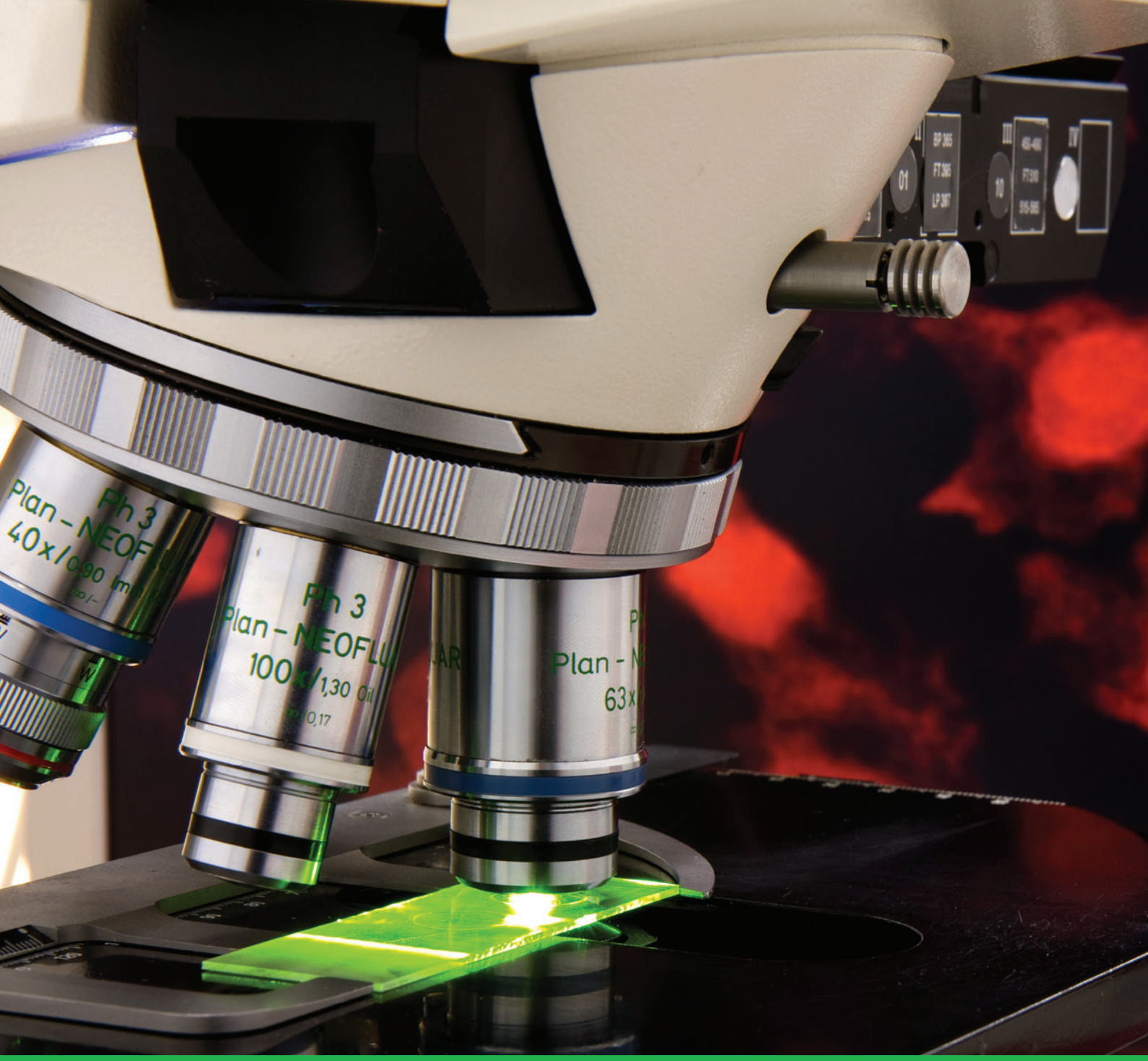


# IAG ring test animal proteins 2017

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- All participants of the ring test
- European Fat Processing and Renderers Association (EFPRA; C. van Vuuren)
- International Fishmeal and Fish oil Organisation (IFFO; A. Jackson, I. Pike)
- Ministry of Economic Affairs (EZ; E.L.J.M. Pierey, K. Zwaagstra)
- Dutch Food and Consumer Product Safety Authority (NVWA; R. Herbes)

# Contents

# Summary

The annual ring test for the detection of animal proteins in animal feed of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy was organized by RIKILT - Wageningen UR, The Netherlands. The aim of the ring study was to provide the participants information on the performance of the local implementation of the detection method for their local quality systems. A further aim was to gather information about the current practices in the application of the microscopic method. The current 2017 version of the IAG ring test for animal proteins facilitated the full scenario with the methods for microscopy and PCR as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs.
The four samples of the ring test were based on two different matrices. Samples A and B were based on an artificial formulation mimicking a ruminant feed. The samples C and D were based on a broiler feed. Three samples were labelled as fish feed (A, B and C). Adulteration was achieved by adding 0.01% ruminant bone meal in sample B (representing an MBM at a level of 0.03%), and 0.2% fish meal in sample D. Two samples were left blank.
A total of 41 participants subscribed to the ring test animal proteins. Four participants did not submit their results. Of the remaining 37 participants, two applied exclusively PCR, leaving 35 sets of microscopic results, accompanied by PCR results in 14 cases.

**Microscopy**
Two participants applied an incorrect number of determination cycles for one or more samples as required by the EU protocol. In total nine participants (26% of 35 participants) included incorrect interpretations of the encountered number of particles (e.g. "below threshold" for zero particles, "present" for 5 particles) or submitted incomplete reports.
Incorrect positive results (positive deviations) were expressed in a specificity score and incorrect negative results (negative deviations) were expressed in a sensitivity score. An optimal score is 1.0. The results are analysed in two ways: numbers below threshold (between 1 and 5 particles per determination cycle inclusive) have been considered negative (for matching the official way of reporting) and as alternative approach has been considered positive (complying to the zero tolerance). The latter representation allows to evaluate the performance of the participants, and to compare with results in previous years.
For all samples a few participants did not detect animal particles when present (sensitivity between 0.83 and 0.92, or between 0.92 and 0.97, depending on the evaluation of the results below the threshold as negative or positive, respectively) or erroneously reported a few animal material particles when absent (specificity between 0.94 and 1.0, or between 0.86 and 0.97, respectively). Spiking of exclusively bone fragments means that no animal protein material can be found in the flotation or raw material, since muscle fibres are absent. A further investment in documentation and training for correct identification of particles of animal origin could be beneficial.

**PCR**
PCR analysis for detecting ruminant material is required for those samples where terrestrial animal material was detected microscopically. In the framework of the current ring test the application of PCR was voluntary. Ruminant material was correctly detected in sample B in all 16 cases where PCR was applied. In samples C and D three erroneous reports of presence of ruminant DNA were submitted. In all cases were PCR results were delivered without necessity according to the SOP "operational schemes v3.0" (other samples) no adjustment of the microscopic results was carried out. This is in concordance with the official procedures.

A further investment is necessary in documentation for and training of microscopists for correct identification of particles of animal origin, which would enhance the specificity of the microscopic method. Evaluation of some aspects of the application of the current microscopic methods would be beneficial.

# 1 Introduction

The monitoring of the presence of animal proteins in feed for prevention of mad cow disease is an important part of the required active monitoring by member states of the European Union. A range of official control methods was combined in 2009 in Regulation (EC) 152/2009. The new method for microscopic detection of animal proteins entered into force on 12 February 2013 (Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009: EC, 2013a, and its corrigendum EC, 2013b). at the same time an official method for DNA identification of ruminant material by means of PCR was published. The changes in the microscopic method implement a more detailed procedure. The modifications were directed by the desire to gain in reproducibility and in harmonization (e.g. Veys et al., 2010). A Limit of Detection (LOD [1]) of five particles was set per determination cycle based on a laboratory sample of 10 grams. As of 1 June 2013 non-ruminant material is allowed as ingredient in aquafeed (Regulation (EC) 56/2013 amending Annex IV of Regulation (EC) 999/2001). Ruminant material remains prohibited, which needs a specified identification method, which was implemented by a PCR method. The combined application of the microscopic and PCR methods needs guidance, which is implemented in a Standard Operational Procedure (SOP) "operational schemes v3.0", developed by European Union Reference Laboratory (EURL). Other SOPs supporting the new method include details of the microscopic and PCR procedures

The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy organises annually a ring test for animal proteins in feeds for all their members. RIKILT – Wageningen UR organises this ring test on behalf of the IAG section Microscopy. Overviews of past results are presented in the annual reports of the ring tests (latest version: van Raamsdonk et al., 2016) and in an evaluation of the detection of fish material (van Raamsdonk et al., 2017). These overviews revealed that erroneous identification of particles from non-animal origin occurs, resulting in specificity scores lower than one. Therefore, the current 2017 version of the IAG ring test for animal proteins includes two different matrices, each of which is represented as a blank. The other two samples are chosen to be spiked at levels close to the technical limit of animal proteins in feed (0.1%; Regulation (EC) 152/2009). The final intention is, as in previous years, to provide the participants data on the performance of their own way of implementation, and to document the application of the two enforcement methods.

In this report the ring test for animal proteins 2017 is presented.

[1] The term Limit of Detection suggests that findings below that level (here: 5 particles) are unreliable or even cannot be achieved properly. Instead, the issues of the presence of individual particles at low levels after microscopic examination might be related to possible lab contamination or to erroneous identification (specificity). Since the term LOD is originally defined in the framework of chemical analysis related to technical limitations instead of solving contamination or specificity issues, the term "threshold" is used here in the report.

# 2 Methods

## 2.1 Materials

The IAG ring test for animal proteins 2017 was based on two different feed compositions: one artificially produced feed and one poultry feed. The artificial feed contained cornglutenfeed (30%), citruspulp (20%), beetpulp (12%), palmkernelmeal (15%), wheat (7%), wheat semolina (7%), soybeanmeal (7%) and mineral mix (2%). All ingredients were bought as pure materials and tested. The poultry feed consisted of wheat (44%), corn (25%), sunflowerexpeller (12%), rapeseed expeller (5%), soy expeller (5%), oat hulls (2.5%), corn DDGS (2,5%), mineral mix (2%).
The IAG ring test for animal proteins 2017 was combined with the IAG ring test for botanic composition (sample 2017-B) and for label control (samples 2017-C and 2017-D). The results of this ring test are being published in a separate report (van Raamsdonk et al., 2017).
The design of the ring test animal proteins allowed to apply the full method for the detection of animal proteins as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 (EC, 2013a), its corrigendum (EC, 2013b) and the accompanying SOPs. The samples were chosen to be fit for detection by both microscopy and PCR. The choice and order of the methods was part of the study. The composition of the four samples is listed in Table 1.

***Table 1*** *Composition of the samples in the NRL-IAG ring trial animal proteins 2016.*

| Label | Sample type | Content |
|---|---|---|
| 2017-A | ruminant feed | blank |
| 2017-B | ruminant feed | ruminant BM (0.01% sediment of a ruminant meal) |
| 2017-C | poultry feed | Blank |
| 2017-D | poultry feed | 0.2% fish |

The fish meal used for spiking were samples from practice, which were examined in the RIKILT regular control program and found to be negative for land animal material.
The ruminant meat and bone meal was collected from a Dutch trading company. It contained primarily ruminant material. The f-factor, based on five sedimentation, was 0.355 (0.272 – 0.466). The sediment fraction of this material was spiked at a level of 0.01%, representing a spike level of approx. 0.03% of the full MBM. Previous results indicated that such levels can reasonably be detected (Engling et al., 2000; van Raamsdonk et al., 2008, 2012; Veys et al., 2010).
All materials were checked on purity (absence of any contamination) and identity, and were all found to be fit for application.

## 2.2 Procedure for production

Since only two of the four samples contained animal proteins at relatively low levels, it was chosen to spike every jar individually. Taking a sample size of 50 grams, an amount of 5 mg bone meal or 100 mg fishmeal, respectively, was individually weighted and added to each jar. The material in each jar was then homogenized. This procedure implies that control of the homogeneity of the batch material for filling the jars was not necessary. Nevertheless, three jars each were investigated for approving the contents of the jars.

## 2.3 Homogeneity study

Two RIKILT microscopists examined independently all basic materials and three jars of all four samples according to the procedure of Regulation (EC) 152/2009. A PCR was check was not performed.

***Table 2*** *Results of the homogeneity study. Sediment amounts are based on 10 grams. Microscopy: three replicates, independently investigated by two microscopists.*

| Sample | | Sediment amount | Microscopy | |
|---|---|---|---|---|
| | | | MBM | fish |
| 2017-A | blank | 229-257 mg/10 g | absent | absent |
| 2017-B | 0.01% ruminant bones (0.03% MBM) | 253-290 mg/10 g | present | absent |
| 2017-C | blank | 85-120 mg/10 g | absent | absent |
| 2017-D | 0.2% fish | 106-112 mg/10 g | absent | present |

The microscopic results were correct in all cases (Table 2).
The microscopy research group and the PCR research group of RIKILT did not participate in the further laboratory analysis of this ring test.

## 2.4 Organization of the ring trial

All IAG members, all NRLs, participants of former ring tests and a series of putative interesting laboratories were informed about the ring test for 2016 by means of an invitations in the IAG Newsletter of 2016 (http://www.iag-micro.org/files/newsletter_2016_iag_section_feed_microscopy_version_2.1_final.pdf). Until the beginning of March a total of 38 participants for the ring test animal proteins were listed. Participants outside Europe were informed to be aware of possible problems with custom regulations. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on Thursday 23rd of March 2017. On Friday March 31st an E-mail message was sent to all participants, together with a file containing a sheet with instructions (see Annex 2) and the electronic report forms (see Annex 3 and 4), and the request to confirm the receipt of the package.
The closing date for reporting results was fixed at Friday May 5$^{th}$. Several requests were received to extent the period for analysis. Results received after the date at which the evaluation of the results was started were ignored. The analysis of the results was carried out between 16$^{th}$ and 26$^{th}$ May.

Since the new Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 is fully operational for both microscopy and ruminant PCR, the reporting form was designed to accommodate both types of results. The report form consisted of four elements:

- Laboratory and sample numbers.
- Results of the microscopy analysis for up to three analyses. Depending on the results of this first determination the cells for the second determination were made active, and depending on the sum of first and second determination the cells for the third and last determination were made active. The final line consisted of a sum of particles found.
- Results of ruminant EURL-AP PCR method.
- The final conclusion of the participant.

The report form was interactive. The decision rule to make the set of cells active for the second determination was made as follows:

IF [#terr.an. IS between 1-5] OR [#fish IS between 1-5] THEN second determination

The decision rule for the third determination was based on the sum after two determinations. The text of the new method requires to perform an extra determination when the number of particles is

between 1 and 5, which interpreted as directing a second (or third) analysis when ONLY one of the types of material was found to be within this range.
The draft report was finalised at May 29th.

## 2.5 Analysis of results

As in every analytical method, several types of results exist, such as duplicate results, intermediate results and final results (conclusion). For the detection of animal proteins two methods are combined: detection by microscopic analysis and identification by PCR analysis, which also imply the existence of intermediate results. The entire strategy is a combination of Annex IV of Regulation (EC) 152/2009 and the binding SOP "operational schemes v3.0". The parts applicable for the evaluation of the current ring test are shown in Figure 1.

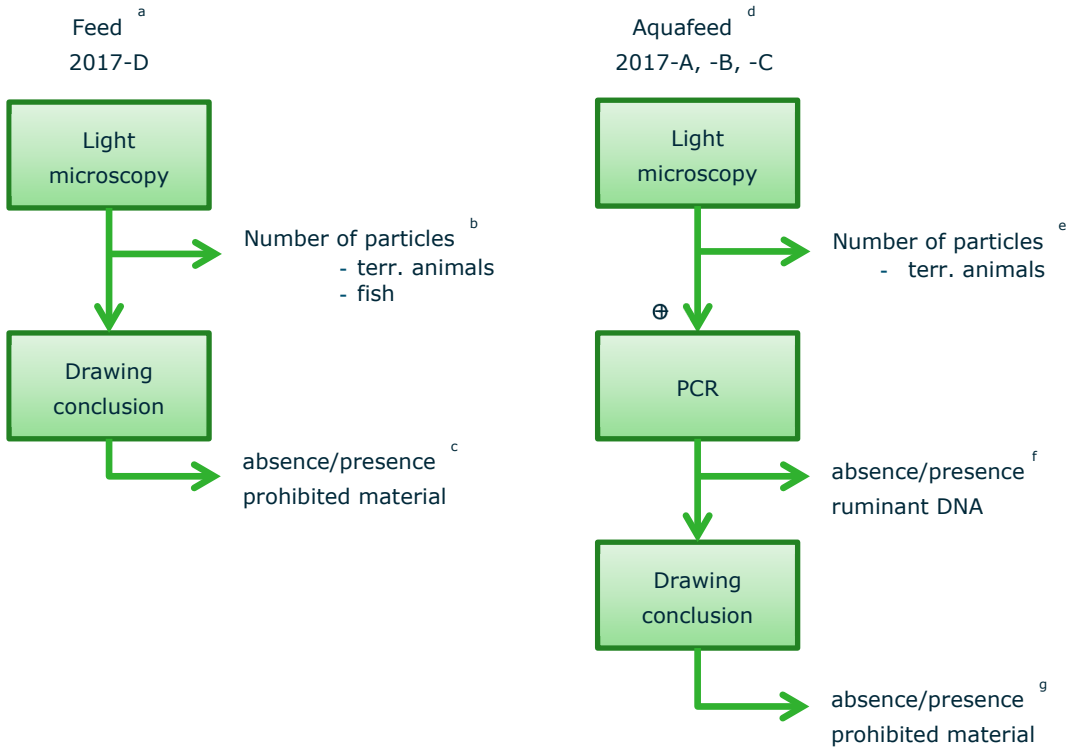


***Figure 1*** *Overview of activity blocks and intermediate or final results obtained after each activity for the analysis of the samples in the IAG ring test animal proteins 2016. Meaning of indices: a: scheme according to SOP, Figure 1; b: Regulation 152/2009, paragraph 2.1.4, diagram 1; c: Regulation 152/2009, paragraph 2.1.5, reporting texts; d: scheme according to SOP, Figure 2; e: Regulation 152/2009, paragraph 2.1.4, diagram 2; f: Regulation 152/2009, paragraph 2.2.6, interpretation of results; g: Regulation 152/2009, paragraph 2.1.5, reporting texts and paragraph 2.2.6.1, reporting texts.*

Since light microscopy is the primary method in the IAG ring test animal proteins, emphasis will be given to the results as indicated in Figure 1 with index "b" and "e". Secondly, the other results ("c", "f" and "g") will be presented.

The results are analysed in two ways: numbers below threshold (between 1 and 5 inclusive) have been considered negative and as alternative approach considered as positive. The choice to consider all reported numbers positive is based on the aim of the ring test to provide all participants information on their performance. In the view of this aim any threshold is avoided in the alternative approach. The principle that any particle correctly identified as of animal origin is apparently present

fits to the legal principle of zero tolerance and it allows a way to compare the present results with those of previous years.

For binary results (yes/no, positive/negative, etc.) standard statistics are accuracy, sensitivity and specificity. The accuracy is the fraction of correct results, either positive or negative. The sensitivity is the ability of the method used, to detect the contaminant when it is present, whereas the specificity is the ability to not detect the contaminant when it is absent. The following equations have been used to calculate the statistics:

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

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

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

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics are presented as fractions. Accuracy (specificity or sensitivity) has been calculated for each sample type.
As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

# 3 Results

A total of 41 participants subscribed for the ring test animal proteins. Three participants did not submit their results. Of the remaining 38 participants, two applied exclusively PCR, leaving 36 sets of microscopic results, accompanied with PCR results in 15 cases. The participants originated from 15 countries: 12 member states of the European Union, and three other countries (China, Norway and Switzerland). The list of participants is presented in Annex 5. Five member states have been involved with three or more participating laboratories: Germany (18 labs), the Netherlands (4), France (4), Belgium (4) and Italy (3).
All results were received by E-mail, in most cases by means of a scan and the original report file. Not in all cases a scan as pdf-file was submitted although clearly requested. In all those cases that a participant sent in several versions of the report sheet the most recent version was used. All full reports were included.
The full results are presented in the tables of Annex VI, VII and VIII.

## 3.1 Application of the method

One participant (12) carried out too many determination cycles for one or more samples, and also one participant (9) carried out too less cycles. In total ten participants (28% of 36 participants: 1, 2, 6, 9, 16, 19, 30, 33, 34, 41) included incorrect interpretations of the encountered number of particles (e.g. "below threshold" for zero particles, "present" for 5 particles) or submitted incomplete reports. In order to be able to evaluate correctly the results, all these participants were informed about this situation, although without mentioning the precise type of error. If the second submitted version still contained any erroneous evaluation of their own results, this was accepted as such. Inconsistencies remained in four cases.

## 3.2 Microscopic procedure

An inventory of nine different parameters was added to the report sheet of the actual results of the four samples. These results are shown in Annex 6 and summarised in Table 3. The main purpose of this inventory was to provide benchmark information for the individual participants for comparison with the general application of the method. Although this has to be considered additional information only, a ring test with a random set of participants provides a good opportunity to collect meta-data on the application of the method. The current results provide the opportunity to discuss some parameters of the microscopic method.
The results as presented in Table 3 generally show a good application of the method, except for the number of determinations applied and for the embedding agent. Differences with previous years will be presented in the next chapter (Discussion).
The minimum share of the total amount of sediment declared to be used was 8% (participant 41). Several other participants used shares between 13 and 20%. Most participants applied moderate portions, in some cases up to 97%.

***Table 3*** *Inventory of parameters for microscopic detection and their application. Pink cells indicate deviations from the official method. *: different types of glassware are in use, which could be summarised of glassware as "champagne glass". The correct indication needs further examination.*

| Parameter | parameter state | number of participants | amount |
|---|---|---|---|
| Correct application of the number of determinations | yes | 33 | |
| | no; too many determinations | 1 | |
| | no; insufficient determinations | 1 | |
| Extra milling step (<1.0 mm) | no | 26 | |
| | yes | 7 | |
| type of glassware | chemical sedimentation funnel | 18 | |
| | conical glass with cock | 8 | |
| | champagne glass * | 2 | |
| | beaker (flat bottom) | 4 | |
| | other | 3 | |
| sedimentation agent | TCE | 34 | |
| | TCE/Petroleumether | 1 | |
| use of staining of sediment | no | 23 | |
| | yes | 12 | |
| use of binocular for examination at lower magnifications | yes | 25 | |
| | no | 10 | |
| size of cover glass used | small (e.g. 20 x 20 mm) | 27 | |
| | medium | 2 | |
| | large (e.g. 26 x 50 mm) | 5 | |
| share of the total sediment used for examination | minimum | | 8% |
| | maximum | | 97% |
| embedding agent for the sediment | glycerine / glycerol | 12 | |
| | paraffin oil | 12 | |
| | immersion oil | 7 | |
| | Norland Adhesive | 4 | |
| | other (water, glycerol:water mixture, mineral oil) | 0 | |

## 3.3 Microscopic detection

After analysis of the results it appeared that all except three participants correctly interpreted their own encountered numbers in terms of absent, below threshold or positive. Two participants (9, 41) reported presence based on numbers below threshold. This would not make a difference when considering number of particles below threshold positive: results in terms of numbers (Figure 1 index "b") and in terms of the final conclusion (Figure 1 index "c") are identical. One participant (40) reported below threshold with an actual number of particles of zero.
The results of the application of the microscopic detection are presented in Table 4; full results are listed in Annex 7. The results are suboptimal for both specificity and sensitivity situations. One participant did not find the fish material in sample 2017-D and three participants overlooked the bone material in Sample 2017-B. For both samples several reports below threshold were submitted, causing scores of 0.91 for fish and 0.83 for terrestrial animals, respectively, when results below threshold are considered negative. The erroneous report of the presence of either terrestrial animal or fish material in the situation that the other type was actual present resulted in the lowest specificity scores: 0.86 for terrestrial animal in sample 2017-D and 0.91 for fish in sample 2017-B.

***Table 4*** *Sensitivity and specificity scores for the detection of animal proteins by the* ***microscopic*** *method of four samples (top row: values below the threshold considered positive; bottom row in italics: values below the threshold considered negative). Abbreviations: n: number of participants. Capitals A to D: sample indication. *: spike level of bone particles, representing 0.03% of the full MBM.*

| | | Terrestrial animal | | | | Fish | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | *D* | A | B | C | *D* |
| n | | 0% | 0.01%* | 0% | *0%* | 0% | 0% | 0% | *0.2%* |
| 35 | specificity | 0.89 | | 0.94 | *0.86* | 0.94 | 0.92 | 0.97 | |
| | | *1.0* | | *0.97* | *0.94* | *0.97* | *0.94* | *0.97* | |
| | sensitivity | | 0.92 | | | | | | *0.97* |
| | | | *0.83* | | | | | | *0.92* |

There is no relationship between the level of the scores for specificity and the type of feed applied (samples 2017-A and –B: artificial ruminant feed, 2017-C and -D: broiler feed).
The results were stratified according to several of the parameters as presented in Table 3. No significant differences were found among the different states for each of these parameters. Also for the grinding of the sample prior to analysis no significant difference in final results is found.

## 3.4 Detection by PCR

Participants were invited to perform DNA analysis targeted for ruminants (EURL-AP Method) and to submit their results together with the results for microscopy. The PCR results are presented in Table 5 and in Annex 8. Four participants correctly reported only a PCR result for sample 2017-B, since bone material was found in a fish feed (SOP "operational schemes v3.0" Figure 2). One participant (31) mentioned that they applied PCR to the other samples for own documentation, but left it out of the official report. Another participant (30) applied PCR to sample 2017-D as well and found a positive result. The results for the PCR method are acceptable.

***Table 5*** *Results for DNA analyses (PCR) for four samples. Target: ruminant.*

| | | Ruminant | | | |
|---|---|---|---|---|---|
| | | A | B | C | *D* |
| | | 0% | 0.01% | 0% | *0%* |
| | specificity | 1.0 | | 0.92 | *0.83* |
| | sensitivity | | 1.0 | | |
| | n | 11 | 17 | 12 | *12* |

## 3.5 Combination of methods

The current ring test consisted of samples with label information that allowed the participants to make their own decision whether or not PCR should be applied in combination with the microscopic method. Guidance is provided by the SOP "operational schemes v3.0". The PCR results are complementary for legal enforcement and not intended to correct possible erroneous microscopic results. An erroneously positive PCR result was reported for three samples: participant 13 for samples 2017-C and 2017-D, and participant 30 for sample 2017-D. In all these cases the initial microscopic result was not modified based on these PCR findings. This was correct, since the mentioned SOP does not include the application of PCR in these situations.

# 4 Discussion

## 4.1 Application of the microscopic method

The current microscopic method was introduced in 2013 (EC, 2013a). Although in previous years several participants had difficulties identifying the correct application of the method, especially the establishment of the correct number of cycles (2014: 33% of the total participants applied incorrect number of cycles, 2015: 12%, 2016: 13%, 2017: 6%; Table 6), the application of a correct number of cycles is increasing over the years (van Raamsdonk et al., 2014, 2015, 2016). The report form of the IAG ring tests provides guidance when results have been entered by changing colour for the cycles still to perform (Annex 4). This could be extended to routine analyses.
Despite of the correct number of cycles, the required embedding agent glycerol is still reported to be applied by a minority of participants (Table 6). This can be clarified by the difference in viscosity of glycerol (dynamic viscosity 1200 cP) and of paraffin oil (preferably 68-81 cP). A lower viscosity causes a faster penetration of embedding agent in the bone structure, resulting in a higher transparency of the bone mass. The drawback of paraffin oil, however, is the variety in the composition of alkanes and hence a variety of available versions with differing viscosities.
Another aspect of correct application of the microscopic method is the interpretation of the numbers of bones. This appeared to be difficult in specific cases, as can be deducted from the report form of several participants. The text of paragraph 2.1.5 of Regulation (EC) 56/2013 amending Annex IV of Regulation (EC) 999/2001 provides the requirements for three sets of reporting texts. This paragraph needs be applied in combination with the previous paragraph (2.1.4.3 settling the number of cycles based on the number of fragments found). A flow chart would assist in the proper interpretation of the Regulation.

***Table 6*** *Comparison between some parameter distributions in the IAG ring studies between 2008 and 2017. *: number of cycles since 2014.*

| parameter | parameter choice | 2008 | 2009 -2016 | 2017 |
|---|---|---|---|---|
| correct number of cycles * | | | 67.3% - 86.7% | 94.3% |
| amount of material used for sedimentation | 5 grams | 16 | 5-0 | 0 |
| | 10 grams | 26 | 41-50 | 35 |
| | other | 3 | 3-0 | 0 |
| share of the total sediment used for examination | minimum | 4% | 0.2%-3% | 8% |
| | maximum | 100% | 100% | 97% |
| embedding agent for sediment | glycerine / glycerol | 8 | 10-25 | 12 |
| | paraffin oil | 18 | 12-23 | 12 |
| | immersion oil | 8 | 7-14 | 7 |
| | Norland Adhesive | 0 | 2-7 | 4 |
| | chloral hydrate | 3 | 1-0 | 0 |
| | other (e.g. Depar 3000, water) | 8 | 5-0 | 0 |

## 4.2 Specificity and sensitivity

The two types of feed applied differ primarily for the presence vs. absence of the pulps (ruminant based: 32%), palmkernelmeal (ruminant based: 15%), sunflowerexpeller (poultry feed: 12%) and rapeseed expeller (poultry feed: 5%). The erroneous reports of the presence of animal material in the two blank samples were at comparable levels, and in total seven reports exceeding the threshold (5 particles) were delivered (Table 4; Annex 7). There seems to be no apparent relationship between the composition of the feed and the results.

The actual level of 0.01% of bone material representing 0.03% of MBM (sample 2017-B) is in an acceptable range of detection. Levels at or below 0.05% usually result in scores for sensitivity higher than 0.95 (Table 7). Spiking of exclusively bone fragments means that no animal protein material can be found in the flotation or raw material, since muscle fibres are absent. It should hardly be a problem, since four out of the six required slides are based on sediment material (EC, 2013a). The spike level for the fish meal (0.2% in 2017-D) is above the technical limit of 0.1% (EC, 2013a). In the large study of the EURL for quantification of fish meal, one out of 22 participants missed the fish meal at a level of 0.15%, equalling a score of 0.95 (Veys and Baeten, 2008).
Grinding of a sample prior to analysis results logically in a larger amount of (smaller) particles. All participants applying the step of extra grinding except one (35) were able to find the spiked materials sufficiently. The effect of grinding is two-fold: smaller particles are more difficult to recognise, and contaminations at low spike levels will likely exceed the threshold. Although grinding can be used for homogenization, this extra step in the procedure should be reconsidered in the view of the side effects.

***Table 7*** *Results for detection of material of terrestrial animals and of fish in feed samples based on sediments of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008-2017) on behalf of the IAG section Microscopy. Results have been communicated in the framework of this Section. Results indicate specificity in the case of the blank, and sensitivity in the case of the other sample types.*

| Detection of : | Land animals | | | | | | Fish | | |
|---|---|---|---|---|---|---|---|---|---|
| Content: fish | 0 | 2-5% | 2% | 0 | 2% | 0 | 0 | 0 | 0 |
| year land animal | 0 | 0 | 0.1% | 0.1% | 0.05% | ≤0.05% | 0 | 0.1% | ≤0.05% |
| 2003 (n=29) | 0.86 | | | 1.0 | | | | | |
| 2004 (n=30) | 0.93 | | | | | 0.97 | 0.97 | | 0.93 |
| 2005 (n=42) | | | 0.95 | 0.95 | | | | 0.76 | |
| 2006 (n=43) | 0.98 | | 1.0 | | | | 0.93 | | |
| 2007 (n=45) | | 0.89 | 0.93 | | | | | | |
| 2008 (n=45) | 0.93 | | | 0.98 | | 0.96 | 0.98 | 0.91 | 0.84 |
| 2009 (n=49) | 0.96 | 0.98 | | 1.0 | | | 0.96 | 0.88 | |
| 2010 (n=53) | 0.96 | | 0.98 | | 0.91 | | 0.98 | | |
| 2011 (n=56) | 1.0 | | | | | 0.98 | 0.98 | | 0.91 |
| 2012 (n=53) | 0.94 | | | 0.98 | | 0.98 | 0.94 | 0.96 | 0.92 |
| 2013 (n=53) | 0.94 | 0.98 | | 0.94 [1)] | | 1.0 | 0.96 | 0.94 | 0.96 |
| 2014 (n=52) | 0.96 | | 0.94 | | | | 0.96 | | |
| 2015 (n=42) | 0.95 | | | 0.93 | | | 0.88 | 0.90 | |
| 2016 (n=45) | 0.96 | | 0.96 | | | | 0.98 | | |
| | | | 0.91 | | | | | | |
| 2017 (n=36), current results | 0.89 | | | | | 0.91 [2)] | 0.94 | | |
| | 0.94 | | | | | | 0.97 | | |

1) TCP used as contaminant for land animal material

2) 0.01% of bone meal representing 0.03% MBM

The IAG ring test sensitivity scores would be lower when considering findings below threshold as negative (Table 4). By principle, the application of a threshold and considering results below that threshold as negative will result in all cases in higher scores for specificity and in lower scores for sensitivity (Table 4; Lindenmayer and Brugman, 2005). When attempting to find an optimal level for the threshold, both the zero tolerance of the European legislation (EU, 2001), and the opportunity to avoid false positives in another way then by fixing a threshold, has to be taken into account.
Several other aspects has to be considered when evaluating the results of the microscopic method. The number of full reports has dropped from 56 in 2011 to 36 in the current test. At lower numbers of basic datasets one erroneous report has a larger effect on the scores. Furthermore a ring test for microscopy basically does not test the performance of the laboratory but rather that of the laboratory technician(s). Shifts in the personnel involved in microscopy and the need of relatively intensive

training might influence the results. Therefore, the results of the current ring test are not principally deviating from those of previous years (Table 6).

## 4.3 Combination of microscopy and PCR

SOP "operational schemes v3.0" provides documentation and flow schemes for specific situations in which PCR should be applied complementary to microscopy or where PCR should be applied as only method. The PCR results are in no situation intended to correct possible erroneous microscopic results. In the case where a PCR analysis was demanded based on the microscopic results (sample 2017-B), ruminant material was detected. In all cases where PCR results were delivered without necessity according to the mentioned SOP (other samples) no adjustment of the microscopic results was carried out. This is in concordance with the official procedures.

# 5 Conclusions and recommendations

## 5.1 Conclusions

The application of the microscopic method as reported in this study revealed several elements, which require further attention. These elements include grinding of feed samples containing already ground material and the use of different types of embedding agents.
The results indicate that certain particles can be erroneously identified as animal material, and confusing particles of animal origin can be overlooked. The principle of a threshold, currently fixed at five particles, below which results are considered negative, has a positive effect on specificity scores, even with spike levels around the technical limit of 0.1%. Alternatively, considering low number results as positive would result in higher sensitivity scores. The sensitivity of the current test is between 0.83 and 0.92 and the specificity is between 0.94 and 1.0 when considering the scores below the threshold of five as negative. The aim of the ring test is to provide the participants information on their performance. In the view of this goal, an alternative approach for presenting the results is applied by considering all reported particles as positive. This approach for evaluation results in sensitivity scores between 0.92 and 0.97, and specificity scores between 0.86 and 0.97.
No indication for an apparent relationship between the composition of the feed and the specificity was found. In order to improve the specificity, further documentation of the appearance of animal particles is required for better identification.

## 5.2 Recommendations

- A further investment is necessary in documentation for and training of microscopists for correct identification of particles of animal origin, which would enhance the specificity of the microscopic method.
- Evaluation of some aspects of the application of the current microscopic methods would be beneficial.

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# Annex 1 Introduction to the test

**Test 2017-A: animal proteins in feed**

The IAG ring test animal proteins in feeds is designed to apply both the microscopic method and the PCR ruminant method. The procedures to be followed are described in Annex VI of Regulation (EC) 152/2009 from the European Union, amended by (EC) 51/2013, and the related SOPs. All the documentation can be found on the website of the EURL AP: http://eurl.craw.eu/index.php?page=187.
The jars contain 50 gram of feed, which is sufficient for carrying out three cycles of the microscopic method and/or for carrying out the PCR analysis, if necessary according to the SOPs. Take care to homogenise the content of each vial before taking the amount for analysis.
The samples are prepared in such a way that you can start with the procedure as described in "EURL-AP SOP operational schemes", followed by the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). If more than 16 fragments per category are found in any cycle, just choose "16" from the drop-down list.
Based on the average number of particles found, you have to make the decision whether each of the two types is absent in a sample (zero particles on average), below LOD (between 1 and 5 particles on average) or present (6 or more particles on average).
In addition to the work flow as presented in the paragraphs 2.1.3.4 until and including 2.1.4.3, it is mandatory to weight the sediment BEFORE and AFTER the analysis as performed in every determination.
All results can be entered in the report form with "animal proteins" in the name, which will be send to you separately.

# Annex 2 Basic instructions for the test procedure

**IAG ring test 2017 animal proteins**

Instructions for the IAG ring test

1 You have received a box with an introduction letter and four vials containing 50 grams of possibly contaminated animal feed. Please report the receipt of your package as soon as possible by E-mail to the address mentioned below.

2 The samples have to be analysed according to Annex VI of Regulation (EC) 152/2009 from the European Union, modified by (EC) 51/2013. The consolidated version and the SOPs can be found on the EURL website. The sample design allows to carry out the PCR ruminant analysis, but follow the SOPs carefully!. **Take care to homogenise the content of each vial before taking the amount for analysis**.
The samples are prepared in such a way that you can start with the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse three determinations of 10 grams as indicated in paragraph 2.1.4.3. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle and a final conclusion.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, horn, skin, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). If more than 16 particles are found in any category, please enter the value 16.
**The report form is interactive: if the results in the first determination cycle make it necessary to perform a second or third analysis according to the requirements of the Regulation, additional cells will turn pink.**
The final conclusion, according to Regulation (EC) 152/2009, can be reported in three ways, depending on the average number of particles found per category:
= Zero particles: animal proteins **absent**. If the first determination reveals no particles in any category, a second determination is not necessary.
= More than 5 particles on average per determination: **present**.
= Between 1 and 5 particles on average: sample is positive but a risk of a false positive result cannot be excluded. For the sake of the framework of the current report form the term '<LOD' has to be chosen.

Click here for the Regulation and connected SOPs

3 Reporting consists of the following steps:

3a Please fill in the questionnaire on the page "Procedure".
Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.
Your unique lab number is mentioned in the introduction letter, enclosed in the box.
All the fields with a drop-down list have to be completed.

| | |
|---|---|
| 3b | Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after your have entered it at the page Procedure. Enter yourself the four unique labels of the vials.<br>All fields with a drop-down list have to be completed. Please add the exact sediment weight in milligrams, without a decimal sign, of the total amount just before analysis and the remaining amount just after analysis. |
| 4 | After completing the two forms "Procedure" and "Results", they have to be sent to the organisers in two ways: |
| 4a | Save the Excel file by using "Save as …", add your unique lab code to the end of name (replace the ## signs with your lab number). The forms have to be sent by E-mail as Excel file and as a scan (*.PDF) to leo.vanraamsdonk@wur.nl AND to Bruno.hedemann@wur.nl. |
| 4b | Results will be included in the final analyses and report only if both forms are send in by electronic mail, and after the proper receipt of the requested fee. |
| 5 | Direct any questions to leo.vanraamsdonk@wur.nl |
| 6 | Closing date is Friday May 5th, 2017. |
| | RIKILT Wageningen UR, the Netherlands |

# Annex 3 Report form for procedure details

| **Please complete at least all the cells with a drop down list** | select your choice from a drop down list | type in your answer if necessary |
|---|---|---|
| **IAG ring test 2017 animal proteins** | | |
| Please select your unique lab number | **-- select --** | |
| Have you read the ring test instructions? | -- select -- | |
| Did you apply PCR ruminant detection method?: | -- select -- | |
| Did you apply grinding before performing the detection procedure? | -- select -- | |
| Indicate your glassware for sedimentation | -- select -- | |
| if other, please specify | | |
| Describe your sedimentation agent | -- select -- | |
| if other, please specify | | |
| Did you apply staining of the sediment (e.g. alizarin staining) as standard procedure? | -- select -- | |
| Did you examine at lower magnifications (using a binocular)? | -- select -- | |
| Indicate the size of cover glass | -- select -- | |
| Please describe your embedding agent for the sediment material | -- select -- | |
| if other, please specify | | |
| Did you use the expert system ARIES for identification of particles? | -- select -- | |

# Annex 4 Report form: results

**Please complete all the cells which are pink coloured. Additional cells will turn pink depending on your results. If more than 16 particles were found in any category, please enter the value 16.**

**IAG ring test 2017 animal proteins**

| | | | | |
|---|---|---|---|---|
| lab number | | | | |
| sample number | | | | |
| **First determination** | | | | |
| weight of sediment before analyses (in mg) | | | | |
| weight of sediment after analyses (in mg) | | | | |
| sediment % used for analyses | - | | - | |
| | land | fish | land | fish |
| Result of first determination cycle | - select - | - select - | - select - | - select - |
| **Second determination** | | | | |
| | | | | |
| | | | | |
| | - | | - | |
| | land | fish | land | fish |
| | | | | |
| **Third determination** | | | | |
| | | | | |
| | | | | |
| | - | | - | |
| | land | fish | land | fish |
| | | | | |
| Total number of particles per category | 0 | 0 | 0 | 0 |
| **PCR results** | | | | |
| Ruminant (EURL method) | | | | |
| | land | fish | land | fish |
| **Final conclusion** | - select - | - select - | - select - | - select - |
| Type of particles | | | | |
| Comment, if necessary | | | | |

# Annex 5 List of participants

| | |
|---|---|
| Austrian Agency for Health and Food Safety-AGES | Austria |
| FLVVT | Belgium |
| Laboratorium ECCA nv | Belgium |
| LFSAL | Belgium |
| China Agricultural University (East campus) | China |
| Croatian Veterinary Institute | Croatia |
| Danish Veterinary and Food Administration | Denmark |
| AdGène Laboratoire | France |
| Inovalys-Nantes | France |
| Laboratoire Départemental d'Analyse & de Recherche | France |
| S.C.L. Laboratoire de Rennes | France |
| Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit | Germany |
| CVUA-RRW | Germany |
| Futtermittelinstitut Stade (LAVES) | Germany |
| Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt | Germany |
| Landeslabor Berlin-Brandenburg | Germany |
| LLFG Landesanstalt für Landwirtschaft | Germany |
| LUFA Nord-West | Germany |
| LUFA Rostock | Germany |
| LUFA-Speyer | Germany |
| SGS Germany GmbH | Germany |
| Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62 | Germany |
| Veravis GmbH | Germany |
| WESSLING GmbH | Germany |
| Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories | Ireland |
| Equine Centre | Ireland |
| Istituto Zooprofilattico della Sicilia | Italy |
| Ministero delle politiche agricole alimentari e forestali, Laboratorio di Modena | Italy |
| CCL - Nutricontrol | Netherlands |
| Eurofins Food Testing Rotterdam BV | Netherlands |
| Nutreco Nederland BV - Masterlab | Netherlands |
| TLR | Netherlands |
| Alcontrol StjØrdal | Norway |
| Nofima AS | Norway |
| Cargill Poland | Poland |
| Lab. Regional de Veterinária | Portugal |
| University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene | Slovenia |
| Laboratorio Agrario Reginal Castilla y Leon | Spain |
| Trouw nutrition Espana | Spain |
| National Veterinary Institute, SVA | Sweden |
| Agroscope (ALP), Swiss Research Station | Switzerland |

# Annex 6 Details of procedures applied, microscopic method

| lab nr | grinding | Glassware | | agent | staining | binocular | size | embedding |
|---|---|---|---|---|---|---|---|---|
| 1 | no | special conical glass with cock | bottom | TCE | no | no | small (21 x 26 mm) | immersion oil |
| 2 | no | conical champagne glass | bottom | TCE | no | yes | small (20 x 20 mm) | immersion oil |
| 3 | no | beaker (flat bottom) | bottom | TCE | no | yes | medium | paraffin oil |
| 4 | yes | special conical glass with cock | bottom | TCE | no | yes | small (20 x 20 mm) | glycerine |
| 5 | no | chemical sedimentation funnel | top | TCE | yes | no | small (20 x 20 mm) | glycerine |
| 6 | yes | chemical sedimentation funnel | top | TCE | no | yes | large (22 x 50 mm) | glycerine |
| 7 | no | other | | TCE | no | no | small (20 x 20 mm) | glycerine |
| 9 | | beaker (flat bottom) | bottom | TCE | no | yes | small (20 x 20 mm) | immersion oil |
| 10 | no | chemical sedimentation funnel | top | TCE | no | no | small (21 x 26 mm) | paraffin oil |
| 11 | no | other | | TCE | no | no | large (26 x 50 mm) | paraffin oil |
| 12 | | beaker (flat bottom) | bottom | TCE | yes | yes | small (20 x 20 mm) | immersion oil |
| 13 | no | beaker (flat bottom) | bottom | TCE | no | no | small (20 x 20 mm) | glycerine |
| 14 | no | chemical sedimentation funnel | top | TCE | no | yes | small (20 x 20 mm) | glycerine |
| 16 | no | special conical glass with cock | bottom | TCE | no | yes | small (20 x 20 mm) | paraffin oil |
| 18 | no | special conical glass with cock | bottom | TCE | no | yes | small (20 x 20 mm) | glycerine |
| 19 | yes | chemical sedimentation funnel | top | TCE | no | no | small (20 x 20 mm) | immersion oil |
| 20 | no | chemical sedimentation funnel | top | TCE | yes | yes | medium | immersion oil |
| 21 | no | chemical sedimentation funnel | top | TCE | yes | yes | small (20 x 20 mm) | Norland adhesive 65 |
| 22 | no | chemical sedimentation funnel | top | TCE | yes | yes | small (20 x 20 mm) | glycerine |
| 23 | no | chemical sedimentation funnel | top | TCE | no | yes | -- select -- | paraffin oil |
| 25 | no | chemical sedimentation funnel | top | TCE | yes | yes | small (20 x 20 mm) | Norland adhesive 65 |
| 27 | no | chemical sedimentation funnel | top | TCE | yes | yes | small (20 x 20 mm) | Norland adhesive 65 |
| 28 | yes | special conical glass with cock | bottom | TCE | no | yes | small (21 x 26 mm) | paraffin oil |
| 29 | yes | chemical sedimentation funnel | top | TCE | yes | yes | small (20 x 20 mm) | glycerine |
| 30 | no | chemical sedimentation funnel | top | TCE | yes | no | small (20 x 20 mm) | paraffin oil |
| 31 | no | chemical sedimentation funnel | top | TCE | no | yes | small (20 x 20 mm) | glycerine |
| 32 | no | chemical sedimentation funnel | top | TCE | no | yes | small (20 x 20 mm) | Norland adhesive 65 |
| 34 | no | chemical sedimentation funnel | top | TCE | no | yes | large (22 x 50 mm) | paraffin oil |

| lab nr | grinding | Glassware | | agent | staining | binocular | size | embedding |
|---|---|---|---|---|---|---|---|---|
| 35 | yes | chemical sedimentation funnel | top | TCE | no | no | small (20 x 20 mm) | paraffin oil |
| 36 | no | other | | TCE | no | yes | small (20 x 20 mm) | paraffin oil |
| 40 | no | special conical glass with cock | bottom | TCE | no | yes | small (20 x 20 mm) | glycerine |
| 41 | no | special conical glass with cock | bottom | TCE/pe | yes | yes | small (20 x 20 mm) | glycerine |
| 42 | no | special conical glass with cock | bottom | TCE | yes | yes | large (22 x 50 mm) | paraffin oil |
| 43 | no | conical champagne glass | bottom | TCE | no | no | small (20 x 20 mm) | immersion oil |
| 47 | yes | chemical sedimentation funnel | top | TCE | yes | yes | large (26 x 50 mm) | paraffin oil |

# Annex 7 Results: presence of animal proteins, microscopic detection

| lab nr | | Sample numbers | | | | land | | | | fish | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCR | | | | | A | B | C | D | A | B | C | D |
| 1 | yes | 51 | 263 | 66 | 278 | absent | present | absent | absent | absent | absent | absent | present |
| 2 | no | 41 | 343 | 16 | 28 | absent | <LOD | absent | <LOD | <LOD | absent | absent | present |
| 3 | no | 101 | 3 | 237 | 18 | absent | present | absent | absent | present | absent | present | present |
| 4 | no | 171 | 353 | 167 | 68 | absent | present | absent | absent | absent | absent | absent | present |
| 5 | no | 111 | 93 | 207 | 58 | absent | present | absent | absent | absent | absent | absent | present |
| 6 | yes | 151 | 314 | 126 | 228 | <LOD | present | absent | absent | absent | absent | absent | present |
| 7 | part | 31 | 324 | 186 | 258 | absent | present | absent | absent | absent | absent | absent | present |
| 9 | no | 161 | 304 | 26 | 229 | absent | <LOD | absent | absent | absent | absent | absent | <LOD |
| 10 | no | 211 | 333 | 86 | 239 | absent | present | absent | absent | absent | absent | absent | present |
| 11 | yes | 21 | 243 | 136 | 8 | absent | present | absent | absent | absent | absent | absent | present |
| 12 | no | 81 | 294 | 326 | 178 | absent | absent | absent | absent | absent | present | absent | present |
| 13 | yes | 231 | 323 | 206 | 188 | absent | present | absent | absent | absent | absent | absent | present |
| 14 | no | 201 | 113 | 246 | 288 | absent | absent | absent | absent | absent | absent | absent | present |
| 16 | no | 141 | 13 | 116 | 248 | absent | present | present | present | absent | absent | absent | present |
| 18 | no | 11 | 354 | 187 | 268 | absent | present | absent | absent | absent | absent | absent | present |
| 19 | yes | 71 | 264 | 6 | 269 | absent | present | absent | absent | absent | absent | absent | present |
| 20 | no | 131 | 124 | 146 | 78 | absent | present | <LOD | absent | absent | absent | absent | present |
| 21 | yes | 221 | 274 | 196 | 158 | absent | present | absent | <LOD | absent | absent | absent | present |
| 22 | yes | 191 | 303 | 336 | 38 | absent | present | absent | absent | absent | absent | absent | present |
| 23 | no | 162 | 134 | 266 | 308 | <LOD | present | absent | absent | absent | absent | absent | present |
| 25 | partly | 61 | 284 | 256 | 359 | absent | present | absent | absent | absent | absent | absent | present |
| 27 | partly | 121 | 103 | 36 | 318 | absent | present | absent | absent | absent | absent | absent | present |
| 28 | no | 172 | 83 | 217 | 329 | absent | present | absent | absent | absent | absent | absent | present |
| 29 | no | 142 | 293 | 17 | 279 | absent | present | absent | absent | absent | absent | absent | present |
| 30 | yes | 132 | 254 | 197 | 259 | absent | present | absent | absent | absent | absent | absent | present |
| 31 | partly | 152 | 244 | 127 | 148 | absent | present | absent | absent | absent | absent | absent | present |
| 32 | no | 181 | 233 | 227 | 299 | absent | present | absent | absent | absent | absent | absent | present |

| lab nr | | Sample numbers | | | | land | | | | fish | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PCR | | | | | A | B | C | D | A | B | C | D |
| 34 | yes | 122 | 223 | 156 | 238 | <LOD | present | absent | absent | absent | absent | absent | present |
| 35 | no | 232 | 163 | 157 | 289 | absent | absent | absent | present | absent | present | absent | absent |
| 36 | no | 192 | 253 | 166 | 339 | absent | present | absent | absent | absent | absent | absent | present |
| 40 | no | 261 | 63 | 216 | 98 | absent | present | absent | absent | absent | absent | absent | present |
| 41 | no | 222 | 213 | 296 | 208 | <LOD | <LOD | absent | <LOD | absent | <LOD | absent | <LOD |
| 42 | no | 281 | 53 | 316 | 348 | absent | present | absent | absent | absent | absent | absent | present |
| 43 | no | 241 | 43 | 177 | 349 | absent | present | absent | absent | absent | absent | absent | present |
| 47 | yes | 351 | 23 | 7 | 138 | absent | present | absent | absent | absent | absent | absent | present |

# Annex 8 Results: PCR ruminant

| lab nr | ruminant | | | |
|---|---|---|---|---|
| | A | B | C | D |
| 1 | absent | present | absent | absent |
| 6 | absent | present | absent | absent |
| 7 | | present | | |
| 8 * | absent | present | absent | absent |
| 11 | absent | present | absent | absent |
| 13 | absent | present | present | present |
| 19 | absent | present | absent | absent |
| 21 | absent | present | absent | absent |
| 22 | absent | present | absent | absent |
| 25 | | present | | |
| 26 * | absent | present | absent | absent |
| 27 | | present | | |
| 30 | | present | | present |
| 31 | | present | | |
| 33 | | present | absent | |
| 34 | absent | present | absent | absent |
| 47 | absent | present | absent | absent |

*: exclusively PCR; no microscopic results.

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