IAG proficiency test animal proteins 2021

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Cover photo: a bone fragment of salmon

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Summary

The annual proficiency 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 Wageningen Food Safety Research, The Netherlands. The proficiency test was intended to provide the participants information on their performance of the implementation of the monitoring methods as well as to gather information about the current practices in the application of the microscopic method. The current 2021 version of the IAG proficiency test for animal proteins addressed all analytical sections of the methods for microscopy and PCR as published in Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs. Regulation (EU) 2020/1560, in force from 16th November 2020, introduced labelling information as extra parameter for decision on actions in the procedure, and changed the maximum determination cycles to two.

The samples used in the proficiency test contained salmon meal (2%), ruminant processed animal protein (PAP, 0.1%) and milk powder (5%). A fourth sample was left blank. The matrix was artificially produced, mimicking a ruminant feed, for avoiding any nonintentional contamination with ruminant DNA. The labels of the samples did not show an indication of the composition nor of the intended target animal.

A total of 41 participants subscribed to the proficiency test animal proteins. One participant did not submit their results and two submitted PCR results only, leaving 38 sets for microscopic evaluation. 16 sets of ruminant PCR results were submitted as well. The organisation and evaluation of the test and its results followed the Quality Guidance for Visual Research in Feed and Food.

Microscopy
All participants were requested to determine the presence or absence of land animal and/or fish, to indicate the type of material found and to describe the method used to achieve these results. Participants made a choice to follow either the old or the new protocol. Their choice to apply a second determination cycle would correctly match either one of these choices in all cases. Three participants made incorrect interpretations of the encountered number of particles (e.g. “suspect” for zero particles, “present” after a positive PCR result without any microscopical observation), and two participants did not report a final conclusion. Therefore, all evaluations were based on the actual number of particles reported by all participants.

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 positive (complying to the zero tolerance) and as alternative considered as negative (for matching the official evaluation).

The sensitivity for both basal ingredients were good (sample D, ruminant PAP: 0.97; sample B, salmon meal: 0.95). The correct indication of absence of both types of ingredients in the blank (specificity) was good as well (Sample A: 0.95 for both terrestrial animal and fish material). Issues were found for the detection of absence of terrestrial animal material in the presence of salmon meal (sample B: 0.66). Most participants did not report 5% milk powder (sample C: 0.32). Milk powder is a legal ingredient for all feeds and this sample was intended to be a challenger without giving an indication of the performance of the participants.

Either the lack of awareness of the probable presence of milk powder or the difficulty to recognise this material or both can cause the low performance of its detection. It can be considered to remove ingredients that are allowed according to Regulation (EC) 1069/2009 from the scope of the method for detection of animal proteins and restrict this method to particles included in the definition of PAP.

PCR

The ruminant material in sample (D) was detected by all participants. Two false positives were reported for the blank (sample A). One participant reported a false positive for the 5% milk powder in sample C. False positive in this context means that milk powder, as legal ingredient, should not be reported as a presence of a prohibited animal product.

Conclusions

Good results were achieved for the sensitivity score for 0.1% of ruminant MBM and for specificity scores (both terrestrial animal material and fish) for the blank sample. The discrimination between salmon meal and terrestrial animal material remains a challenge. It can be considered to remove ingredients that are allowed according to Regulation (EC) 1069/2009 from the scope of the method for detection of animal proteins and restrict this method to particles included in the definition of PAP.

The current protocol includes a check of the label for legally declared ingredients. If found and declared, a second determination cycle is not required. This is a welcome improvement of the microscopic method, certainly in the view of new relaxations. However, the logical consequence to label PT samples and in that way declare the contents to the participants, would reduce the PT to a label declaration test. An option could be to request the examination of specific types of ingredients.

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. With a long historical track record, microscopic detection of animal by-products is an important method for monitoring. The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy, serves as a platform for exchange of information, knowledge building and quality control. This international group organises annually a proficiency test for animal proteins in feeds for all their members. Wageningen Food Safety Research (WFSR) [1] is managing this proficiency test on behalf of the IAG section Microscopy. Overviews of past results are presented in the annual reports of the proficiency tests for monitoring animal proteins in feed (latest version: van Raamsdonk et al., 2019).

The current version of the microscopic method, together with an official method for DNA identification of ruminant material by means of PCR, was implemented by Regulation (EU) 2020/1560 (EU, 2020) amending Annex VI of Regulation (EC) 152/2009 (EC, 2009; consolidated version: 16-11-2020). A combined application of the microscopic and PCR methods is installed. Guidance is implemented in a Standard Operational Procedure (SOP) "operational schemes v5.0", developed by European Union Reference Laboratory (EURL). Other SOPs supporting the new method include details of the microscopic and PCR procedures, slide preparation among them. A Decision Limit of five particles per determination cycle is set. In the view that the IAG proficiency test is intended to monitor the technical performance of the participants, no filtering of results below a certain threshold is applied. As alternative besides this basic choice for evaluation, the Decision Limit of five particles per cycle is applied as well. This alternative approach is applied for comparison with other tests. Although the IAG proficiency test has a primary focus in microscopy, the participants were invited to submit their PCR results as well.

As for every other qualitative detection method, sensitivity (frequency of false negatives) and specificity (frequency of false positives) are important indicators for the performance of the method. Although specific elements of a method can be installed for improving one of these two indicators, there is a statistical relationship. In statistical terms, a decrease of a type I error (false positives) would imply an increase of a type II error (false negatives) or vice versa (Sheskin, 2004: page 88-89; Sedgwick, 2014). An important issue is the correct and precise discrimination between terrestrial animal material and fish material, which are currently the major categories for monitoring. Confusion among particles of these two categories (see van Raamsdonk et al., 2017), or with plant material or minerals will contribute to specificity and sensitivity scores lower than one.

The current 2021 version of the IAG proficiency test for animal proteins includes four samples with basic spike levels. Besides a blank sample two other samples have been spiked with 0.1% ruminant PAP or 2% of salmon meal. The fourth sample has been spiked with 5% milk powder intended as challenger. The results would not count for the performance of the participants. 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 monitoring methods.

In this report the proficiency test for animal proteins 2021 is presented.

[1] Formerly RIKILT. WFSR started 1 June 2019 as the merger of WFSR and the food safety laboratory of the Dutch Food and Consumer Product Safety Authority.

2 Methods

2.1 Materials

The IAG proficiency test for animal proteins 2021 was based on an artificially produced composition mimicking a cattle feed. The composition consisted of citruspulp (25%), wheat semolina (25%), soybeanmeal (15%), palmkernelmeal (15%), rapeseedmeal (10%), sugar beetpulp (8%), and a mineral mix (2%) consisting of monocalciumphosphate, bicalciumphosphate and calciumcarbonate in equal portions (Figure 1). The choice to produce a matrix from exclusively single ingredients was based on the situation of feed unintentionally contaminated with traces of ruminant material in the 2019 version of the IAG proficiency test (van Raamsdonk et al., 2019).

The design of the proficiency test animal proteins allowed to apply the full analytical part of the method for the detection of animal proteins as published in Regulation (EU) 2020/1560 (EU, 2020) amending Annex VI of Regulation (EC) 152/2009 (EC, 2009; consolidated version: 16 November 2020) with additional procedures in accompanying SOPs.

The new version includes several modifications, with most importantly the maximum of one repetition (a total of two sedimentations maximum) and the option to skip the second sedimentation when an ingredient of animal proteins is declared. None of the samples was labelled in this PT, with the consequence that a second analysis was required in all cases of a positive first result for animal material. The composition of the four samples is listed in Table 1.

Table 1 *Composition of the samples in the NRL-IAG proficiency trial animal proteins 2021.*

Label	Sample type	Content
2021-A	artificial feed	Blank
2021-B	artificial feed	2% (w/w) salmon meal
2021-C	artificial feed	Blank with 5% milk powder; challenger
2021-D	artificial feed	0.1% (w/w) ruminant PAP

A ruminant meat and bone meal (MBM) was chosen for the terrestrial PAP in order to test the performance of the PCR test in a regular situation of a prohibited contaminant. The salmon meal was selected from the WFSR collection of salmon meals for its low level of confusion particles. The composition of sample 2021-C was chosen to monitor the performance of the method for several aspects. Milk powder is a legal ingredient in all types of animal feed, but is included in the list of targeted particles in the Principle of the light microscopic method (EU, 2020: point 2.1.1). In the situation that the milk powder was not traced and, for reasons of analysis in the framework of a proficiency test, a PCR analysis was carried out, the final result should not be modified because of the positive result of that PCR test. The results of sample 2021-C, indicated as challenger, will not be part of the evaluation of the performance of the laboratories. The jars were labelled with an order number without a declaration of the content. Sample 2021-C was used for the IAG PT composition as well.

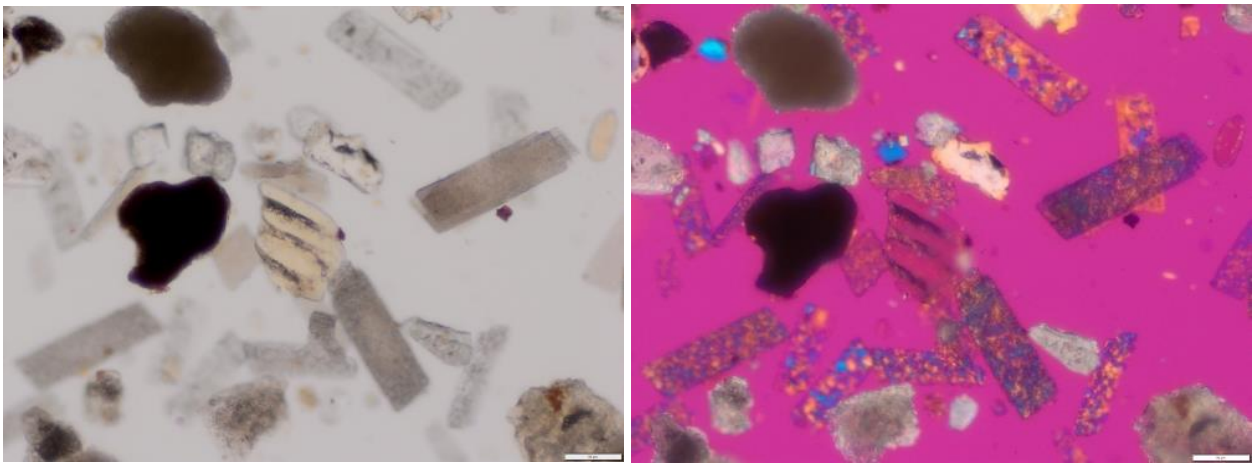

Figure 1 *Impression of the mineral mix used for the production of the samples at a magnification of 100x. Left: without polarization, right: with polarization. Bar: 50 µm.*

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order: 2021-A - 2021-B - 2021-C - 2021-D. All samples were prepared in a laboratory that is located at a distance from the WFSR microscopy laboratory. A sample size of 50 grams was chosen, which was sufficient for applying both determinations as mentioned for the full method in Regulation (EC) 152/2009 (amended by Regulation (EU) 2020/1560).

Jars for sample 2021-A were filled with 50 grams of the pure feed, closed and set aside. The other samples were produced by step-wise dilution of the dedicated contaminants down to a level of either 2% or 0.1%. The production scheme is presented in Figure 2. The milk powder was added and mixed in one step.

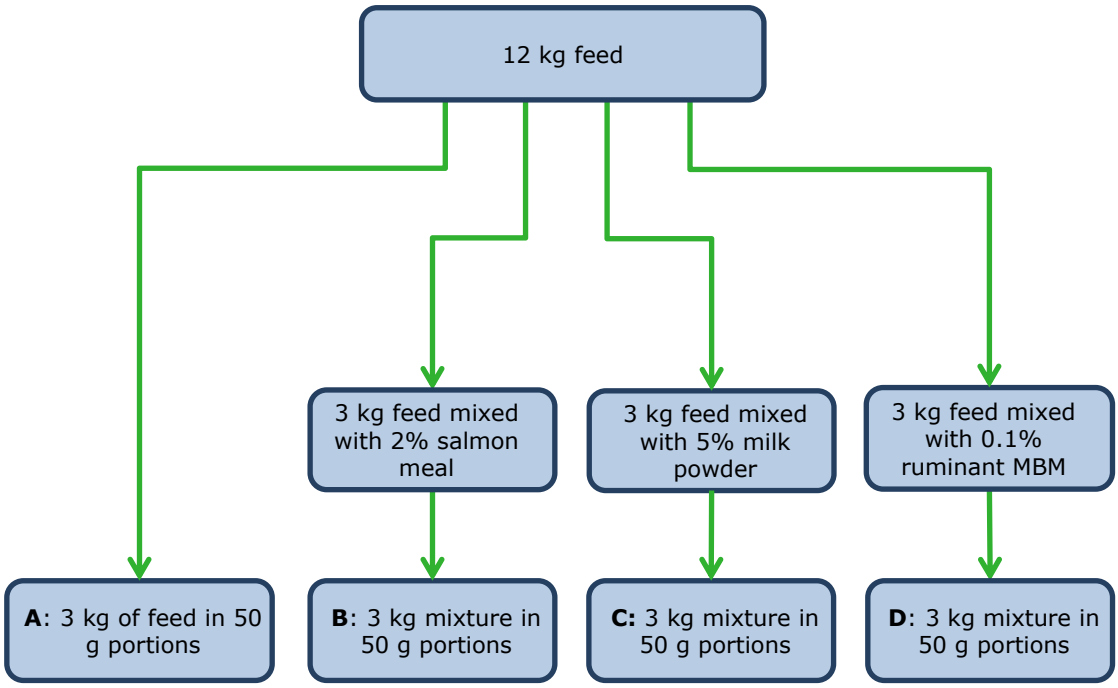

Figure 2 *Flow diagram for the production of the samples in order from left to right.*

2.3 Homogeneity study

Two WFSR microscopists independently examined all basic materials and five jars of all four samples according to the procedure of Regulation (EC) 152/2009 amended by Regulation (EU) 2020/1560. PCR was carried out according to the EURL-AP protocol and SOPs for ruminant. Cut-off: Cq = 38.06.

Table 2 *Results of the homogeneity study carried out by WFSR. Sediment amounts are based on 10 grams. Microscopy: five replicates. PCR: four replicates for ruminant, Cq values for the two undiluted analyses given.*

		Sediment amount	**Microscopy**		**PCR**
Sample			**terrestrial**	**fish**	**Ruminant; Cq values**
2021-A	Blank	217 mg/10 g	absent	absent	absent; 39.07, 40.89
2021-B	2% (w/w) salmon meal	213-227 mg/10 g	absent	present	absent; 39.25, 39.27
2021-C	Blank with 5% (w/w) milk powder; challenger	176-203 mg/10 g	present	absent	present; 24.96, 24.98
2021-D	0.1% (w/w) ruminant MBM	191-212 mg/10 g	present	absent	present; 29.29, 29.21

The microscopic results were correct in all cases (Table 2).

The microscopy research group and the PCR research group of WFSR did not participate in the further laboratory analysis of this proficiency test.

2.4 Organization of the proficiency test

All IAG members, all NRLs, participants of former proficiency tests and a series of putative interested laboratories were informed about the proficiency test for 2021 by means of mailing using distribution lists. Until the beginning of April, a total of 41 participants for the proficiency test animal proteins were listed. Two participants applied exclusively for PCR. 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 Tuesday 25th of May 2021. The file with instructions and the report form were sent to the participants on Friday 28th of May (see Annex 3 and 4). The closing date for reporting results was 24th of June. This date was postponed with one week for all participants after some requests of participants. The analysis of the results was carried out 28th of July. The report was distributed in draft to the Board of IAG section Feed microscopy and the participants on Wednesday the 1st of September.

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 current method requires to perform an extra determination cycle when the number of particles is between 1 and 5, which should be interpreted as directing a second (or third) analysis when ONLY one of the types of material, either fish or terrestrial animal, was found to be within this range.

2.5 Evaluation of results

As in every analytical method, several types of results exist, such as duplicate results, intermediate results and final results (conclusion). Since none of the samples was indicated as aquafeed, light microscopy is the only method for reaching the final conclusion, as stated in the SOP "Operational schemes v3.0". It is the intention of the proficiency test to establish primarily the analytical capability of the participants. Therefore, in those cases where the final conclusion as provided by a participant violates with the actual number of particles encountered, that number is used as basis for the evaluation.

The results are analysed in two ways: numbers below threshold (between 1 and 5 inclusive) have been considered positive and as alternative considered as negative. The choice to consider these number positive was based on the principle that any particle correctly identified as of animal origin is apparently present. This approach 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:

$$CS = \frac{TP+TN}{TP+FN+FP+TN}$$

$$SE = \frac{TP}{TP+FN}$$

$$SP = \frac{TN}{FP+TN}$$

where TP is the number of correct positive identifications (true positives), NA the number of correct negative identifications (true negatives), FP the number of false positives and FN the number of false negatives. The statistics are presented as fractions. The term Correctness replaces the term Accuracy as used in the past for avoiding confusion with the general application of "Accuracy". The parameters have been calculated for each sample and type of contaminant, either terrestrial animal or fish. As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

The organisation and evaluation of the IAG proficiency test animal proteins follows the Quality Guidelines for visual research in food and feed (van Raamsdonk et al., 2022).

3 Results

A total of 41 participants subscribed for the proficiency test animal proteins. One participant did not submit their results. Of the remaining 40 participants, two applied exclusively PCR results, leaving 38 sets of microscopic results, accompanied with PCR results in 17 cases. The participants originated from 16 countries: 13 member states of the European Union, and three other countries (Norway, Peru and Switzerland). The list of participants is presented in Annex 5. Five member states have been involved with three or more participating laboratories: Germany (15 labs), the Netherlands (7), France (3) and Belgium (3).

All results were received by E-mail, in most cases by means of both a scan and the original report file. A total of 16 participants (41.0%) did not submit the two reporting files with the correct name and 11 participants (28.2%) did not mention the lab number in one or both files. Additionally, in two cases the original Excel file was not submitted. File names according to the format and with lab number for both reporting files were clearly requested in order to avoid administrative errors during evaluation. In all those cases that a participant sent in several versions of the report sheet the most recent version was used. All full and correct reports were included. The draft report was finalised at August 29th, 2021. The full results are presented in the tables of Annex 6, 7 and 8.

3.1 Application of the method and reporting

The protocol of the method for detection of animal proteins has been changed in November 2020. A considerable part of the participants followed the former protocol for deciding to perform a second determination cycle (n=23, 62.2%). Of these 23 participants, 5 applied correctly a second determination cycle for the observation of 5 particles or less. Fourteen participants followed correctly the current protocol by applying a second determination cycle for some or all samples in the absence of a label declaration. Two participants (8.1%: 10, 13) included incorrect interpretations of the encountered number of particles (e.g. "suspect" for zero particles, "present" after a positive PCR result without any microscopical observation).

Incomplete or non-conclusive reports were submitted by seven participants (3, 16, 18, 39, 42, 43, 44). The errors included missing Excel file, missing final conclusions for one or more samples, switch of the numbers of particles reported for terrestrial animal and fish, or report of fictional numbers of particles. For five cases the results could not be processed, and new report sheets were requested. In terms of proficiency test procedures, a range of participants used other file names than requested, or the amount of sediment used was not indicated (42, 43).

The official method includes basically several steps: the analytical procedure including the determination of the number of cycles, the drawing of the conclusion and filing the report. The latter part, the use of the official texts for reporting, is excluded from the procedure in this proficiency test (see Annex 2). An evaluation of the final conclusion as reported would combine the analytical and a part of the "administrative" procedure. This evaluation would include the wrong interpretations of three participants and would be hampered by the missing conclusions of two other participants. In the view that the analytical performance of the participants should be the primary focus, the numbers of particles as reported are chosen as basis for the evaluation of the results. This approach also fits in the strategy to consider all results below the threshold as positive. As in previous years, the results with all results below threshold as negative will be shown as well.

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 2. 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 proficiency 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 previous version of the official method included a maximum of two repetitions resulting in a maximum of three possible determination cycles. The decisions to perform an extra cycle were only based on the results of the previous cycle. The current official version has a limit of two cycles, and a presence of an ingredient included in the label declaration does not need to be confirmed in a second determination cycle. The jars of the samples in this PT were not labelled because a declaration would reduce this PT to a label control test. The consequence is that every sample with a presence of an animal product needed to have been inspected twice. Considering the chosen action after the finding of more than 5 particles by each participant, the old protocol was followed by 21 participants, and 13 participants followed the new protocol. Two participants switched between the two versions for the several samples. Finally, two participants did not achieve any result exceeding 5 particles, which makes it impossible to find out their strategy. In addition, seven participants applied a second analysis after a blank result, in all cases in combination with the new protocol. This was and is not necessary for neither the old nor the new version.

The results as presented in Table 3 generally show a good application of the method. Differences with previous years will be presented in the next chapter (Discussion). Eleven participants applied a second determination cycle for all positive samples, which is indicative of a correct application of the new procedure. Two participants applied a second determination cycle for all four samples.

The minimum share of the total amount of sediment declared to be used was 1% for three samples (participant 23). In general, the portions of the sediment material used were between 10 and 50%.

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".*

Parameter	parameter state	number of participants	amount
Correct application of the number of determinations	Yes, according to 2013 protocol	21	
	Yes, according to 2020 protocol	13	
	Mixed application or inconclusive	4	
	Additional: repetition for the blank	7	
type of glassware	chemical sedimentation funnel	16	
	conical glass with cock	8	
	champagne glass *	5	
	beaker (flat bottom)	4	
	other	5	
sedimentation agent	TCE	38	
	Chloroform	0	
	TCE/Petroleumether	0	
use of staining of sediment	no	24	
	yes	14	
use of binocular for examination at lower magnifications	yes	27	
	no	11	
size of cover glass used	small (e.g. 20 x 20 mm)	31	
	medium	3	
	large (e.g. 26 x 50 mm)	4	
share of the total sediment used for examination	minimum		1%
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	15	
	paraffin oil	11	
	immersion oil	8	
	Norland Adhesive	4	
	other (water, glycerol:water mixture, mineral oil)	0	

3.3 Microscopic detection

The results of the application of the microscopic detection, expressed on the basis of declared numbers of particles, are presented in Table 4; full results are listed in Annex 7. The amount of added material, 0.1% (w/w) of terrestrial animal material or 2% (w/w) of fish material, would theoretically be sufficient for the application of one determination cycle at all times to reach a conclusive result.

The sensitivity for both basal ingredients were good (samples D: terrestrial animal material; sample B: fish). The correct indication of absence of both types of ingredients in the blank was good as well (sample A). Issues were found for the detection of terrestrial animal material in the presence of salmon meal (sample B; Figure 3). Thirteen participants reported the presence of terrestrial animal material. Most participants did not report the milk powder (5% in sample C).

The results after considering the reported numbers of particles below the decision limit as negative are by principle higher for specificity and lower for sensitivity compared to their evaluation as positive results. Four participants reported less than 5 particles for terrestrial animal material in sample B. After considering these negative the specificity was 0.76, higher that 0.65 when taking these results as positive. With the same principle, the sensitivity score for milk powder in sample C was higher when declaring a number of particles below 5 as positive.

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. Sample C contained 5% milk powder and will be evaluated as challenger.*

		Terrestrial animal				Fish			
		A	**B**	**D**	**C**	**A**	**B**	**D**	**C**
n		**0%**	**0%**	**0.1%**	**5%**	**0%**	**2%**	**0%**	**0%**
36	specificity	0.95	0.66			0.95		0.84	0.92
		0.97	*0.76*			*0.97*		*0.87*	*0.92*
	sensitivity			0.97	0.32		0.95		
				0.92	*0.29*		*0.92*		

The results were stratified according to several of the parameters as presented in Table 2. No significant differences were found among the different states for each of these parameters.

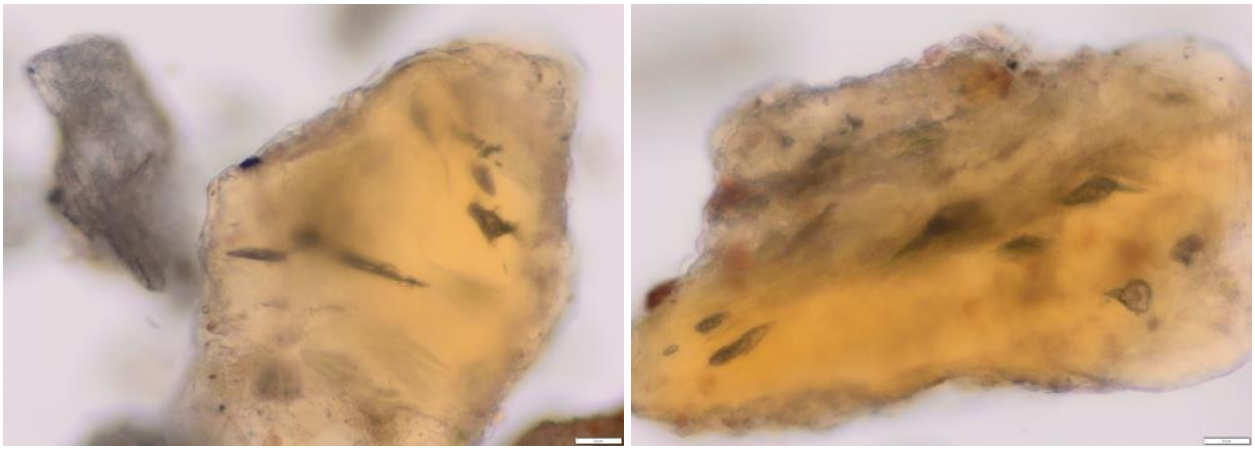

Figure 3 *Two particles of salmon meal at a magnification of 200x. Bar: 20 µm.*

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. Sixteen participants reported results for the sample (D) when found positive after microscopic analysis. Eleven of them also reported results for one or more of the other samples. The overall results are shown in Table 5. Full results are shown in Annex 8. The ruminant material in sample (D) was detected by all participants. Two false positives were reported for the blank (sample A). One participant reported a false positive for the 5% milk powder in sample C.

Table 5 *Results for DNA analyses (PCR) for four samples. Target: ruminant. *: results based on the presence of 5% milk powder.*

		Ruminant			
		A	**B**	**D**	***C***
		0%	**0%**	**0.1%**	**5% ***
	specificity	0.82	0.92		
	sensitivity			1.0	0.91
	n	11	12	17	11

4 Discussion

4.1 Samples for performance monitoring

The samples were all based on an artificial composition of ingredients. The mineral fraction was based on equal shares of monocalciumphosphate, bicalciumphosphate and calciumcarbonate. The appearance was different from that of mineral mixtures in regular compound feeds from practice (Figure 1). Despite this, confusion is not likely, especially when applying polarization.

The performance in this proficiency test for the blank sample (A: 0.95 and 0.97 for terrestrial animal material and for fish, respectively) and for the sample exclusively containing 0.1% of ruminant PAP (0.97 and 0.86) is comparable to the scores as achieved in previous years (Table 6). The slight underperformance for fish in the presence of an MBM can be compared to the results of past PTs in (2005: 0.76 and 2009: 0.88). The sample in the 2009 version of the PT was based on pig material produced by an approved producer (DAKA, Denmark). Other scores in the 2009 version of the IAG PTs were at a level of 0.96 or above (van Raamsdonk, 2009). It is apparently possible that, in some years, additional false positives for fish detection in the presence of an MBM can occur.

Table 6 *Results for detection of material of terrestrial animals and of fish in feed samples based on sediments of previous proficiency tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and WFSR (2008-2021) on behalf of the IAG section Microscopy. Results have been communicated in the framework of the annual meetings 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)		0.89					0.91 [2)]	0.94		
		0.94						0.97		
2018 (n=43)		0.91	0.84	0.95	1.0			0.93	0.95	
2019 (n=41)		0.90	0.93	0.95	1.0			0.98	0.90	
2021 (n=37), current results		0.95	0.66		0.97			0.95	0.84	

1) TCP used as contaminant for land animal material.

2) 0.01% of bone meal representing 0.03% MBM.

Salmon meal has been proven to cause confusion for the detection of terrestrial land animal material in several past PTs (overview in Table 4 of van Raamsdonk et al., 2017). The proper recognition of the absence of MBM in the presence of salmon meal resulted in specificity scores of 0.70 (sample fishmeal fortified with 10% of salmon) or 0.65 (compound feed with 0.5% salmon). Two different fish meals originating from the North Sea (Denmark) and from the southern Pacific (Chile/Peru) have been used in the IAG PT of 2015 at a level of 0.1% in the absence of MBM (van Raamsdonk et al., 2015: Table 3). The specificity scores were 0.76 and 0.93, respectively. Fish meals from the North Sea are known for their high content of bone fragments without lacunae. Particles from certain fish meals can be confused for particles from terrestrial animal materials. The common feature shared by salmon meal and the fish meal from the North Sea is the relatively large share of bone fragments without lacunae. This issue of confusion appears to remain.

4.2 Challenger

The presence of milk powder is a confusing ingredient for both the detection of animal materials as well as for the estimation of the composition. Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 (EC, 2013) did not include milk globules in its scope, although blood particles were mentioned. The new method implemented by Regulation (EU) 2020/1560 (EU, 2020) amending Annex VI of Regulation (EC) 152/2009 includes milk globules and lactose crystals in its scope. Despite this, milk and products thereof are correctly excluded from the description of PAP in the EURL-AP Standard Operational Procedure "Operational protocol for the combination of light microscopy and PCR" according to the definition of PAP in Regulation (EC) 142/2011 Annex I (EC, 2011, consolidated version: 17 October 2021).

The situation of milk powder as a legitimate ingredient with an animal origin can be compared to the presence of feather meals in several PTs organised by the EURL Animal Proteins. Feather meal in compound feed at a level of 0.5% resulted in a sensitivity score of 0.98 (Veys et al., 2009) and at a level of 0.1% in a score of 0.52 (Fumière et al., 2017). Feather meal in fish feed containing fish meal resulted in sensitivity scores of 0.22 at a level of 0.1% (Veys et al., 2014) and 0.31 at a level of 0.5% (Veys et al., 2012).

Two factors can cause the low performance for ingredients such as milk powder and feather meal. First, they are difficult to recognise. With respect to milk powder, the examination with chloral hydrate of the raw material would result in dissolving of the milk globules in the water-based embedding agent. Second, these ingredients might not fit in the range of more common ingredients expected to have a probability to be present. For example, several EURL AP PTs in the years 2014 to 2016 consisted of samples containing 0.1% or 0.5% of milk powder, 1% of blood meal (in two PTs) or 3% of blood plasm and in all cases the expected result was "absence" (Veys et al., 2015, 2016; Fumière et al., 2017). In order to test the probable causes of the low performance for this type of ingredients, labelled samples with an indication of the presence of milk powder, blood products or feather meal in a PT design could shed light on these issues. This would force the technician to check specifically for such an ingredient. Absence or lack of recognition would result in rejection of the declaration, and in a conclusion on recognisability, since ignorance was avoided as factor in such a design.

4.3 Method implementation

A major difference between the old protocol (Regulation (EU) 51/2013 amending Regulation (EC) 152/2009) and the new protocol (Regulation (EU) 2020/1560 amending Regulation (EC) 152/2009) is the inclusion of the label declaration as decision factor. In the new version a second determination cycle is required if the presence (more than 5 particles) does not confirm the label declaration or in the absence of any declaration. The samples in the current PT were not labelled in order to avoid reducing the PT to a label declaration test. The practical consequence is that any sample in which 1 or more particles were found, either from terrestrial animal or from fish, should be subjected to a second determination cycle. With one blank, this would imply seven analyses in all cases, assuming correct results. According to the old protocol a final set of results could have been achieved after four analyses under the same assumption. Eighteen participants (48.6%) made the logical choice to apply one determination cycle for all samples in the absence of any positive result at or below 5 particles.

It has been chosen that the IAG PTs for animal proteins follow the analytical part of the official method, except for the sample preparation (minimal 50 g of sample material per determination cycle, pre-sieving) and the reporting procedures (use of official reporting sentences). The choice for one or two determination cycles can be exempted as well from the scope of a PT, but this is an integrated part of the analytical procedure in the new method. The EURL AP PT of 2020 (closing date 16th November 2020; Fumière et al., 2021) was organised under the 2013 version of the protocol since the new protocol came into force at the 16th of November. Besides these organisational issues for PTs, problems do exist in practice as well. Label information (including information on the animal for which the feed is intended) is not transferred to the laboratory by every Competent Authority. An alternative for proficiency testing could be to request the examination of specific types of ingredients.

The embedding agent used can be supposed to influence the appearance of animal particles, especially those in the sediment. The expertise of a technician, achieved after years of training, is presumably based on one or a few specific types of embedding agents. This assumption is likely to be reflected in the numbers of participants using different types of embedding agents (Table 7). In general, the different steps in a visual method can be organised in three type of elements: technical actions (e.g. grinding, sieving, preparing the slides, types of equipment used for observations), aspects related to personal preferences for optimal performance (e.g. embedding agent, aperture of condenser, use of polarisation), and administrative actions (e.g. drawing conclusions from the observations, reporting). Where harmonisation is necessary for the technical and administrative actions (the procedure), the specific circumstances for the observations should meet the precise skills of the observer (the expertise). Precisely the importance of the expertise of the technician for the identification of particles is one of the principal differences between visual methods at one side and chemical analytical methods at the other.

Table 7 *Comparison between some parameter distributions in the IAG proficiency studies between 2008 and 2018. *: number of cycles since 2014.*

parameter	**parameter choice**	**2008**	**2009 -2019**	**2021**
correct number of cycles *			67.3% - 94.3%	100%
share of the total sediment used for examination	minimum	4%	0.2%-3%	1%
	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-25	15
	paraffin oil	18	12-23	11
	immersion oil	8	7-14	8
	Norland Adhesive	0	2-7	4
	chloral hydrate	3	1-0	0
	other (e.g. Depar 3000, water)	8	5-0	0

4.4 PCR

The presence of 0.1% of ruminant MBM was detected correctly by all participants (n=16). Some false positives occurred. One participant reported a negative result in the presence of milk powder. Further conclusions cannot be drawn in the absence of the Cq values. Several participants (n=6) followed the rule that PCR is not allowed to be used for samples reported negative after microscopic evaluation.

5 Conclusions and recommendations

5.1 Conclusions

Good results were achieved for the sensitivity score for 0.1% of ruminant MBM and for specificity scores (both terrestrial animal material and fish) for the blank sample. Several issues were addressed in this version of the IG PT for animal proteins.

The suboptimal score for specificity for terrestrial animal material in the presence of salmon meal (0.65) is comparable to other situations, such as fish meal originating from the North Sea. The common feature shared by salmon meal and the fish meal from the North Sea is the relatively large share of bone fragments without lacunae. This issue of confusion appears to remain.

Milk powder is not found in a majority of cases (sensitivity 0.30). Still it is part of the scope of the current protocol, as are blood products and feather meal (Regulation (EU) 2020/1560 (EU, 2020) amending Annex VI of Regulation (EC) 152/2009). Either the lack of awareness of the probable presence of these ingredients or the difficulty to recognise these materials or both can cause the low performance of their detection. In order to test the probable causes of the low performance for this type of ingredients, labelled samples with an indication of the presence of milk powder, blood products or feather meal could shed light on these issues. It can be considered to remove ingredients that are allowed according to Regulation (EC) 1069/2009 from the scope of the method for detection of animal proteins and restrict this method to particles included in the definition of PAP.

The current protocol includes a check of the label for legally declared ingredients. If found and declared, a second determination cycle is not required. This is a welcome improvement of the microscopic method, certainly in the view of new relaxations. However, the logical choice to label PT samples and in that way declare the contents to the participants, would reduce the PT to a label declaration test. This check on label declaration is part of the analytical protocol, which is the subject of PTs. An option could be to request the examination of specific types of ingredients.

5.2 Recommendations

- The documentation for and training of microscopists for correct identification of particles of animal origin would deserve further attention in order to improve the performance.
- The approach of implementing the new protocol in the design of PTs need further evaluation.
- The scope of the method needs revision. It is recommended to restrict the method to PAP in order to facilitate the current and future relaxations.

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Annex 1 Introduction to the test

Test 2021-A: animal proteins in feed
The IAG proficiency 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 Commission Implementing Regulation (EU) 2020/1560, 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. PLEASE NOTE: this new version implies to apply a maximum of two cycles. The jars do not have a label declaration, and consequently the second determination need to be applied in all cases where one or more particles of animal origin were found.

The jars contain 50 grams of feed, which is sufficient for carrying out two 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 2.1.3.3.4: use a portion of 10 g for sedimentation etc. The process of analysis as included in this proficiency 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 20 fragments per category are found in any cycle, just choose "20" 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 the decision limit (between 1 and 5 particles on average) or present (6 or more particles on average).

In addition to the workflow as presented in the paragraphs 2.1.3.3.4 until and including 2.1.4.3, it is mandatory to weight the sediment BEFORE and AFTER the analysis as performed in each of the two 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 proficiency test 2021 animal proteins

Instructions for the IAG proficiency 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, amended by (EU) 2020/1560. The consolidated version and the SOPs can be found on the EURL website. The sample design allows to carry out the PCR ruminant analysis. **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 proficiency 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 20 particles are found in any category, please enter the value 20.

The report form is interactive: if the results in the first determination cycle make it necessary to perform a second 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 'suspect' 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 you have entered it at the page "Procedure". Enter yourself the four unique labels of the vials.

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 microscopie.WFSR@wur.nl.
4b	Results will be included in the final analyses and in the report only if both forms are sent 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 Thursday June 24th, 2021.
	WFSR 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 proficiency test 2021 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the proficiency 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 proficiency test 2021 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
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
SGS Bulgaria	Bulgaria
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
Inovalys-Nantes	France
Laboratoire Départemental d'Analyse & de Recherche	France
S.C.L. Laboratoire de Rennes	France
CVUA-RRW	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Landeslabor Berlin-Brandenburg	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
LTZ Augustenberg	Germany
LUFA Nord-West	Germany
LUFA Rostock	Germany
LUFA-Speyer	Germany
SGS Germany GmbH, Hamburg	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
SYNLAB Analytics and Services GmbH, Jena	Germany
Thüringer Landesanstalt für Landwirtschaft Jena	Germany
WESSLING GmbH	Germany
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Equine Centre	Ireland
Ministero delle politiche agricole alimentari e forestali, Laboratorio di Modena	Italy
CCL - Nutricontrol	Netherlands
Eurofins Food Testing Rotterdam BV	Netherlands
ForFarmers	Netherlands
Labora	Netherlands
Nutreco Nederland BV - Masterlab	Netherlands
Nutrilab BV	Netherlands
TLR	Netherlands
Synlab Stjørdal	Norway
SGS del Perú S.A.C.	Peru
Lab. Regional de Veterinária	Portugal
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
National Veterinary Institute, SVA	Sweden
Agroscope (ALP), Swiss Research Station	Switzerland

Annex 6 Details of procedures applied, microscopic method

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

lab nr	prior							
33	no	chemical sedimentation funnel	TCE	yes	no	small (20 x 20 mm)	glycerine	no
34	yes	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
36	no	chemical sedimentation funnel	TCE	yes	no	small (20 x 20 mm)	Norland adhesive 65	no
37	yes	beaker (flat bottom)	TCE	no	yes	small (20 x 20 mm)	immersion oil	no
38	no	chemical sedimentation funnel	TCE	no	no	small (21 x 26 mm)	paraffin oil	no
39	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65	yes
40	no	other	TCE	no	no	small (20 x 20 mm)	glycerine	no
41	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
42	no	chemical sedimentation funnel	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65	no
43	no	other	TCE	no	yes	medium	paraffin oil	no
46	no	special conical glass with cock	TCE	yes	yes	small (20 x 20 mm)	glycerine	no

Annex 7 Results: presence of animal proteins, microscopic detection

lab nr		sample number				land				fish			
	PCR					A	B	C	D	A	B	C	D
1	yes	423	165	376	307	absent	absent	absent	present	absent	present	absent	absent
2	no	304	270	481	314	absent	absent	absent	suspect	absent	suspect	absent	absent
3	no	318	480	306	426	absent	absent	absent	absent	suspect	present	present	present
4	yes	486	319	397	440	absent	absent	absent	present	absent	present	absent	absent
5	yes	164	347	453	153	absent	present	absent	present	absent	present	absent	absent
6	no	437	298	460	496	absent	suspect	absent	suspect	absent	present	absent	present
7	no	479	354	432	195	absent	absent	present	present	absent	present	absent	present
8	no	409	473	390	202	absent	suspect	present	present	absent	present	absent	absent
10	yes	143	228	439	265	absent	absent	present	present	absent	present	absent	absent
11	no	430	144	425	356	absent	absent	present	present	absent	present	absent	absent
12	yes	311	452	369	216								
13	yes	458	410	418	237	suspect	suspect	present	present	absent	present	absent	absent
14	no	367	326	355	118	absent	absent	absent	present	absent	present	absent	absent
15	no	444	368	404	244	absent	present	absent	present	absent	present	absent	absent
16	no	402	200	488	258	absent	absent	absent	present	absent	present	absent	absent
18	no	395	305	138	349	absent	suspect	suspect	present	absent	present	absent	absent
19	no	178	221	348	461	absent	absent	absent	present	absent	present	absent	absent
20	no	472	277	362	223	absent	absent	absent	present	absent	present	absent	absent
21	yes	199	291	467	377	absent	absent	present	present	absent	present	absent	absent
22	no	451	116	411	489	absent	absent	present	present	absent	present	absent	absent
23	no	213	207	117	398	absent	present	absent	present	absent	present	absent	suspect
24	no	416	375	278	230	absent	present	absent	present	absent	present	absent	absent
25	no	374	340	341	209	absent	absent	absent	present	absent	present	absent	absent
26	yes	465	256	334	328	absent	absent	absent	present	absent	present	absent	absent
28	yes	388	235	383	167	absent	absent	present	present	present	absent	present	present
29	yes	276	466	327	125	absent	present	absent	present	absent	present	absent	absent
30	no	325	179	250	468	absent	present	present	present	absent	present	absent	absent
31	yes	360	151	320	419								
32	yes	339	361	180	335	absent	absent	present	present	absent	present	absent	absent

lab nr		sample number				land				fish			
	PCR					A	B	C	D	A	B	C	D
33	yes	262	487	208	391	absent	absent	absent	present	absent	present	absent	absent
34	no	353	333	187	475	absent	absent	absent	present	absent	present	absent	absent
36	yes	290	389	299	454	absent	absent	absent	present	absent	present	absent	absent
37	no	171	424	173	188	absent	present	absent	present	absent	present	absent	absent
38	no	157	431	166	321	absent	absent	absent	present	absent	present	absent	absent
39	yes	122	242	159	447	absent	absent	absent	present	absent	present	absent	absent
40	yes	297	137	131	405	absent	absent	absent	present	absent	present	absent	absent
41	no	234	403	124	272	absent	absent	absent	present	absent	present	absent	absent
42	yes	248	263	257	279	absent	absent	absent	present	absent	present	absent	absent
43	no	269	396	194	251	present	present	absent	present	absent	absent	absent	present
46	no	150	193	215	286	absent	present	present	present	absent	present	present	absent

Annex 8 Results: PCR ruminant

lab nr	sample				ruminant			
	A	B	C	D	A	B	C	D
1	423	165	376	307	present	present	present	present
4	486	319	397	440				present
5	164	347	453	153		absent		present
10	143	228	439	265	absent	absent	present	present
12	311	452	369	216	absent	absent	present	present
13	458	410	418	237	absent	absent	present	present
21	199	291	467	377	absent	absent	present	present
26	465	256	334	328	absent	absent	present	present
28	388	235	383	167	absent	absent	present	present
29	276	466	327	125	absent	absent	present	present
31	360	151	320	419	absent	absent	present	present
32	339	361	180	335	absent	absent	present	present
33	262	487	208	391				present
36	290	389	299	454				present
39	122	242	159	447				present
40	297	137	131	405				present
42	248	263	257	279	present	absent	absent	present

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