Combined microscopy-PCR EURL-AP Proficiency Test 2019

Final version – version 02

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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. The study also evaluated the correct application of the SOP (Standard Operating Procedure) on operational protocols for the combination of light microscopy and PCR with respect to the type of feed and the composition of the samples.

The total number of participating laboratories was 30 (26 NRLs and 4 labs outside the NRL network). On the exception of one NRL, all laboratories delivered results. The study was based on a set of nine 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.

The percentage of the NRLs that were complying with the correct implementation of the SOP on operational protocols for the combination of light microscopy and PCR was of 76 %. The study as well as the comments made by the participants stressed the difficulty of interpreting this SOP properly in absence of a declared destination or full labelling. Some PCR analyses which would have been realised after the detection of terrestrial particles are missing whereas PCR was able to give an additional information on the presence of forbidden ruminant material. This demonstrates that the SOP in its current version needs further improvements, notably by new protocols based on the type and origin of raw materials.

Regarding the detection of PAPs by light microscopy the overall results indicated an excellent and satisfactory level of global performance for 88 % of the NRLs. Only 12% of the NRLs revealed to be underperforming. The composition of the sample set allowed pointing some sensitivity issues for terrestrial animal remains in premix matrices as well as for fish particles in artificial milk.

Concerning the PCR results, 60 % of the NRLs (15 out 25) performed excellently. Six NRLs (24 %) returned satisfying results and 4 laboratories (16 %) were considered as underperforming. Deviations are still due to a lack of sensitivity of the light microscopy leading to stop the investigations. Nevertheless, analytical deviations were also present. More surprisingly, no PCR analysis was performed for some samples despite the detection of terrestrial particles by light microscopy.

Keywords :

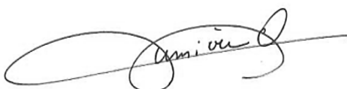
Processed animal proteins – Light microscopy – PCR - Proficiency test – Qualitative analysis

This report identified by an ISBN repeals and replaces the version identified by the ISBN 2-87286-112-2. This version was amended after reception of contests from two participants. Their remarks concerning the samples labelled as haemoglobin powder (sample types 2 and 3) were considered as relevant. These samples were thus retrieved from the legal compliance evaluation. Modifications to the first version are indicated by vertical bars on the right margin.

A first version of this report identified by ISBN 2-87286-112-2 has been prepared from a draft version sent for revision and comments to the participants on the 28th February 2020. After reception of the comments on the 13th March 2020, and the conclusions of the Appeal Commission on the 1st April, 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]. Since 2016, the proficiency tests conducted by the EURL-AP are organised under the ISO17043 standard.

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 10th September 2019 to all invited participants. On the 4th October 2019 participants were also informed on a delay of the timing initially planned for the study. They were officially informed of a new agenda for the study on the 21st October 2019 (Annex 3).

On the 25th October 2019, the sample sets were shipped to the participants. On the same day the Excel report forms containing the instructions (Annex 4) 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 22nd November 2019.

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 4). 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 NRL, which did not deliver its results, all results were delivered on time to the organiser.

Twenty eight participants returned results for both microscopic and 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

Nine different test materials were prepared for the study. 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 aquafeeds as matrices for assessing the detection capabilities of terrestrial PAPs because since the 1st June 2013 non-ruminant PAPs are authorized in aquafeeds according to Commission Regulation EU/56/2013 [6].
- As it can occur in real control analyses, ingredients and premixes without any indication about the destination were also included in the set.

Each participating lab received nine samples of about 50 g each. 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

Sample	Label	Destination	Material	Nr of replicates	Expected results *		
					Microscopy		PCR
					Terrestrial particles	Fish particles	Ruminant DNA
1	Soybean meal	No	soybean meal	1	-	-	na
2	Haemoglobin powder	No	porcine Hb meal	1	na (+)$	na (-)$	-
3	Haemoglobin powder	No	porcine Hb meal + 0.1 % ruminant PAP	1	na (+)$	na (-)$	+
4	Premix for aquafeed	Yes	premix I + 1 % TCP	1	+	-	-
5	Compound milk	Yes	artificial milk	1	- †	-	na
6	Pig feed	Yes	pig feed	1	-	-	na
7	Aquafeed with haemoglobin powder	Yes	aquafeed + 3 % bovine plasma powder	1	na	na	+
8	Aquafeed with animal proteins	Yes	aquafeed + 1 % porcine PAP	1	na (+)$	na (+)$	-
9	Premix	No	premix II	1	-	-	na
Total				**9**			

*** Explanations on expected results are described in section 3.4; $ When the option of performing microscopic analyses was chosen by the participants the results expected are in brackets; † If milk particles are reported a positive result was accepted; na = not applicable; Hb = haemoglobin; TCP = tricalcium phosphate**

Expected results were internally determined based on the known composition of the samples (presence of 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

Seven commercial matrices were used:

- **Soybean meal**. This feed was analytically free of any terrestrial PAP. Its sediment was of 0.1 %. It was used for preparing sample 1.
- **Porcine haemoglobin powder**. Three pure porcine haemoglobin powders were mixed and used for preparing sample 2. A fourth porcine haemoglobin powder has been added to the mixture for preparing sample 3. Their purities were checked by microscopy and PCR. They were free from sediment and PCR analyses revealed them as from porcine origin.
- **Premix I**. The composition is unknown but it was analytically free of any terrestrial PAP. Its sediment was of 85 %. It was used for preparing sample 4.
- **Artificial milk** was a complete feeding for calves made of skimmed milk powder, lactoserum, palm oil, copra oil, dextrose, calcium carbonate, magnesium sulphate, sodium bicarbonate. Its sediment was of 0.3 %. It was used only for sample 5.
- **Feed for fattening pig** was a compound feed for pig. It contained barley, wheat, maize, soybean flour calcium carbonate, lard, lysine, bicalcium phosphate, salts and premix. Its sediment was of 1.1%. It was used for preparing sample 6.

- **Aquafeed** was a compound feed for salmon. It was composed of soya protein concentrate, vegetable oil, fish oil, fish meal, faba beans, wheat gluten, maize gluten meal, wheat, mono-ammonium phosphate, monocalcium phosphate and yeast. Its sediment content was of 1.1%. This feed was used for preparing samples 7 and 8.
- **Premix II**. Its composition was unknown but was analytically free of any terrestrial PAP (see section 3.5). Its sediment was of 64 %. It was used only for sample 9.

Adulterant material used:

- A **pure ruminant PAP** was used for preparing sample 3. This PAP presenting a high bone content of 60.7 % was used in previous proficiency test 2017 and 2018 [9, 10]. Its purity was controlled by PCR.
- A **tricalcium phosphate** heat treated in a muffle furnace at 500 °C to destroy any potential remaining presence of DNA was used for preparing sample 4. PCR analyses proved no detection of ruminant DNA.
- A **bovine plasma powder** was used for preparing sample 7. It was free from any sediment. PCR analyses revealed it from ruminant origin and free from porcine DNA.
- A **pure porcine PAP** was used for preparing sample 8. This PAP was used in previous proficiency test 2015, 2016, 2017 and 2018 [7, 8, 9, 10]. Its bone content was of about 14 % and its purity was checked by microscopy and PCR.

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 matrix was conditioned first in order to avoid contamination.

On the exception of the matrices for samples 2, 3, 5 and 9, all other matrices were ground at 2 mm before any other treatment.

Adulteration of samples 4, 7 and 8 was performed by successive dilutions.

Sample 3 was directly spiked with the adulterant.

3.3. Qualitative analysis

Analyses of qualitative proficiency testing were applied following ISO 13528 [11].

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 [11].

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 participants 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. As well, any absence of PCR result due to a false negative microscopic result was considered as incorrect for PCR 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 [12] (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.

According to the decision of the Appeal Commission, a distinction was made between minor errors and critical errors. Minor errors are incorrect interpretations of the SOP with no consequence on the feed ban in terms of risk, while critical errors are interpretations of the SOP leading to possible breaches in the feed ban.

The performance criteria for the legal implementation were decided, on recommendation of the Appeal Commission, as:

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

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	Soybean meal	Farmed animals other than aquaculture and fur animals (microscopy)
2 & 3*	Haemoglobin powder	Aquaculture animals (microscopy and PCR, or PCR only)
4	Premix for aquafeed	Aquaculture animals (microscopy and PCR)
5	Compound milk	Farmed animals other than aquaculture and fur animals (microscopy)
6	Pig feed	Farmed animals other than aquaculture and fur animals (microscopy)
7	Aquafeed with haemoglobin powder	Aquaculture animals (PCR only)
8	Aquafeed with animal proteins	Aquaculture animals (microscopy and PCR, or PCR only)
9	Premix	Farmed animals other than aquaculture and fur animals (microscopy)

*** The origin of the haemoglobin powder cannot be determined visually, PCR is the only method to use to determine the presence of ruminant material. Nevertheless a participant argued rightly that a microscopic analysis may provide additional information on the presence of forbidden PAP if the final destination is not the aquaculture (e.g. porcine PAP if the destination is to feed pigs).**

3.4.2. Light microscopy

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

Samples 1, 5, 6 and 9 are to be declared negative for both terrestrial and fish material presence.

Sample 4 is to be declared positive for terrestrial material presence and negative for fish material presence.

Samples 2 and 3 are intended to be analysed by PCR. However, as argued by a participant, since the destination of this product may not exclusively be limited to fish, it was accepted that microscopic analyses could also be performed. Nevertheless the results of such microscopic analyses were neither considered

in the present study for the evaluation of the participant performance nor for the assessment of the legal compliance implementation (at least for the decision of performing microscopic analysis).

Sample 7 shall not be analysed by light microscopy but only by PCR.

Sample 8 is intended to be analysed by PCR. However since the term 'animal proteins' is not necessarily detailed it can be analysed first by light microscopy and then by PCR, or directly by PCR only. The microscopic results on this sample were not considered for the evaluation of the participant performance.

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

- **Excellent** level of global performance = consolidated AC superior or equal to 0.90 with no ND for terrestrial material.
- **Satisfying** level of global performance = consolidated AC superior or equal to 0.90 with one ND for terrestrial material OR a consolidated AC of 0.8 with no ND for terrestrial material.
- **Underperforming** level of global performance = consolidated AC of 0.80 with one ND for terrestrial material OR consolidated AC equal or inferior to 0.70.

3.4.3. PCR

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

Samples 2, 4 and 8 are to be declared negative for the presence of ruminant DNA.

Samples 3 and 7 are to be declared positive for the presence of ruminant DNA. Sample 3 contains 0.1 % of ruminant PAP. Sample 7 was adulterated with 3 % of bovine plasma powder.

Samples 1, 5, 6 and 9 should not be analysed by PCR because the sample label mentions that they are feed other than aquafeed (samples 5 and 6) or are not supposed to contain PAP detectable by microscopy.

Concerning the PCR, the performance criteria were decided as:

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

3.5. Homogeneity study

Homogeneity study has been carried out for all materials used. Table 3 on next page summarizes the results.

The homogeneity was studied by light microscopy on 10 g of sample material for each replicate, on the exception of the premixes where only 3 g were used. Analyses of replicates were performed following strictly EC/152/2009 regulation. For PCR analysis of each replicate a double DNA extraction was performed on 100 mg of sample material.

Sample 1 (soybean meal) was microscopically free from any trace of animal origin. On the exception of one test portion from one replicate (1/10) giving a late signal before the cut-off value, all test portions tested gave negative results for the presence of ruminant DNA. In the case of this sample, this result is not a problem because this sample is only for microscopy analysis.

Sample 2 (porcine Hb meal) was free of ruminant DNA but positive for porcine DNA.

Sample 3 (porcine Hb meal + 0.1 % ruminant PAP) was positive for the presence of ruminant and pig DNA.

Sample 4 (premix I + 1 % TCP) showed systematically the presence of terrestrial bones. No particles that could be interpreted as from fish origin were observed. PCR analyses revealed the sample as negative for ruminant DNA.

Table 3: Homogeneity study – Results

Sample	Material	Light microscopy			PCR			
		Nr of replicates	Terrestrial	Fish	Nr of replicates	Ruminant	Porcine	Fish
1	soybean meal	10	-	-	10	-	nt	nt
2	porcine Hb meal		nt	nt	10	-	+	nt
3	porcine Hb meal + 0.1 % ruminant PAP		nt	nt	10	+	+	nt
4	premix I + 1 % TCP	10	+	-	10	-	nt	nt
5	artificial milk	10	-	-	10	+	nt	nt
6	pig feed	10	-	-	10	-	nt	nt
7	aquafeed + 3 % bovine plasma powder	10	<LOD	+	10	+	nt	+
8	aquafeed + 1 % porcine PAP	10	+	+	10	-	+	+
9	premix II	10	-	-	10	-	nt	nt

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

Sample 5 (artificial milk) on the exception of milk and fat globules, as well as lactose crystals, no trace of other terrestrial animal material or fish material was found. PCR analyses showed the sample positive for ruminant DNA.

Sample 6 (pig feed) was negative for both terrestrial PAP particles and fish fragments. On the exception of one test portion from one replicate (1/10) giving a late signal before the cut-off value, all test portions tested gave negative results for the presence of ruminant DNA. In the case of this sample, this result is not a problem because this sample is only for microscopy analysis.

Sample 7 (aquafeed + 3 % bovine plasma powder) was positive for fish particles. On the exception of a single bone fragment, interpreted as from terrestrial origin, not other terrestrial PAP fragment was detected. Ruminant DNA was systematically detected using PCR.

Sample 8 (aquafeed + 1% porcine PAP) was systematically positive for both terrestrial animal and fish particles. PCR analyses revealed the sample negative for ruminant DNA but positive for porcine and fish DNA.

Sample 9 (premix II) was free from any trace of animal origin. The PCR analyses confirmed the absence of ruminant DNA.

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

3.6. Stability of the samples

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

4. Results

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

4.1. Legal compliance

Nineteen NRLs (76 %) are compliant with the SOP on operational scheme.

The cases of non-compliance were found and reported in Table 4.

Table 4: Non-complying laboratory list

Sample	Label	Light microscopy	PCR
1	Soybean meal	/	/
2	Haemoglobin powder	/	8, 9
3	Haemoglobin powder	/	8
4	Premix for aquafeed	/	7, 21
5	Compound milk	/	/
6	Pig feed	/	/
7	Aquafeed with haemoglobin powder	5, 14	/
8	Aquafeed with animal proteins	/	/
9	Premix	/	/

Six labs (labs 5, 7, 8, 9, 16 and 19) decided to perform microscopic analyses for samples 2 and 3, both samples being labelled identically as ”Haemoglobin powder”, a blood product which may be used for non-ruminant feed or aquafeed. Although the destination for the use of this product is not mentioned, the option chosen by the organizers was to check by PCR for the prohibited ruminant origin. As rightly argued by lab 7, the microscopic analysis can be useful to detect the presence of terrestrial PAP (bone and muscle particles) that would not be detected by the ruminant PCR test (porcine or poultry PAP) and that are forbidden in pig and poultry feed. Nevertheless, these microscopic analyses resulted in most cases in reporting false negative results for terrestrial animal presence for sample 2 (5 out of the 6 labs). Among these labs, participant 7 explained having declared the sample as negative for terrestrial since milk and haemoglobin particles observed by microscopy were not considered as terrestrial animal particles. As already underlined in previous proficiency test reports [10], this statement cannot be accepted by the organizers as Annex VI of Regulation (EU) No 51/2013 [3] mentions explicitly in its point 2.1.1 that all constituents of animal origin are identified by microscopy with blood cited as example. Nevertheless participant 7 performed rightly the PCR analyses of these samples. Participants 8 and 9 also did not consider the haemoglobin particles as terrestrial animal particles and stopped the investigations without having any information about the origin of sample 2. For sample 3, only participant 8 considered that a ruminant PCR test was not to perform despite the detection of terrestrial bones.

Sample 4 was labelled as premix for aquafeed and PCR must be used to determine whether ruminant DNA is present. Lab 21 stopped its investigations after light microscopic observations while a PCR analysis would have been necessary to determine the species origin of the terrestrial particles detected. Lab 7 confirmed the PCR analysis after the detection of terrestrial particles by light microscopy but omitted to report it.

Some other comments need to be added.

Although no non-conformity had to be reported, it must be mentioned that the compound milk (sample 5) which is not a product for aquaculture was analysed using PCR by lab 20. This is only a minor error but which could be a source of unjustified blocking if the positive PCR result is not well understood.

For sample 7, labs 5 and 14 performed unnecessary microscopic analyses although the label clearly referred to aquafeed.

Nine labs chose to start the second protocol of the SOP with analyses by light microscopy for sample 8, the aquafeed mentioning to contain animal proteins. Although the label did not strictly refer to ‘processed animal proteins’, as argued by lab 5, the relevant issue is determining if the declared proteins are from ruminant origin and therefore PCR should be applied first. After reception of the indicative tabulation form,

lab 23 also commented in this sense preferring to perform microscopic examination followed by PCR testing. All results from the microscopic analyses were correct; their decision had thus no impact on the feed ban control policy since the positive results conducted to the PCR analysis.

For the current study, as it was for past studies, the outcome of the interpretation of the SOP had not impact on the analytical proficiency assessment. For next coming studies, errors resulting from misinterpretation of the SOP could be fully included in the proficiency assessment. Nevertheless before achieving such performance evaluation, a more detailed version of the SOP with clearer protocols needs to be produced. In its present version (v.0.3) the SOP is based mainly on the final destination of the feed. More precisions regarding the ingredients and their origin should be developed as presented in the discussion.

4.2. Microscopy results

4.2.1. Qualitative analyses from the NRLs

4.2.1.1. Results and performance of the network

Table 5 summarizes the results submitted by the 25 NRLs for the sample types submitted to microscopic analysis.

The overall results, expressed in terms of global accuracy (AC) reveal the high quality of the NRL network for the detection of PAPs. The percentage of total error only accounted for 14 % of the total responses. On the contrary to what was observed in previous studies, there is a ventilation of these errors over the whole set of samples.

Table 5: Global results expressed as accuracy (AC) – light microscopy

Sample	Material	n	AC	
			Terrestrial	Fish
1	soybean meal	25	0.960 (1)	0.920 (2)
2	porcine Hb meal	na	na	na
3	porcine Hb meal + 0.1 % ruminant PAP	na	na	na
4	premix I + 1 % TCP	25	0.920 (2)	0.840 (4)
5	artificial milk	25	1.000	0.840 (4)
6	pig feed	25	0.960 (1)	1.000
7	aquafeed + 3 % bovine plasma powder	na	na	na
8	aquafeed + 1 % porcine PAP	na	na	na
9	premix II	25	0.920 (2)	0.960 (1)

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).

With regard to the composition of the samples intended to be microscopically analysed, the specificity for fish was challenged as fish ingredients were absent from these samples.

Four cases of false positive results were noted for sample 4 and 5, respectively the premix fortified with tricalcium phosphate and the artificial milk. The emergence of such false positive fish findings should be investigated: observations made on the premix and the artificial milk showed them as free from any animal particles. For sample 4, the origin of this misinterpretation can only be linked to use of tricalcium phosphate although the homogeneity study did only reveal particles which could only be categorised as bone fragments from terrestrial origin (since tricalcium phosphate, prohibited in ruminant feed, is precisely obtained by calcination of bones). From the detailed analyses it appears also that in sample 4 one participant observed muscles. It remains unexplained because the tricalcium phosphate was placed in a muffle furnace at 500°C to destroy the potential remaining presence of DNA. Interestingly one participant (lab 9) reported in the artificial milk the presence of particles “like in mussels, oysters” probably leading to the conclusion of fish presence. The most likely explanation for such particle is a confusion of polarised lactose tomahawk like crystals with nacre fragments from bivalve shells.

Two other reports of false positive fish particles originated from the pure soybean meal with descriptions of starfish particles, fish bones, scales and even muscles. Finally a last false positive fish detection was

found in the second premix sample with a description of findings like fish bones, scales and again muscles.

Some issues were also noted for the detection of terrestrial animal particles: four cases of specificity problems and two sensitivity failures.

4.2.1.2. Detailed review of results per sample

Sample 1 : soybean meal

PD for terrestrial particles:

- Lab 19 reported bones and muscles

PD for fish particles:

- Lab 20 reported starfish particles
- Lab 19 reported fish bones, scales and muscles

One <LOD case impacting both terrestrial and fish detection:

- Lab 25 reported the findings of muscles only (6 muscle fibres on a total of three repetitions)

Some comments were also made by participants. Lab 5 found particles identified as from insect but declared the sample as negative for both terrestrial and fish. Lab 10 mentioned presumably the presence of arthropod (shrimp meal) but confirmed the absence of insect structures and declared the sample also negative for both terrestrial and fish.

Sample 2: porcine haemoglobin meal

No microscopic analysis was supposed to be realised.

Sample 3: porcine haemoglobin meal + 0.1 % ruminant PAP

No microscopic analysis was supposed to be realised.

Sample 4: premix I + 1 % TCP

ND for terrestrial particles

- Labs 5 and 28 failed at detecting any terrestrial fragments

PD for fish particles

- Labs 5 and 9 declared having found fish bones
- Lab 28 identified cartilage particles
- Lab 19 declared fish bones, scales and muscles.

Some additional comments need to be reported. Some laboratories (labs 13 and 17) have mentioned the presence of blood even supported by a positive blood test (lab 13). However the type of test was not mentioned.

Sample 5: artificial milk

PD for fish particles:

- Lab 9 reported fish bones, particles of mussels and oysters
- Lab 13 reported bones and cartilage
- Lab 16 observed particles similar to fish skull fragments
- Lab 19 observed fish bones and muscles

The findings of particles described as originating from bivalves probably corresponds to the abundance of lactose crystals as observed during the homogeneity study. The difference of birefringence pattern between lactose crystals and nacre fragments is helping in the differentiation of these two types of structures. In addition the trapezoidal side shape of the lactose crystals is also determining. Two participants (labs 13 and 17) declared the sample as positive for terrestrial material based on the identification of milk powder. These results were assimilated to correct answers.

Sample 6: pig feed

PD for terrestrial particles:

- Lab 19 found feathers and muscles, and reported one bone.

A number of <LOD cases were reported for either terrestrial or fish material presence. The findings leading to this conclusion were:

- One hair (lab 4)
- Feathers like particles (lab 17)
- Muscles (labs 4 and 25)

Sample 7: aquafeed + 3 % bovine plasma powder

No microscopic analysis had to be realised.

Sample 8: aquafeed + 1 % porcine PAP

No microscopic analysis had to be realised.

Sample 9: premix II

PD for terrestrial particles:

- Lab 13: blood particles
- Lab 16 declared the sample as positive based on the observation of particles resembling mono- and di-calcium phosphate and which were stained by Alizarin Red.

PD for fish particles:

- Lab 19 reported fish bones, muscles and scales.

Some participants, although concluding on the absence of terrestrial and fish material, nevertheless reported a positive reaction to blood-test (lab 17) but without mentioning the type of test used, the finding of blood-like particles (lab 8) and finally the presence of insect fragments (lab 19).

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 6 and 7 (next page). A ranking of the labs was prepared based on the consolidated accuracy.

A general ranking of the NRLs was also performed on a consolidated evaluation including their proficiency in detecting both terrestrial and fish materials through the set of blind samples (Table 8 next page).

Nineteen labs out of 25 NRLs (76 %) performed very well.

Three NRLs performed satisfyingly (12 %) and three other NRLs (12 %) were underperforming for microscopic analyses.

In agreement with the EURL-AP SOP for managing underperformances (available on the EURL-AP intranet since 18 January 2012), the underperforming participants (labs 5, 28 and 19) are asked to report on the origin of their errors as well as on the actions they will undertake in order to solve the problems.

Tables 6 (left) and 7 (right): NRL proficiencies regarding the detection of terrestrial and fish material respectively. For terrestrial material detection, ranking follows AC values for primary key and SE for second key

Terrestrial			
lab code	AC	SE	SP
1	1.000	1.000	1.000
2	1.000	1.000	1.000
4	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
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
25	1.000	1.000	1.000
27	1.000	1.000	1.000
13	0.800	1.000	0.750
16	0.800	1.000	0.750
5	0.800	0.000	1.000
28	0.800	0.000	1.000
19	0.600	1.000	0.500

Fish	
lab code	SP
1	1.000
2	1.000
4	1.000
6	1.000
7	1.000
8	1.000
10	1.000
11	1.000
12	1.000
14	1.000
17	1.000
18	1.000
21	1.000
22	1.000
23	1.000
24	1.000
25	1.000
27	1.000
5	0.800
13	0.800
16	0.800
20	0.800
28	0.800
9	0.600
19	0.200

Table 8: 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 results and lines in red to underperforming results

Consolidated			
lab code	AC	SE	SP
1, 2, 4, 6, 7, 8, 10, 11, 12, 14, 17, 18, 21, 22, 23, 24, 25 and 27	1.000	1.000	1.000
20	0.900	1.000	0.889
9	0.800	1.000	0.778
13	0.800	1.000	0.778
16	0.800	1.000	0.778
5	0.800	0.000	0.889
28	0.800	0.000	0.889
19	0.400	1.000	0.333

4.2.2. 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 9 and 10.

Tables 9 (left) and 10 (right): non-EU lab proficiencies regarding the detection of terrestrial and fish material respectively. For terrestrial material detection, ranking follows AC values for primary key and SE for second key. (Legend: na, not applicable)

Terrestrial			
lab code	AC	SE	SP
30	1.000	1.000	1.000
32	0.800	0.000	1.000
33	0.800	0.000	1.000
31	0.600	1.000	0.500

Fish	
lab code	SP
30	1.000
33	1.000
31	0.500
32	na

The error details are described per sample:

Sample 1 : Soybean meal

No errors were noted.

Sample 2: porcine haemoglobin meal

No microscopic analysis was supposed to be realised.

Sample 3: porcine haemoglobin meal + 0.1 % ruminant PAP

No microscopic analysis was supposed to be realised.

Sample 4: premix I + 1 % TCP

ND for terrestrial particles

- Labs 32 and 33 failed at detecting any terrestrial fragments

PD for fish particles

- Labs 31 reported scales and bones

One case of <LOD for fish presence was reported by lab 33 without further details.

Lab 31 also reported a positive reaction to tetramethylbenzidine / hydrogen peroxide and reported finding of blood.

Sample 5: artificial milk

No errors were noted.

Lab 33 reported the sample as <LOD for fish without further details.

Sample 6: pig feed

PD for both terrestrial and fish particles:

- Lab 31 found terrestrial bones, fish bones and scales.

Sample 7: aquafeed + 3 % bovine plasma powder

No microscopic analysis had to be realised.

Sample 8: aquafeed + 1 % porcine PAP

No microscopic analysis had to be realised.

Sample 9: premix II

PD for terrestrial and fish particles:

- Lab 31 declared blood, due to a positive reaction to tetramethylbenzidine / hydrogen peroxide, fish bones and scales.

A general ranking as for the NRL network was established (Table 11):

Table 11: 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 and lines in red refer to underperforming results

Consolidated			
lab code	AC	SE	SP
30	1.000	1.000	1.000
33	0.900	0.000	1.000
32	0.800	0.000	1.000
31	0.556	1.000	0.500

One participant performed excellently and another one performed satisfyingly (line in blue in Table 11). The two other participants were classified as underperforming (lines in red in Table 11) according to the applied criteria.

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. No deviation is to notice.

4.3.1.2 Overview of results and global performance of the network

Table 12 (next page) summarizes the results provided by 25 NRLs for the five sample types submitted to qualitative PCR analysis.

Sample 2 was a porcine haemoglobin meal. The PCR result expected for the presence of ruminant DNA was negative. Three out of the 6 positive deviations were false positive PCR results. Among the three remaining deviations, two were due to missing results and the last one could probably be attributed to an inversion of results made by lab 11 with sample 3 also labelled as haemoglobin meal. The second deviation observed for sample 3 was a missing PCR result.

Table 12: Global results expressed as accuracy (AC) – PCR

Sample	Material	n	AC
1	soybean meal	na	na
2	porcine Hb meal	25	0.76 (6)
3	porcine Hb meal + 0.1 % ruminant PAP	25	0.92 (2)
4	premix I + 1 % TCP	25	0.80 (5)
5	artificial milk	na	na
6	pig feed	na	na
7	aquafeed + 3 % bovine plasma powder	25	1.00
8	aquafeed + 1 % porcine PAP	25	0.92 (2)
9	premix II	na	na

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; na = not applicable)

Five positive deviations were also recorded for sample 4. Four of them were PCR results absent (labs 5, 7, 21 and 28) whereas terrestrial particles were detected by labs 7 and 21. The last deviation was a false positive result reported by lab 6.

Sample 8 was declared twice positive for ruminant DNA. One of these positive deviations was reported by lab 6 that cumulated 3 false positive deviations.

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 13 that summarizes the results obtained by the participants for the analyses of sample types 2, 3, 4, 7 and 8 representing a total of 5 samples.

Table 13: 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, 2, 4, 10, 13, 14, 16, 17, 18, 19, 22, 23, 24, 25, 27	1.000	1.000	1.000
5*	0.800	1.000	0.667
7*	0.800	1.000	0.667
9*	0.800	1.000	0.667
12	0.800	1.000	0.667
20	0.800	1.000	0.667
21*	0.800	1.000	0.667
28	0.600	1.000	0.333
8*	0.600	0.500	0.667
11	0.600	0.500	0.667
6	0.400	1.000	0.000

*** The absence of PCR result(s) is assimilated to deviation(s)**

Excellent performances were recorded for only 15 labs out of 25 NRLs (60 % of the NRLs) having no false result.

Six labs were satisfying: lab 5 stopped the analyses and did not report an expected PCR result after failing to detect terrestrial particles in sample 4; labs 7and 21 succeeded to detect terrestrial particles in sample 4 but did not report any PCR result for the sample. According to lab 7, it is an error of reporting whereas no explanation was delivered for lab 21; lab 9 did not analyse samples 2 and 3; labs 12 and 20 delivered one PD.

Four labs were underperforming: lab 8 reported two PD; lab 11 had two deviations (1 PD + 1 ND). An inversion of the two results cannot be excluded. Lab 28 cumulated one PD and a missing PCR result. Lab 6 reported 3 PD.

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.

This year 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 was 56,0 % (65.4 % in 2018 [10]; 64.0 % in 2017 [9]; 59.3 % in 2016 [8]; 65.4 % in 2015 [7] ; 70.4 % in 2014 [13] ; 55.6 % in 2013 [14]).

4.3.2. Qualitative analyses from the non-EU participants

4.3.2.1. Individual performances

Individual performances were assessed for only two non-EU participants who reported PCR results by calculating the accuracy, sensitivity and specificity over the samples. Their results are to be found in Table 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
33*	0.400	0.500	0.333

*** The absence of PCR result(s) is assimilated to deviation(s)**

Labs 30 obtained excellent results (no deviation).

Concerning Lab 33, three deviations are recorded with the sample type 2 (Porcine haemoglobin meal), 3 (Porcine haemoglobin meal + 0.1 % ruminant PAP) and 4 (Premix for aquafeed +1 % TCP). The samples were not analysed by PCR. No terrestrial particles were identified by light microscopy. Under the assumption that this participant was following the SOP related to the operational protocols, it was justified stopping the analyses.

4.3.2.2. Assessment of the cut-off values

Labs 30 and 33 have cut-off values that comply with the minimum criterion of 9 copies set by the EURL-AP.

5. Discussion and conclusions

This combined proficiency test involving both the detection of animal traces by light microscopy and/or PCR delivered good expected scores.

Concerning microscopic results, the number of excellent and satisfactory scores obtained within the network of NRLs reached 88 %. Only three NRLs (12%) were underperforming for the present study.

The set of samples designed to be analysed by light microscopy was intended to test the potential specificity issues for fish. Indeed none of these samples were containing ingredients from fish origin. The problem of false positive fish detection is known for years as illustrated in Table 15 which is summarizing this type of error through the EURL-AP studies over the last ten years.

Table 15 : Overview of the percentages of specificity issues (PD) for fish over the last 10 years of EURL-AP proficiency tests

Year	Feed matrix	% of PD for fish
2017	Poultry feed	4
2016	Compound feed for laying hens	29
2014	Horse feed	4
	Compound feed for mini-pigs	4
2013	Horse feed	7
	Rabbit feed	7
2012	Rabbit feed	7
	Home produced feed	3
	Pheasant feed	9
	Pheasant feed with terrestrial PAPs	15
	Pure poultry PAP	22
2011	Compound feed	8
	Beet pulp	8
2010	Pig compound feed	8
	Feed supplement for bovine	6
	Bovine feed	8
2009	Compound feed	12
	Feed supplement for bovine	15

On the exception of 2016, where the erroneous identification of fish material was linked to the presence of shell grids, the explanation for this background lack of specificity generally still remains unexplained. The morphological diversity of fishbone fragments [15] partly accounts for the difficulty of differentiating such particles from other ones.

Concerning the detection of terrestrial animal remains, the study evidenced also two sensitivity issues in presence of tricalcium phosphate. For reminder a morphological distinction between terrestrial bone fragments and tricalcium phosphate cannot be achieved. Although the high concentration level, the lack of sensitivity observed could have been explained by the high percentage of sediment from the premix matrix. However it does not seem to be the main factor since the two labs failing at detecting terrestrial fragments reported fish bones and cartilage particles proving an interpretation error rather than a low detection level.

Premixes used in the study also highlighted some unexpected findings interpreted as a presence of blood. Although the composition of the premixes was unknown, the homogeneity study showed them free from any blood particles. After reception of the results, tests with tetramethylbenzidine/hydrogen peroxide (TMB + H_2O_2) were performed to verify the reactivity to this test. Effectively a turquoise coloration was found but without the immediate oxygen release as observed in presence of blood. Furthermore the absence of immediate reactivity (within some seconds) in presence of TMB + H_2O_2 definitively excluded

the presence of blood. The implementation of the SOP related to the staining reagents [16] would prevent from such misleading interpretation.

Concerning non-EU participants, they encountered problems which were similar to the ones of the NRL network, notably concerning the premix based samples.

This year, it has to be noticed that in two cases the detection of terrestrial particles by light microscopy was not followed by a PCR analysis whereas it is foreseen in the SOP. A third case was explained by a reporting error (omission to report the PCR result).

For the PCR results, only 15 laboratories out of 25 (60 %) performed excellently. Six participants returned satisfying results and 4 labs were considered as underperforming. During the previous proficiency tests combining light microscopy with PCR a majority of the deviations were due to the lack of sensitivity from the microscopic observations leading to a stop of the investigations and the absence of PCR analyses. It is still the case for three participants (labs 5, 9 and 28 respectively). But analytical deviations (PD) were also recorded for labs 6 (3 times), 12 and 28. More surprising and dangerous for the feed ban are cases where no PCR analysis was performed while terrestrial particles were detected by light microscopy (labs 7, 8 and 21) or when there is no other analytical tool to determine the origin of the haemoglobin powder (labs 8 and 9). Two unexpected PCR results were also recorded (labs 16 and 19) but there were justified by PD during the light microscopy analysis and were analytically correct. Finally the compound milk was analysed by PCR by lab 20 while it is not authorised by the operational schemes, this is nevertheless a minor interpretation error.

Only 2 non-EU participants returned PCR results. As it was already the case in 2018, one performed excellently and the other one was considered as underperforming. This year again, this negative evaluation has to be nuanced as it is still due to an absence of PCR results consecutive to negative microscopic results.

In 2018, the interpretation of the SOP on operational protocols for the combination of light microscopy and PCR seemed to be improved. This year, the participants were faced to complex but realistic situations with 4 samples for which the final destination was not mentioned (one soybean meal, two haemoglobin powders and one premix). The case of the soybean meal is probably the clearest one: it is a plant material into which no animal particles have to be detected. The worst case scenario for the premix is the presence of PAP whatever the destination. For these two samples, microscopic investigation was the best method to apply. Concerning the haemoglobin powders, the option approved by DG Sante when blood products or blood meals have to be controlled is the mandatory use of PCR when it is the only method available for demonstrating the absence of ruminant origin. The statement that the use of microscopy is useless for this type of material was contradicted by one participant considering the case of the presence of non-ruminant PAP that cannot be detected by the ruminant PCR method. Depending on the final destination of the sample, the presence of non-ruminant PAP is authorised (aquafeed) or forbidden (non-ruminant feed) and the use of microscopy can therefore be justified. Nevertheless, some participants declared haemoglobin powder as negative for terrestrial animal after microscopic analysis, therefore even excluding the possibility to perform PCR when considering that this material could be used for aquaculture.

Another sample was labelled ‘aquafeed with animal proteins’ without specified reference to the term ‘processed’. Such a lack of precision can be confusing for the decision on the methods to apply.

These difficulties were taken into consideration for the legal compliance assessment which resulted in a compliance of 76% of the NRLs about the choice of the method(s) according to the SOP. Nevertheless, regarding the SOP on the operational protocols for the combination of light microscopy and PCR, it is clear that there is a need to improve this binding complement to the regulation. In its preceding and current versions, the SOP determines the protocol to follow according to the sole final destination of a compound feed or a feed material: for all farmed animals other than aquaculture and fur animals (Protocol 1) and for aquaculture animals (Protocol 2). If it can be considered that the document is fit for purpose for compound feeds, because they are usually referring to the type of farmed animal for which they are produced, it is far incomplete for feed materials. Effectively feed ingredients may be used for entering in the composition of feeds of several farmed animals species. It means that their final destination is not necessarily declared. In such case the ‘worst case’ scenario has to be the rule to fit the requirements of the feed ban but mainly to prevent from breaches or violations.

Therefore complementary protocols should be introduced into this SOP, not based on the final destination but on the type, the animal or plant origin of the materials or even the composition of some ingredients (premixes for instance may be prepared for porcine, poultry or even fish feed formulations). Such additional protocols should be introduced based on a reasonable risk assessment and realistic considerations in order to select the method(s) which should be applied. This recommendation for improvement of the SOP on the combination of methods will be transferred to the competent authorities.

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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
Botswana	*Botswana National Veterinary Laboratory*
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
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 Institute of Marine 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 87 52 28 32 (0) 81 87 40 19
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

Announcement of the EURL-AP proficiency test 2019/01 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 9 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.

Time schedule

- Official announcement of the study to the NRLs by way of the intranet and e-mail : **10 September 2019**
- Sending of the sample boxes and communication of the instructions : **11 October 2019**

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

- Deadline for returning of results to the organizer : **15 November 2019**

Further information

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

or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager
  ☎32 (0) 81 87 52 28
  32 (0) 81 87 40 19
  E-mail: p.veys@cra.wallonie.be

or

- Dr Olivier FUMIERE
  Head of EURL-AP Molecular biology team
  ☎32 (0) 81 87 52 40
  32 (0) 81 87 40 19
  E-mail: o.fumiere@cra.wallonie.be

Annex 3

Change of agenda

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 87 52 28 32 (0) 81 87 40 19
e-mail: secretary@eurl.craw.eu Internet : http://eurl.craw.eu

EURL-AP proficiency test 2019/01
for the determination of Processed Animal Proteins (PAPs) in feed:

SENDING OF SAMPLES and NEW AGENGA

Gembloux, 21 October 2019

Dear Participant,

As announced on the 4 October, we had to delay the sending of the sample set for the proficiency test.

We are now pleased to inform you that we are in the position of sending the samples. Please take also note of the new timing :

- Sending of the sample boxes and communication of the instructions : 25 October 2019
- Deadline for returning of results to the organizer : 22 November 2019

We thank you for your understanding and wish you a successful participation.

Yours Truly.

Further information

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

  or

- Dr Pascal VEYS
  EURL-AP NRL Network Manager
  ☎32 (0) 81 87 52 28
  32 (0) 81 87 40 19
  E-mail: p.veys@cra.wallonie.be

  or

- Dr Olivier FUMIERE
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  E-mail: o.fumiere@cra.wallonie.be

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Annex 4

Excel result report form

	A	B	C	D	E	F	G	H	I	J	K
1	Proficiency Test Microscopy-PCR 2019/01										
2	**Laboratory identification**										
3	Laboratory code :	1									
5	Responsibility agreement :	No									
6	*"Yes" means you have read carefully the "Instructions" worksheet and its accurate application through the present study.*										
7	**Report**										
9		Lab code	1	1	1	1	1	1	1	1	1
10		Sample rank	1st	2nd	3th	4th	5th	6th	7th	8th	9th
11		**Sample N°**									
12		**Method of analysis**	**Light microscopy**								
14	Light microscopy analyses	Terrestrial animal particles									
15		details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*									
17		Fish particles									
18		details of particles *Only to fill in if in the cell above "present" or "< LOD" is chosen.*									
20		Analyses performed on									
21		Number of determinations									
23		Free comment *Example : presence of unusual fragments,...*									
25		**Method of analysis**	**PCR**								
27	PCR analyses	Ruminant DNA									
30		**Cut-off at 15 copies of the PCR platform used (in cycles)**									
31		**Copy number at the cut-off of the PCR platform used (in copies)**									
33		Dilution 1 (e.g. 1 fold)									
34		Ct value replicate 1									
35		Ct value replicate 2									
37		Dilution 2 (e.g. 10 fold)									
38		Ct value replicate 1									
39		Ct value replicate 2									
42		Comment *Example : PCR inhibition,...*									

EURL Animal Proteins

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
6	71	Absent		Absent		Sed. + Raw	2
9	134	Absent		Absent		Sed. + Flot.	2
3	251	Not to perform		Not to perform			
4	254	Present	Bones	Absent		Sed. + Flot.	1
2	491	Not to perform		Not to perform			
5	824	Absent		Absent		Sed. + Flot.	2
7	965	Not to perform		Not to perform			
1	1163	Absent		Absent		Sed. + Raw	2
8	1427	Present	Bones	Present	Bones	Sed. + Flot.	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
8	374	Present	terestrial bones	Present	fisch bones	Sed. + Flot.	2
9	377	Absent		Absent		Sed. + Flot.	2
5	797	Absent		Absent		Sed. + Flot.	2
6	908	Absent		Absent		Sed. + Flot.	2
4	1334	Present	terestrial bones	Absent		Sed. + Flot.	1
1	1406	Absent		Absent		Sed. + Flot.	2
7	1451	Not to perform		Not to perform			
2	1517	Not to perform		Not to perform			
3	1520	Not to perform		Not to perform			

Laboratory identification code :	4

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
2	626	Not to perform		Not to perform			
3	1277	Not to perform		Not to perform			
7	1289	Not to perform		Not to perform			
8	1481	Present	bones, muscles	Present	fish bones, scales, otolites, muscles	Sed. + Flot.	1
4	1523	Present	bones	Absent		Sed. + Flot.	1
9	242	Absent		Absent		Sed. + Flot.	1
5	392	Absent		Absent		Sed. + Raw	1
1	974	Absent		Absent		Sed. + Flot.	1
6	1232	< LOD	1 hair (possible rat), 2	< LOD	2 muscle	Sed. + Raw	3

In sample No1232 totaly was detected 3 particles: 1 hair and 2 muscles.

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
1	2	Absent		Absent		Sed. + Flot.	2
8	104	Present	bones, blood particles	Present	fish bones, scales, muscle fibres.	Sed. + Flot.	2
3	305	Present	blood particles	Absent		Sed. + Flot.	2
7	425	Present	blood particles	Present	fish bones, scales, muscle fibres.	Sed. + Flot.	2
6	530	Absent		Absent		Sed. + Flot.	2
5	608	Absent		Absent		Sed. + Flot.	2
2	653	Present	blood particles	Absent		Sed. + Flot.	2
4	902	Absent		Present	fish bones	Sed. + Flot.	2
9	1484	Absent		Absent		Sed. + Flot.	2

sample n.2: presence of insect particles; sample n. 104: figure 2 of operation schemes shows that PCR should be performed when feed is known to contain terrestrial PAP. Sample 104 label claims "aquafeed with animal proteins" with no specification about the protein origin (i.e. terrestrial or fish). Therefore we decided to perform microscopy test first; sample n. 425: presence of shells

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
9	80	Absent		Absent		Sed. + Flot.	1
3	143	Not to perform		Not to perform			
2	572	Not to perform		Not to perform			
6	935	Absent		Absent		Sed. + Flot.	1
8	968	Not to perform		Not to perform			
4	1037	Present	Bones	Absent		Sed. + Flot.	1
5	1175	Absent		Absent		Sed. + Flot.	1
1	1487	Absent		Absent		Sed. + Flot.	1
7	1559	Not to perform		Not to perform			

For the 1487 sample, it was also performed analysis on raw material.

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
9	53	Absent		Absent		Sed. + Flot.	1
5	230	Absent		Absent		Sed. + Flot.	1
2	383	Absent		Absent		Sed. + Flot.	1
4	389	Present	Bones	Absent		Sed. + Flot.	1
3	845	Present	Bones	Absent		Sed. + Flot.	1
7	1019	Not to perform		Not to perform			
1	1028	Absent		Absent		Sed. + Flot.	1
8	1076	Not to perform		Not to perform			
6	1205	Absent		Absent		Sed. + Flot.	1

Milk and haemoglobine observed by microscopy is not considered as terrestrial animal particles

Laboratory identification code :	8

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
6	395	Absent		Absent		Sed. + Raw	1
8	428	Present	bones, muscle fibers, cartilage	Present	fish bones, scales, gills, muscle fibers, cartilage	Sed. + Raw	1
1	434	Absent		Absent		Sed. + Raw	1
5	635	Absent		Absent		Sed. + Raw	1
7	749	Not to perform		Not to perform			
4	848	Present	bones	Absent		Sed. + Raw	1
9	1025	Absent		Absent		Sed. + Raw	1
2	1490	Absent		Absent		Sed. + Raw	1
3	1547	Present	bones	Absent		Sed. + Raw	1

2nd/428: shrimp/krill-like particles (wheel spoke). 7th/1025: a few blood-like particles

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
9	26	Absent		Absent		Sed. + Flot.	3
5	311	Absent		Present	fish bone, particles like as mussels, oysters	Sed. + Flot.	3
4	335	Present	bones	Present	fish bones	Sed. + Flot.	3
2	464	Absent		Absent		Sed. + Flot.	3
8	482	Not to perform		Not to perform			
7	830	Not to perform		Not to perform			
1	866	Absent		Absent		Sed. + Flot.	3
6	1097	Absent		Absent		Sed. + Flot.	3
3	1250	Present	bones	Absent		Sed. + Flot.	3

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
9	701	Absent		Absent		Sed. + Raw	1
1	1001	Absent		Absent		Sed. + Raw	1
4	1091	Present	more than 10 fragments of bones.	Absent		Sed. + Raw	1
6	1529	Absent		Absent		Sed. + Raw	1
5	68	Absent		Absent		Sed. + Raw	1
2	59	Not to perform		Not to perform			
8	563	Not to perform		Not to perform			
7	722	Not to perform		Not to perform			
3	1466	Not to perform		Not to perform			

Sample 68: milk powder is not defined as a PAP, and is therefore not a usual matrix for monitoring. Since artificial milk feed based on milk powder can be denaturated by fish meal, the sample was examined by microscopy. An addition of starch was found. Sample 1001: fragments of Arthropods were found, presumably originating from shrimp meal, since specific insect structures were not found. Sample 1529: three chitin fragments of insects found. This is apparently not a subject in the current PT, since it cannot be reported as result. Although below the LOD of the official PAP method, a second sedimentation cycle was therefore not carried out.

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
9	161	Absent		Absent		Sed. + Flot.	2
2	194	Not to perform		Not to perform			
5	446	Absent		Absent		Sed. + Flot.	2
4	605	Present	bones	Absent		Sed. + Flot.	2
7	641	Not to perform		Not to perform			
8	779	Not to perform		Not to perform			
6	854	Absent		Absent		Sed. + Flot.	2
1	1379	Absent		Absent		Sed. + Flot.	2
3	1439	Not to perform		Not to perform			

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	56	Absent		Absent		Sed. + Flot.	1
7	182	Not to perform		Not to perform			
9	593	Absent		Absent		Sed. + Flot.	1
4	659	Present	Bones	Absent		Sed. + Flot.	1
5	689	Absent		Absent		Sed. + Flot.	1
8	1184	Present	Bones	Present	Bones	Sed. + Flot.	1
3	1358	Not to perform		Not to perform			
6	1394	Absent		Absent		Sed. + Flot.	1
2	1463	Not to perform		Not to perform			

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
5	41	Present	milk powder	Present	bones, cartilage	Sed. + Flot.	1
2	86	Not to perform		Not to perform			
8	617	Not to perform		Not to perform			
1	650	Absent		Absent		Sed. + Flot.	1
9	890	Present	blood particles	Absent		Sed. + Flot.	1
4	1010	Present	blood particles, bones	Absent		Sed. + Flot.	1
3	1088	Not to perform		Not to perform			
7	1208	Not to perform		Not to perform			
6	1448	Absent		Absent		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
9	188	Absent		Absent		Sed. + Raw	
1	272	Absent		Absent		Sed. + Raw	
5	419	Absent		Absent		Sed. + Raw	
4	929	Present	bone particles	Absent		Sed. + Raw	
7	992	Present	blood particles	Present	fish bone, fish scale, othololite	Sed. + Raw	
6	1502	Absent		Absent		Sed. + Raw	
3	359	Not to perform		Not to perform			
2	5	Not to perform		Not to perform			
8	941	Not to perform		Not to perform			

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
4	200	Present	Terrestrial bone fragments	Absent		Sed. + Flot.	1
2	302	Absent		Absent		Sed. + Flot.	
9	404	Present	* see text in free coment	Absent		Sed. + Raw	1
3	575	Present	Terrestrial bone fragments	Absent		Sed. + Flot.	1
7	803	Not to perform		Not to perform			
6	1151	Absent		Absent		Sed. + Flot.	1
1	1352	Absent		Absent		Sed. + Flot.	1
8	1373	Not to perform		Not to perform			
5	1472	Absent		Present	fragments similar to fish skull were observed and stained red when subjected to alizarin red	Sed. + Flot.	1

** sample 404 - fragments similar in appearance to mono-calcium/di-calcium phosphate were observed and strongly stained red with Alizarin red, therefore sample was subjected to PCR to determine if from ruminant source*

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	284	Present	Compound milk	Absent		Sed. + Raw	1
6	368	< LOD	feather like particles	Absent		Sed. + Raw	3
3	467	Not to perform		Not to perform			
2	545	Not to perform		Not to perform			
1	677	Absent		Absent		Sed. + Raw	1
9	782	Absent		Absent		Sed. + Raw	1
8	1049	Not to perform		Not to perform			
4	1415	Present	Bones, blood	Absent		Sed. + Raw	1
7	1478	Not to perform		Not to perform			

Sample782 and 1415 showed positive in blood-test

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
4	119	Present	bones, cartilage	Absent		Sed. + Flot.	1
1	164	Absent		Absent		Sed. + Flot.	1
2	248	Not to perform		Not to perform			
5	257	Absent		Absent		Sed. + Flot.	1
9	674	Absent		Absent		Sed. + Flot.	1
8	833	Present	bones, cartilage	Present	bones, scales	Sed. + Flot.	1
3	953	Not to perform		Not to perform			
6	1124	Absent		Absent		Sed. + Flot.	1
7	1127	Not to perform		Not to perform			

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
5	203	Absent		Present	fish bones and muscles	Sed. + Flot.	2
7	209	Not to perform		Not to perform			
9	323	Absent		Present	fish bones, muscles and scales	Sed. + Flot.	2
6	422	Present	feathers and muscles. 1 bone	Absent		Sed. + Flot.	3
1	542	Present	bones and muscles	Present	fish bones, muscles and scales	Sed. + Flot.	2
3	629	Present	bones	Absent		Sed. + Flot.	2
4	821	Present	bones and muscles	Present	fish bones, muscles and scales	Sed. + Flot.	2
2	1247	Absent		Absent		Sed. + Flot.	2
8	1535	Not to perform		Not to perform			

In sample 323 we have found insect fragments.

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
5	14	Absent		Absent		Sed. + Raw	1
4	92	Present	Bones	Absent		Sed. + Raw	1
2	329	Not to perform		Not to perform			
8	671	Not to perform		Not to perform			
1	1271	Absent		Present	Starfish endoskeleton particles	Sed. + Raw	1
3	1331	Not to perform		Not to perform			
6	1475	Absent		Absent		Sed. + Raw	1
7	1532	Not to perform		Not to perform			
9	1538	Absent		Absent		Sed. + Raw	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
9	107	Absent		Absent		Sed. + Flot.	1
8	158						
1	191	Absent		Absent		Sed. + Flot.	1
3	602						
2	977						
6	989	Absent		Absent		Sed. + Flot.	1
7	1073						
4	1226	Present	animal bones	Absent		Sed. + Flot.	1
5	1283	Absent		Absent		Sed. + Flot.	1

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	152	Absent		Absent		Sed. + Flot.	1
4	227	Present	bones	Absent		Sed. + Flot.	2
3	440	Not to perform		Not to perform			
7	614	Not to perform		Not to perform			
2	842	Not to perform		Not to perform			
1	1298	Absent		Absent		Sed. + Flot.	1
5	1337	Absent		Absent		Sed. + Flot.	1
8	1346	Not to perform		Not to perform			
9	1349	Absent		Absent		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
6	206	Absent		Absent		Sed. + Raw	1
8	293	Present	bones, muscle fibers, cartilage	Present	scales, fishbones, otholithes, tooth, gills, musle fibers, cartilage,	Sed. + Raw	1
4	470	Present	bones, cartilage	Absent		Sed. + Raw	1
2	761	Not to perform		Not to perform			
5	851	Absent		Absent		Sed. + Raw	1
9	1106	Absent		Absent		Sed. + Raw	1
3	1169	Not to perform		Not to perform			
7	1262	Not to perform		Not to perform			
1	1541	Absent		Absent		Sed. + Raw	1

206=REN2019-2249, 293=REN2019-2250, 470=REN2019-2251, 761=REN2019-2252, 851=REN2019-2253, 1106=REN2019-2254, 1169=REN2019-2255, 1262=REN2019-2256, 1541=REN2019-2257

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	38	Present	bones	Absent		Sed. + Flot.	1
7	101	Not to perform		Not to perform			
1	515	Absent		Absent		Sed. + Flot.	1
6	719	Absent		Absent		Sed. + Flot.	1
3	764	Not to perform		Not to perform			
8	1157	Not to perform		Not to perform			
9	1187	Absent		Absent		Sed. + Flot.	1
2	1355	Not to perform		Not to perform			
5	1445	Absent		Absent		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
2	167	Not to perform		Not to perform			
3	197	Not to perform		Not to perform			
7	452	Not to perform		Not to perform			
9	620	Absent		Absent		Sed. + Flot.	1
4	740	Present	Bones	Absent		Sed. + Flot.	1
8	752	Present	Bones	Present	Fishbones,gills,scales,muscles	Sed. + Flot.	1
6	800	< LOD	muscles	< LOD	muscles	Sed. + Flot.	2
5	932	Absent		Absent		Sed. + Flot.	1
1	1325	< LOD	muscles	< LOD	muscles	Sed. + Flot.	3

Sample 800: Were detected 3 particles (Muscles) in the flotate, were carried-out 2 determinations. Sample 932: The milk compound is not mentioned in the terrestrial particles as is indicated in the label, were detected shells in this sample. Sample 1325:Were detected 6 particles (Muscles) in the flotate, were carried-out 3 determinations.

Laboratory identification code :	27

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	110	Absent		Absent		Sed. + Flot.	1
3	116	Not to perform		Not to perform			
6	179	Absent		Absent		Sed. + Flot.	1
9	215	Absent		Absent		Sed. + Flot.	1
2	275	Not to perform		Not to perform			
4	281	Present	bones	Absent		Sed. + Flot.	1
8	509	Not to perform		Not to perform			
5	1067	Absent		Absent		Sed. + Flot.	1
7	1370	Not to perform		Not to perform			

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
3	35	Not to perform					
7	74	Not to perform					
8	131	Not to perform					
9	512	Absent		Absent		Sed. + Flot.	1
2	734	Not to perform					
6	773	Absent		Absent		Sed. + Flot.	1
5	959	Absent		Absent		Sed. + Flot.	1
4	983	Absent		Present	Cartilage dominating	Sed. + Flot.	1
1	1082	Absent		Absent		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
4	173	Present	bones	Absent		Sed. + Flot.	1
7	560	Not to perform		Not to perform			
9	647	Absent		Absent		Sed. + Flot.	1
8	698	Present	bones, muscles	Present	bones, otoliths	Sed. + Flot.	1
3	818	Not to perform		Not to perform			
6	881	Absent		Absent		Sed. + Flot.	1
2	950	Not to perform		Not to perform			
1	1217	Absent		Absent		Sed. + Flot.	1
5	1229	Absent		Absent		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
5	95	Absent		Absent		Sed. + Raw	1
8	266	Present	Bones	Present	Bones	Sed. + Raw	1
3	494	Present	Bones and blood plasma	Absent		Sed. + Raw	2
2	680	Present	Blood plasma	Absent		Sed. + Raw	1
6	98	Present	Bones	Present	Scales and bones	Sed. + Raw	2
4	767	Present	Blood and Bones	Present	Scales and bones	Sed. + Raw	1
7	884	Absent		Present	Bones and scales	Sed. + Raw	1
9	1133	Present	Blood	Present	Scales and bones	Sed. + Raw	2
1	1136	Absent		Absent		Sed. + Raw	1

Tetramethylbenzidine/hydrogen peroxide stain was used in sample EURL No 494 and EURL No 680 in which the mixture reacted for both samples and it became blue-green (torquise) without release of oxygen bubbles,concluding that the samples contained blood plasma. Tetramethylbenzidine/hydrogen peroxide stain was also used for samples EURL No 767 and EURL No 1133 and the mixture reacted and immediately became turquise and released oxygen bubbles, concluding that there was blood in the samples.

Laboratory identification code :	32

Sample type	Sample N°	Terrestrial animal part.	Details of terrestrial part.	Fish part.	Details of fish part.	Fractions used	Number of determinations
1	29	Absent					
2	599	Absent					
7	695	Present					
9	809	Absent					
5	878	Absent					
8	914	Present					
6	962	Absent					
3	1304	Present					
4	1361	Absent					

Samples were processed to determine the presence or absence of Terrestrial animal particles only. No sediment was availble in sample 599 (sample 2) for processing

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
4	65	Absent		< LOD		Sed. + Raw	
7	155	Not to perform		Not to perform			
3	224	Present		< LOD		Sed. + Raw	
1	245	Absent		Absent		Sed. + Raw	
6	287	Absent		Absent		Sed. + Raw	
5	905	Absent		< LOD		Sed. + Raw	
8	1022	Not to perform		Not to perform			
9	1160	Absent		Absent		Sed. + Raw	
2	1220	Absent		Absent		Sed. + Raw	

Annex 6

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

Laboratory identification code :	1
Cut-off at 15 copies :	35.76 cycles
Copy number at the cut-off :	9.09 copies

Sample type	Sample N°	Ruminant DNA	Comment
6	71	Not to perform	
9	134	Not to perform	
3	251	Present	
4	254	Absent	
2	491	Absent	
5	824	Not to perform	
7	965	Present	
1	1163	Not to perform	
8	1427	Absent	

Laboratory identification code :	2
Cut-off at 15 copies :	36.05 cycles
Copy number at the cut-off :	9.78 copies

Sample type	Sample N°	Ruminant DNA	Comment
8	374	Absent	
9	377	Not to perform	
5	797	Not to perform	
6	908	Not to perform	
4	1334	Absent	
1	1406	Not to perform	
7	1451	Present	
2	1517	Absent	
3	1520	Present	

Laboratory identification code :	4
Cut-off at 15 copies :	36.05 cycles
Copy number at the cut-off :	9.78 copies

Sample type	Sample N°	Ruminant DNA	Comment
2	626	Absent	replicate 1 have inhibition
3	1277	Present	no
7	1289	Present	no
8	1481	Absent	no
4	1523	Absent	no
9	242	Not to perform	
5	392	Not to perform	
1	974	Not to perform	
6	1232	Not to perform	

Laboratory identification code :	5
Cut-off at 15 copies :	35.57 cycles
Copy number at the cut-off :	11.25 copies

Sample type	Sample N°	Ruminant DNA	Comment
1	2	Not to perform	
8	104	Absent	
3	305	Present	
7	425	Present	
6	530	Not to perform	
5	608	Not to perform	
2	653	Absent	
4	902	Not to perform	
9	1484	Not to perform	

Laboratory identification code :	6
Cut-off at 15 copies :	37.03 cycles
Copy number at the cut-off :	9.84 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	80	Not to perform	
3	143	Present	
2	572	Present	
6	935	Not to perform	
8	968	Present	
4	1037	Present	
5	1175	Not to perform	
1	1487	Not to perform	
7	1559	Present	

Laboratory identification code :	7
Cut-off at 15 copies :	34.32 cycles
Copy number at the cut-off :	10.79 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	53	Not to perform	
5	230	Not to perform	
2	383	Absent	PCR is done since the animal category of destination is not given (could be for aquaculture).
4	389	Not to perform	
3	845	Present	'PCR is done since the animal category of destination is not given (could be for aquaculture).
7	1019	Present	
1	1028	Not to perform	
8	1076	Absent	Inhibition test is done by addition of plasmid 40cp to the sample : no PCR inhibition detected. Pig
6	1205	Not to perform	

Laboratory identification code :	8
Cut-off at 15 copies :	36.57 cycles
Copy number at the cut-off :	9.64 copies

Sample type	Sample N°	Ruminant DNA	Comment
6	395	Not to perform	
8	428	Absent	The used PCR platform was CFX Maestro Version 4.0.2325.0418
1	434	Not to perform	
5	635	Not to perform	
7	749	Present	
4	848	Absent	
9	1025	Not to perform	
2	1490	Not to perform	
3	1547	Not to perform	

Laboratory identification code :	9
Cut-off at 15 copies :	34.10 cycles
Copy number at the cut-off :	9.48 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	26	Not to perform	
5	311	Not to perform	
4	335	Absent	
2	464	Not to perform	
8	482	Absent	
7	830	Present	
1	866	Not to perform	
6	1097	Not to perform	
3	1250	Present	

Laboratory identification code :	10
Cut-off at 15 copies :	35.35 cycles
Copy number at the cut-off :	10.80 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	701	Not to perform	
1	1001	Not to perform	
4	1091	Absent	
6	1529	Not to perform	
5	68	Not to perform	
2	59	Absent	
8	563	Absent	One extraction positive close to the cut-off and the other negative, also after repetition.
7	722	Present	
3	1466	Present	

Laboratory identification code :	11
Cut-off at 15 copies :	33.06 cycles
Copy number at the cut-off :	10.21 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	161	Not to perform	
2	194	Present	inhibition
5	446	Not to perform	
4	605	Absent	inhibition
7	641	Present	
8	779	Absent	
6	854	Not to perform	
1	1379	Not to perform	
3	1439	Absent	

Laboratory identification code :	12
Cut-off at 15 copies :	34.23 cycles
Copy number at the cut-off :	11.08 copies

Sample type	Sample N°	Ruminant DNA	Comment
1	56	Not to perform	
7	182	Present	
9	593	Not to perform	
4	659	Absent	
5	689	Not to perform	
8	1184	Present	
3	1358	Present	
6	1394	Not to perform	
2	1463	Absent	

Laboratory identification code :	13
Cut-off at 15 copies :	35.92 cycles
Copy number at the cut-off :	10.76 copies

Sample type	Sample N°	Ruminant DNA	Comment
5	41	Not to perform	
2	86	Absent	
8	617	Absent	
1	650	Not to perform	
9	890	Not to perform	
4	1010	Absent	
3	1088	Present	
7	1208	Present	
6	1448	Not to perform	

Laboratory identification code :	14
Cut-off at 15 copies :	36.64 cycles
Copy number at the cut-off :	11.28 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	188	Not to perform	
1	272	Not to perform	
5	419	Not to perform	
4	929	Absent	
7	992	Present	
6	1502	Not to perform	
3	359	Present	
2	5	Absent	
8	941	Absent	

Laboratory identification code :	16
Cut-off at 15 copies :	35.90 cycles
Copy number at the cut-off :	10.76 copies

Sample type	Sample N°	Ruminant DNA	Comment
4	200	Absent	No inibition observed.
2	302	Absent	No inibition observed.
9	404	Absent	No inibition observed.
3	575	Present	No inibition observed.
7	803	Present	No inibition observed.
6	1151	Not to perform	
1	1352	Not to perform	
8	1373	Absent	No inibition observed.
5	1472	Not to perform	

Laboratory identification code :	17
Cut-off at 15 copies :	34.30 cycles
Copy number at the cut-off :	9.26 copies

Sample type	Sample N°	Ruminant DNA	Comment
5	284	Not to perform	
6	368	Not to perform	
3	467	Present	
2	545	Absent	
1	677	Not to perform	
9	782	Not to perform	
8	1049	Absent	
4	1415	Absent	
7	1478	Present	

Laboratory identification code :	18
Cut-off at 15 copies :	31.41 cycles
Copy number at the cut-off :	13.14 copies

Sample type	Sample N°	Ruminant DNA	Comment
4	119	Absent	
1	164	Not to perform	
2	248	Absent	
5	257	Not to perform	
9	674	Not to perform	
8	833	Absent	
3	953	Present	
6	1124	Not to perform	
7	1127	Present	

Laboratory identification code :	19
Cut-off at 15 copies :	36.13 cycles
Copy number at the cut-off :	11.24 copies

Sample type	Sample N°	Ruminant DNA	Comment
5	203	Not to perform	
7	209	Present	Partial inhibition
9	323	Not to perform	
6	422	Not to perform	
1	542	Absent	PCR performed because we don´t know the use of the feed material (farmed animals or aquafeed).
3	629	Present	Partial inhibition
4	821	Absent	At 1 fold: PCR inhibition, At 10 fold no PCR inhibition
2	1247	Absent	PCR performed as previous blood product is positive. At 1 fold: PCR inhibition, At 10 fold no PCR
8	1535	Absent	Partial inhibition.

Laboratory identification code :	20
Cut-off at 15 copies :	33.81 cycles
Copy number at the cut-off :	10.56 copies

Sample type	Sample N°	Ruminant DNA	Comment
5	14	Present	
7	92	Absent	
9	329	Present	
6	671	Absent	
1	1271	Not to perform	
3	1331	Present	
4	1475	Not to perform	
2	1532	Present	
8	1538	Not to perform	

Laboratory identification code :	21
Cut-off at 15 copies :	36.35 cycles
Copy number at the cut-off :	9.46 copies

Sample type	Sample N°	Ruminant DNA	Comment
9	107	Not to perform	
8	158	Absent	
1	191	Not to perform	
3	602	Present	
2	977	Absent	
6	989	Not to perform	
7	1073	Present	
4	1226	Not to perform	
5	1283	Not to perform	

Laboratory identification code :	22
Cut-off at 15 copies :	35.92 cycles
Copy number at the cut-off :	9.30 copies

Sample type	Sample N°	Ruminant DNA	Comment
6	152	Not to perform	
4	227	Absent	
3	440	Present	
7	614	Present	
2	842	Absent	
1	1298	Not to perform	
5	1337	Not to perform	
8	1346	Absent	
9	1349	Not to perform	

Laboratory identification code :	23
Cut-off at 15 copies :	37.70 cycles
Copy number at the cut-off :	9.69 copies

Sample type	Sample N°	Ruminant DNA	Comment
6	206	Not to perform	Label : pig feed
8	293	Absent	Label :aquafeed with animal proteins
4	470	Absent	Label : premix for aquafeed
2	761	Absent	Label : haemoglobin powder
5	851	Not to perform	Label : compound milk
9	1106	Not to perform	Label : premix
3	1169	Present	Label : haemoglobin powder
7	1262	Present	Label : aquafeed with haemoglobin powder
1	1541	Not to perform	Label :soybean meal

Laboratory identification code :	24
Cut-off at 15 copies :	31.91 cycles
Copy number at the cut-off :	10.32 copies

Sample type	Sample N°	Ruminant DNA	Comment
4	38	Absent	
7	101	Present	
1	515	Not to perform	
6	719	Not to perform	
3	764	Present	
8	1157	Absent	
9	1187	Not to perform	
2	1355	Absent	
5	1445	Not to perform	

Laboratory identification code :	25
Cut-off at 15 copies :	35.34 cycles
Copy number at the cut-off :	11.08 copies

Sample type	Sample N°	Ruminant DNA	Comment
2	167	Absent	
3	197	Present	
7	452	Present	
9	620	Not to perform	
4	740	Absent	
8	752	Absent	
6	800	Not to perform	
5	932	Not to perform	
1	1325	Not to perform	

Laboratory identification code : **27**

Cut-off at 15 copies :	37.04 cycles
Copy number at the cut-off :	11.29 copies

Sample type	Sample N°	Ruminant DNA	Comment
1	110	Not to perform	
3	116	Present	
6	179	Not to perform	
9	215	Not to perform	
2	275	Absent	
4	281	Absent	
8	509	Absent	
5	1067	Not to perform	
7	1370	Present	

Laboratory identification code : **28**

Cut-off at 15 copies :	36.73 cycles
Copy number at the cut-off :	9.03 copies

Sample type	Sample N°	Ruminant DNA	Comment
3	35	Present	
7	74	Present	
8	131	Absent	
9	512	Not to perform	
2	734	Present	
6	773	Not to perform	
5	959	Not to perform	
4	983	Not to perform	
1	1082	Not to perform	

Laboratory identification code : **30**

Cut-off at 15 copies :	37.74 cycles
Copy number at the cut-off :	9.29 copies

Sample type	Sample N°	Ruminant DNA	Comment
4	173	Absent	
7	560	Present	
9	647	Not to perform	
8	698	Absent	
3	818	Present	
6	881	Not to perform	
2	950	Absent	
1	1217	Not to perform	
5	1229	Not to perform	

Laboratory identification code : **33**

Cut-off at 15 copies :	37.75 cycles
Copy number at the cut-off :	10.34 copies

Sample type	Sample N°	Ruminant DNA	Comment
4	65	Not to perform	
7	155	Present	
3	224	Not to perform	
1	245	Not to perform	
6	287	Not to perform	
5	905	Not to perform	
8	1022	Absent	
9	1160	Not to perform	
2	1220	Not to perform	