platform used (in copies)	10.90

Sample type	Sample N°	Ruminant DNA
1	2018	
1	2914	
2	2698	Present
3	1698	
4	2658	Absent
4	4450	Absent
5	2722	Present
6	2394	

Laboratory identification code :	8

Cut-off at 15 copies of the PCR platform used (in cycles)	35.72
Copy number at the cut-off of the PCR platform used (in copies)	11.68

Sample type	Sample N°	Ruminant DNA
1	226	
1	4818	
2	1970	Present
3	1418	
4	1762	Absent
4	3610	Absent
5	2050	Present
6	2226	

Laboratory identification code :	9

Cut-off at 15 copies of the PCR platform used (in cycles)	34.14
Copy number at the cut-off of the PCR platform used (in copies)	9.12

Sample type	Sample N°	Ruminant DNA
1	3922	
1	4034	
2	1410	Present
3	2090	
4	698	Absent
4	3946	Absent
5	1434	Present
6	994	

Laboratory identification code :	10

Cut-off at 15 copies of the PCR platform used (in cycles)	37.69
Copy number at the cut-off of the PCR platform used (in copies)	9.06

Sample type	Sample N°	Ruminant DNA
1	2298	
1	3530	
2	2418	Present
3	1474	
4	2770	Absent
4	3554	Absent
5	1826	Present
6	4130	

Laboratory identification code :	11

Cut-off at 15 copies of the PCR platform used (in cycles)	36.86
Copy number at the cut-off of the PCR platform used (in copies)	9.27

Sample type	Sample N°	Ruminant DNA
1	58	
1	1346	
2	2754	Present
3	2146	
4	2882	Absent
4	4786	Absent
5	2106	Present
6	3626	

Laboratory identification code :	12

Cut-off at 15 copies of the PCR platform used (in cycles)	35.14
Copy number at the cut-off of the PCR platform used (in copies)	10.39

Sample type	Sample N°	Ruminant DNA
1	2802	
1	4090	
2	1522	Present
3	1530	
4	250	Absent
4	362	Absent
5	650	Present
6	1386	

Laboratory identification code :	13

Cut-off at 15 copies of the PCR platform used (in cycles)	36.36
Copy number at the cut-off of the PCR platform used (in copies)	10.31

Sample type	Sample N°	Ruminant DNA
1	1122	
1	4874	
2	1186	Present
3	2538	
4	138	Absent
4	4002	Absent
5	2218	Present
6	4634	

Laboratory identification code :	15

Cut-off at 15 copies of the PCR platform used (in cycles)	35.61
Copy number at the cut-off of the PCR platform used (in copies)	10.79

Sample type	Sample N°	Ruminant DNA
1	618	
1	2578	
2	1130	Present
3	1194	
4	2434	
4	4562	
5	1154	Present
6	4354	

Laboratory identification code :	16

Cut-off at 15 copies of the PCR platform used (in cycles)	36.02
Copy number at the cut-off of the PCR platform used (in copies)	10.77

Sample type	Sample N°	Ruminant DNA
1	4706	
1	3866	
2	962	Present
3	746	
4	1034	Absent
4	1930	Absent
5	2498	Present
6	3682	

Laboratory identification code :	17

Cut-off at 15 copies of the PCR platform used (in cycles)	35.89
Copy number at the cut-off of the PCR platform used (in copies)	10.03

Sample type	Sample N°	Ruminant DNA
1	1010	
1	1458	
2	1690	Present
3	2594	
4	866	Absent
4	4226	Absent
5	426	Present
6	2058	Absent

Laboratory identification code :	18

Cut-off at 15 copies of the PCR platform used (in cycles)	35.34
Copy number at the cut-off of the PCR platform used (in copies)	9.58

Sample type	Sample N°	Ruminant DNA
1	506	
1	1682	
2	1018	Present
3	354	
4	3386	Absent
4	2210	Absent
5	1266	Present
6	42	

Laboratory identification code :	19

Cut-off at 15 copies of the PCR platform used (in cycles)	36.48
Copy number at the cut-off of the PCR platform used (in copies)	9.21

Sample type	Sample N°	Ruminant DNA
1	2	Absent
1	1794	Absent
2	2138	Present
3	1306	Absent
4	306	Absent
4	4898	Absent
5	258	Present
6	378	Absent

Laboratory identification code :	20

Cut-off at 15 copies of the PCR platform used (in cycles)	35.49
Copy number at the cut-off of the PCR platform used (in copies)	9.95

Sample type	Sample N°	Ruminant DNA
1	2410	
1	3250	
2	178	Present
3	970	
4	2994	
4	3666	
5	1098	Present
6	3290	

Laboratory identification code :	21

Cut-off at 15 copies of the PCR platform used (in cycles)	36.41
Copy number at the cut-off of the PCR platform used (in copies)	9.35

Sample type	Sample N°	Ruminant DNA
1	170	
1	1850	
2	234	Present
3	914	
4	2322	Absent
4	3442	Absent
5	818	Present
6	546	

Laboratory identification code :	22

Cut-off at 15 copies of the PCR platform used (in cycles)	35.48
Copy number at the cut-off of the PCR platform used (in copies)	10.21

Sample type	Sample N°	Ruminant DNA
1	2354	
1	2522	
2	1466	Present
3	1754	
4	2826	Absent
4	4730	Absent
5	1378	Present
6	770	

Laboratory identification code :	23

Cut-off at 15 copies of the PCR platform used (in cycles)	33.97
Copy number at the cut-off of the PCR platform used (in copies)	10.23

Sample type	Sample N°	Ruminant DNA
1	1178	
1	2858	
2	906	Present
3	410	
4	586	Absent
4	4170	Absent
5	2554	Present
6	2618	Absent

Laboratory identification code :	24

Cut-off at 15 copies of the PCR platform used (in cycles)	34.46
Copy number at the cut-off of the PCR platform used (in copies)	14.38

Sample type	Sample N°	Ruminant DNA
1	3362	
1	4650	
2	1242	Present
3	1250	
4	810	
4	4954	Absent
5	1658	Present
6	3234	

Laboratory identification code :	25

Cut-off at 15 copies of the PCR platform used (in cycles)	34.99
Copy number at the cut-off of the PCR platform used (in copies)	10.33

Sample type	Sample N°	Ruminant DNA
1	786	
1	3698	
2	2194	Present
3	2650	
4	642	Absent
4	2378	Absent
5	314	Present
6	1946	

Laboratory identification code :	26

Cut-off at 15 copies of the PCR platform used (in cycles)	34.23
Copy number at the cut-off of the PCR platform used (in copies)	11.08

Sample type	Sample N°	Ruminant DNA
1	1402	
1	4538	
2	2474	Present
3	2034	
4	1986	Present
4	2938	Present
5	1546	Present
6	3178	Absent

Laboratory identification code :	27

Cut-off at 15 copies of the PCR platform used (in cycles)	35.01
Copy number at the cut-off of the PCR platform used (in copies)	10.22

Sample type	Sample N°	Ruminant DNA
1	1626	
1	4146	
2	738	Present
3	690	
4	1258	Absent
4	3722	Absent
5	90	Present
6	882	

Laboratory identification code :	30

Cut-off at 15 copies of the PCR platform used (in cycles)	37.95
Copy number at the cut-off of the PCR platform used (in copies)	9.86

Sample type	Sample N°	Ruminant DNA
1	1738	
1	4258	
2	850	Present
3	466	
4	1874	Absent
4	4506	Absent
5	762	Present
6	4578	Absent

Laboratory identification code :	31

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)	

Sample type	Sample N°	Ruminant DNA
1	282	Present
1	4986	Absent
2	1914	Absent
3	2762	Absent
4	3218	Absent
4	4282	Present
5	2610	Absent
6	3738	Absent

Laboratory identification code :	32

Cut-off at 15 copies of the PCR platform used (in cycles)	37.75
Copy number at the cut-off of the PCR platform used (in copies)	10.34

Sample type	Sample N°	Ruminant DNA
1	674	
1	3306	
2	1298	
3	130	
4	978	Absent
4	3050	Absent
5	1210	Present
6	3458	

Laboratory identification code :	34

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)	

Sample type	Sample N°	Ruminant DNA
1	2186	Absent
1	4762	Absent
2	402	Present
3	522	Absent
4	922	Absent
4	5010	Absent
5	986	Present
6	1834	Absent