IAG proficiency test animal proteins 2022

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Cover photo: bone fragments of tuna fish.

Contents

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, 2022, 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 fish meal (2%), meat and bone meal (MBM, 0.1%), and milk powder (3%). A fourth sample was left blank. The matrix was artificially produced, mimicking a ruminant feed, for avoiding any nonintentional contamination with ruminant DNA. Two out of the four samples were labelled as containing milk powder. One of these samples did contain milk powder, while the other was purposefully labelled incorrectly.

A total of 50 participants subscribed to the proficiency test animal proteins. Three participants did not submit their results and one submitted PCR results only, leaving 46 sets for microscopic evaluation. 19 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 were asked to follow the included protocol, which asked for a second determination in the case that the content of the sample did not match the label. Out of the 46 participants which sent in microscopic evaluation results, 17 followed the instructed protocol correctly, with the majority of participants performing a second determination when not required, and 12 participants made incorrect interpretations of the encountered number of particles (e.g. “absent” for one or more but less than six particles, “present” when not having more than five particles per determination, not counting particles of milk powder.) 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 excellent (sample B, ruminant MBM: 1.0; sample C, fish meal: 1.0). The correct indication of absence of both types of ingredients in the blank (specificity) was acceptable and excellent for terrestrial and fish material respectively as well (Sample A: 0.93 and 1.0 for terrestrial and fish material respectively.). The specificity of the detection of terrestrial animal material in samples containing fish meal was acceptable (0.93 in sample C.) More varied results were found in the detection of the presence or absence of milk powder. Sample C and D were labelled as containing milk powder, however only sample D actually contained 3% milk powder. The sensitivity for milk powder was low (0.67 in sample D). The specificity for milk powder, on the other hand, was excellent (1.0 in Sample C) Milk powder is a legal ingredient for all feeds and the labelled samples was intended to be a challenger without giving an indication of the performance of the participants. The Labels were added as previous ring tests revealed that the sensitivity for milk powder was low when participants were not informed to look for it. As such, the labels provided a means to test whether or not the low sensitivity was a result of inattentiveness, or a shortcoming

of the method or a lack of expertise. This, because the labels provide a reminder to look for milk powder in the samples that contained it, providing statistics for the sensitivity of the method, and in samples that do not contain milk powder, providing statistics for the specificity of the method, whilst removing inattentiveness as an obfuscating factor.

PCR

The ruminant material in the MBM sample (sample B) was correctly detected by all participants, one false positives was reported in the fish meal sample (sample C), all participants correctly detected ruminant material in the milk powder sample (sample D), and all participants correctly reported the absence of ruminant material in the blank (sample A).

Conclusions

Very good or excellent results were achieved for the detection of processed animal proteins. The situation for detection of other ingredients, exemplified by the presence or absence of milk powder, needs considerable improvement. It is recommended to consider to reduce the scope of the current legally implemented method to PAPs and find adjusted protocols for other types of ingredients. Training for identification of these different particles need to be organised.

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 its members. Wageningen Food Safety Research (WFSR) 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., 2021).

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 v5.1", 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 2022 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 either 0.1% ruminant MBM or 2% of fish meal. The fourth sample has been spiked with 3% milk powder intended as challenger. Of these, two samples were labelled as containing milk powder. One of which did contain milk powder, whilst the other was falsely labelled. This was in order to test the method's effectiveness at finding milk powder, whilst removing inattentiveness as a factor. 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 2022 is presented.

2 Methods

2.1 Materials

The IAG proficiency test for animal proteins 2022 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%) The mineral mix was obtained from Cargill Poland and proven to be animal protein free, including tricalciumphosphate (TCP). The ingredients were ground with a mesh size of 2 mm and thoroughly mixed. (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 double sedimentation procedure for insect detection was officially published 8th June, 2022 (Regulation (EU) 2022/893; EU, 2022). This publication date was after the final reporting date of the experimental phase of this proficiency test, and in the view of absence of a consolidated version of Regulation (EC) 152/2009, the amended version of the method resulting from Regulation (EU) 2020/1560 was used.[1]

A ruminant meat and bone meal (MBM) was chosen for the terrestrial PAP in order to verify the performance of the PCR test in a regular situation of a prohibited contaminant. The fish meal was selected from the WFSR collection of fish meals. The milk powder of sample 2022-D 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. Furthermore, the previous PT showed that milk powder was found through microscopic methods by a minority of the participating labs when not made aware of the possibility of the presence of milk powder in the samples. As such, two samples were labelled as containing milk powder to test whether or not the method of detection was adequate with the factor of inattentiveness removed. The results of sample D, indicated as challenger, will not be part of the evaluation of the performance of the laboratories. 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	Label information
2021-A	artificial feed	Blank	None
2021-B	artificial feed	0.1% (w/w) ruminant MBM	None
2021-C	artificial feed	2% (w/w) fish meal	Milk powder: presence
2021-D	artificial feed	Blank with 3% milk powder; challenger	Milk powder: presence

The applied version of the method asks for the repetition of the determination in the case that the contents found do not match the label or in the absence of a label. The two unlabelled samples and the one incorrectly labelled sample as included this PT would be subjected to a second determination cycle in the situation of correct results. This situation would cause a higher work load than in previous PTs without the production of additional information. For the purpose of this PT the instructions contained a provision that for the unlabelled samples and for the detection of any type of animal material other than milk powder (e.g. bones, fish bones, scales), a second determination was deemed unnecessary (see Annex 1).

[1] This situation was verified at July 20th, 2022.

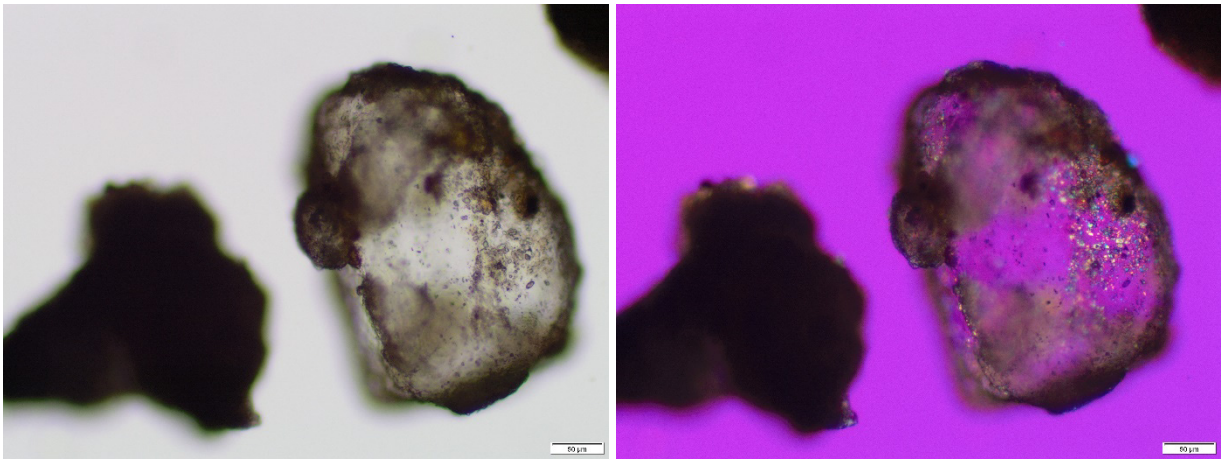

Figure 1 *Impression of the mineral mix used for the production of the samples at a magnification of 100x. Left: without polarization, right: with polarization. Bar: 50 µm.*

The jars were all labelled with an order number, and two jars were further labelled as containing milk powder.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order: 2022-A - 2022-D - 2022-C - 2022-B. 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 and for additional PCR analysis 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 fish meal and MBM samples were produced by step-wise dilution of the dedicated contaminants down to a level of 2% and 0.1%, respectively. The milk powder was added and mixed in one step. The production scheme is presented in Figure 2.

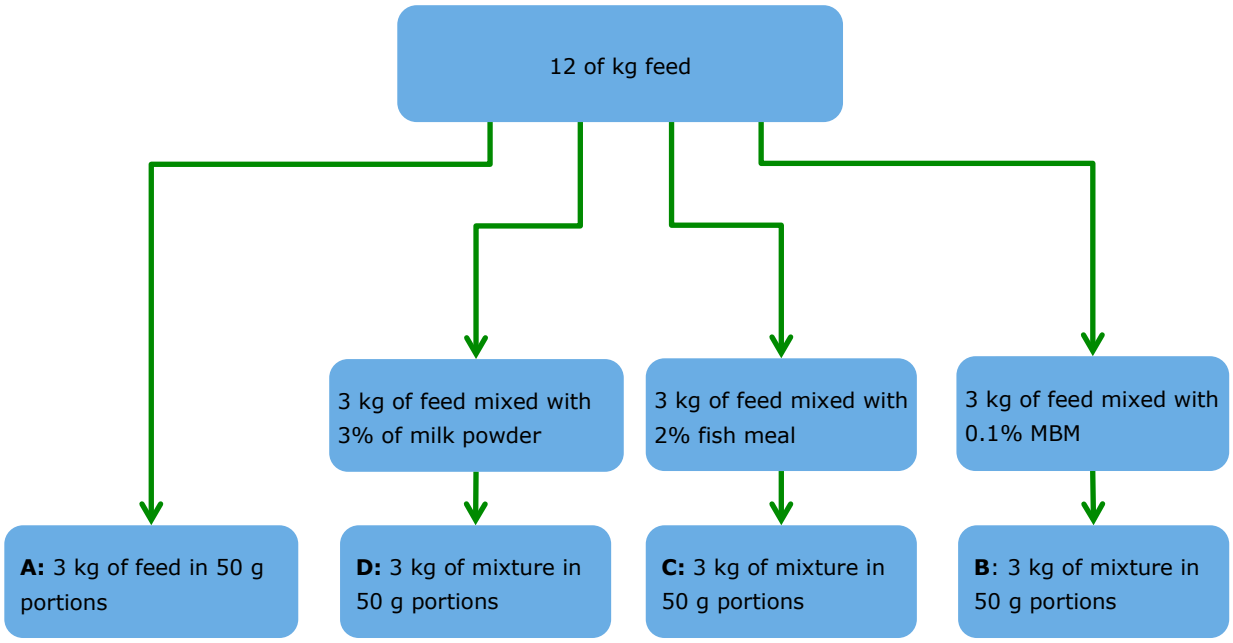

Figure 2 *Flow diagram for the production of the samples in order from left to right.*

2.3 Homogeneity study

Two WFSR microscopists independently examined all basic materials and four 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 = 36.07.

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

Sample	Sediment amount	Microscopy		PCR
		terrestrial	fish	Ruminant; Cq values
2022-A Blank	222-254 mg/10 g	absent	absent	absent; 38.10, 38.75
2022-B 0.1% (w/w) ruminant MBM	242-267 mg/10 g	present	absent	present; 30.17, 29.96
2022-C 2% fish meal	276-296 mg/10 g	absent	present	absent; 37.88, 38.28
2022-D 3% (w/w) milk powder; challenger	195-220 mg/10 g	present	absent	present; 27.24, 26.88

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 2022 by means of mailing using distribution lists. When the samples were sent in the beginning of March, 50 participants had registered for the proficiency test animal proteins. One participant 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 Monday the 7th of March 2022. The file with instructions and the report form were sent to the participants on Friday the 11th of March. (see Annex 3 and 4). The closing date for reporting results was the 14th of April, however this was extended to the 27th of April due to participant requests. The analysis of the results was carried out 9th of May. The report was distributed in draft to the Board of IAG section Feed microscopy and the participants on Monday 30st of May.

The report form consisted of three different sheets:
1. The Introductions sheet, which outlined the actions that needed to be taken to participate in the PT.
2. The Procedure sheet, where participants were asked to answer questions regarding the particular method they use for animal protein monitoring. For example, which embedding agent or what type of glassware was used.
3. The Results sheet, where participants shared the results of their analysis, for up to two analyses. Depending on the results of this first determination the cells for the second determination would need to be filled in. The Results sheet consisted of four main elements
   - The laboratory and sample numbers.
   - The determination results, described as number of particles found and type of particles found.
   - The final conclusion of the microscopy analysis.
   - The Results of ruminant EURL-AP PCR method.

The current method requires to perform an extra determination cycle when the particles found do not match the label. As the labels included in this PT only declared the presence of a type of particle, and not the specific absence, this should be interpreted as directing a second analysis ONLY when the mentioned particle is NOT found in the sample.

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 v5.1". 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 correctness, sensitivity and specificity. The correctness 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 50 participants subscribed for the proficiency test animal proteins. Three participant did not submit their results. Of the remaining 47 participants, one applied exclusively PCR results, leaving 46 sets of microscopic results, accompanied with PCR results in 19 cases. The participants originated from 20 countries: 14 member states of the European Union, and six other countries (China, Norway, Peru, Serbia, Switzerland, and the United Kingdom). The list of participants is presented in Annex 5. Five member states have been involved with three or more participating laboratories: Belgium (4), France (3), Germany (15), The Netherlands (7), Peru (4).

All results were received by E-mail, in most cases by means of both a scan and the original report file. A total of 12 participants (25.5%) did not submit the Excel sheet with the correct name and 3 participants (6.4%) did not mention the lab number in one or both files. File names according to the format and with lab number for the Excel sheet 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 May 30st, 2022.
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. The new method asks for a second determination in case the contents of the sample differ from the contents declared on the label. A considerable part of the participants did not properly follow the protocol for deciding to perform a second determination cycle (n=30, 65.2%). 16 participants correctly followed the current protocol by applying a second determination cycle for the sample which did not contain milk powder, even though the label declared otherwise (sample 2022-C). Twelve participants (26.1%) included incorrect interpretations of the encountered number of particles (e.g. “absent” for one or more but less than six particles, “present” when not having more than five particles per determination, not counting particles of milk powder).

Erroneous or non-conclusive reports were submitted by two participant (27, 40), which had an error in a sample number and missed the final conclusion respectively. For one case the results could not be processed, and a new report sheet was requested. In terms of proficiency test procedures, one participant declared that they did not apply a PCR ruminant detection method while still including PCR test results (40), and one participant did not select an embedding agent used (42).

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 current official procedure requires a second determination cycle if the contents of the sample do not match the label description. Usually the samples in this PT are unlabelled as to not reduce the PT to a label control test. This year, however, two samples were labelled as containing milk powder in order to remove inattentiveness as a factor in milk powder detection. As such, a second determination cycle would have needed to be performed in the case that no milk powder was found. To prevent unnecessary extra work, the PT instructions had a modified reporting flowchart for unlabelled monsters which did not require a second determination (see Annex 1). 17 participants correctly followed this scheme, with 10 participants performing the second determination cycle for all samples, 9 performing the second determination cycle for the sample with ruminant particles as well as the one without milk powder, 7 not performing a second determination cycle at all, and 3 performing a second determination cycle on all samples but the blank.

The results as presented in Table 3 generally show a good application of the method, except for following the correct number of determinations for a sample which seemed to be a source of confusion. 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 2%. Overall, the portion of sediment used was spread out, with no clear preference detected.

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 instructed protocol	17	
	No	29	
type of glassware	chemical sedimentation funnel	23	
	conical glass with cock	10	
	champagne glass *	7	
	beaker (flat bottom)	3	
	other	3	
sedimentation agent	TCE	46	
	Chloroform	0	
	TCE/Petroleumether	0	
use of staining of sediment	no	28	
	yes	18	
use of binocular for examination at lower magnifications	yes	27	
	no	19	
size of cover glass used	small (e.g. 20 x 20 or 21 x 26 mm)	35	
	medium	7	
	large (e.g. 20 x 50 or 26 x 50 mm)	3	
share of the total sediment used for examination	minimum		2%
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	20	
	paraffin oil	10	
	immersion oil	9	
	Norland Adhesive	6	
	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 to find 6 or more particles and thus to reach a conclusive result.

The sensitivity for both basal ingredients were Excellent (sample B: terrestrial animal material; sample C: fish). The specificity of terrestrial animal material in the blank was acceptable, whilst for fish material it was excellent (sample A). The specificity of land animal material in the presence of fish meal was also just acceptable (sample C). The challenger showed a skewed result. With the specificity being excellent (sample C), but the sensitivity not reaching the acceptable level (Sample D).

The results, after considering the reported numbers of particles below the decision limit as negative, are higher for specificity and lower for sensitivity compared to their evaluation as positive results. For example, three participants reported 5 or less particles for terrestrial animal material in sample A. After considering these negative the specificity was 1.00, higher than the 0.93 when taking these results as positive. Applying the same principle, the sensitivity score for terrestrial animal material in sample B was higher when declaring a number of particles 5 or lower as positive, with the sensitivity being 1.0 as opposed to 0.93.

The samples have been evaluated twice: at first for the reported presence of animal proteins except milk powder, and separately for the reported presence of milk powder. Three participants reported the presence of terrestrial animal material in sample C. Also for sample D three positive results have been reported. With respect to the report of the presence of milk powder, only one participant incorrectly declared the presence of milk powder in the erroneously labelled sample C. However, with a correct label declaration for sample D, 19 participants did not detect milk powder. It should be mentioned however, that six of these participants did comment that they detected milk powder in sample D, but failed to count them as particles. As such our analysis, which is based on the number of particles, had to count the result as 'absent'.

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 and D were labelled as containing milk powder and were analysed separately in the context of milk powder found (grey). *: results based on the absence of animal material (e.g. bone fragments, muscle fibres), but excluding the presence of milk powder globules.*

		Terrestrial animal						**Fish**			
		A	**B**	**C**	***D***	**C**	**D**	**A**	**B**	**C**	***D***
n		**0%**	**0.1%**	**0%**	***0%*** *	**0%**	**3%**	**0%**	**0%**	**2%**	***0%***
36	specificity	0.93 *1.00*		0.93 *0.96*	0.93 *0.98*	*0.98* *1.00*		1.00 *1.00*	1.00 *1.00*		*0.96* *1.00*
	sensitivity		1.00 *0.93*				*0.59* *0.59*			1.00 *0.98*	

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. 20 participants reported results for the ruminant PCR analysis. All 20 participants reported samples B and D as positive for ruminant and sample A as negative for ruminant. 1 participant (25) reported a false positive for sample C, whilst the other participants reported sample C as negative. The overall results are shown in Table 5. Full results are shown in Annex 8.

Table 5 *Results for DNA analyses (PCR) for four samples. Target: ruminant. *: results based on the presence of 3% milk powder.*

		Ruminant			
		A	**B**	**C**	***D***
		0%	**0.01%**	**0%**	***3% ****
	specificity	1.00		0.95	
	sensitivity		1.00		*1.00*
	n	20	20	20	*20*

4 Discussion

4.1 Samples for performance monitoring

The samples were all based on an artificial composition of ingredients in order to minimize the risk of natural contamination with PAPs or other animal components.

Overall, the performance in this proficiency test is good. With the results of the PAPs ranging between acceptable and excellent. The selectivity found in the blank sample (sample A), and the fish meal sample (sample C) for ruminant PAPs is comparable to the scores of previous years (Table 6), and the sensitivity was excellent. Both the sensitivity and the selectivity of fish PAPs were excellent in the fish meal sample (sample C) and the blank sample (Sample A), respectively. The lowest specificity scored for PAPs, while still well in the acceptable level, in this PT was the specificity for terrestrial animal materials, which yielded a specificity of 0.93 in all three samples not containing terrestrial animal materials. The slightly lower specificity of terrestrial animal materials can possibly be explained by a lack of regular encounters with samples including terrestrial PAPs in them, as they are rarely used in feed, mainly due to the strong restrictions on their use.

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 0.91				0.98		
2017 (n=36)	0.89 0.94					0.91 [2)]	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)	0.95	0.66		0.97			0.95	0.84	
2022 (n=46), current results	0.93	0.93		1.0			1.0	1.0	

1) TCP use as contaminant for land animal material

2) 0.01% of bone meal representing 0.03% MBM

4.2 Challenger

The 2021 edition of the PT animal proteins consisted of a sample with 5% milk powder without declaration (Van Raamsdonk et al., 2021). In this edition both absence and presence of 3% milk powder combined with a declaration were included in the test design. In both editions blank samples were included. These make the full set of combinations presence/absence and (non-)declaration of milk powder complete. This overview with the achieved performance in terms of specificity or sensitivity is presented in Table 7.

Table 7 *Sensitivity or specificity for the detection of milk powder in four combinations: actual presence of milk powder (rows), presence of a declaration (columns). 2021: see report Van Raamsdonk et al., 2021 (milk powder 5%); 2022: this report (milk powder 3%). *: performance applying to milk powder, excluding incorrect reports of other types of particles (bones fragments, feather filaments).*

Milk powder declared	**no**	**yes**
Milk powder presence		
no (specificity)	1.00 (2021) * 1.00 (2022)	0.98 (2022)
yes (sensitivity)	0.32 (2021)	0.59 (2022)

In the absence of milk powder, whether or not declared, none of the participants did not report milk powder. There is one report of gelatine in 2022, which might be a confusing material, if visible. If its presence is not announced, one third of the participants would detect milk powder, and if declared, this share of positive findings increased to approximately two third of the participants. These results indicate three explanations. At first, milk powder is apparently not part of the classic scope as interpreted by the majority of the microscopists: when not declared, it is generally not detected and overlooked by two-third of the participants. Secondly, confusing with other particles is very rare: milk powder is not reported when not declared. Finally, milk powder is still overlooked if present: more than one third of the participants did not recognise it even when declared. The detection of milk powder cannot be improved by simply paying (frequent) attention to the situation that milk powder is part of the scope according to Regulation (EC) 152/2009, for two reasons. Milk powder is not recognised in 41% of the occasions even in the presence of a positive declaration, and the current method has never been validated for ingredients such as milk powder, HB powder and blood meal. This means that these ingredients should be removed from the scope until the method is properly validated for this extension of the scope and until technicians are properly trained for successful detection.

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 sample does not conform to the label declaration. Two samples in the current PT were labelled as containing milk powder. The practical consequence is that any labelled sample which did not contain milk powder should be subjected to a second determination cycle. With one labelled sample not containing milk powder, this would imply five analyses in all cases, assuming correct results. Seventeen participants (37.0%) correctly performed only one second determination cycle for sample C.

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. 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 8). 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 8 *Comparison between some parameter distributions in the IAG proficiency studies between 2008 and 2018. *: number of cycles since 2014.*

Parameter	parameter choice	2008	2009 -2021	2022
correct number of cycles *			67.3% - 100%	37%
share of the total sediment used for examination	minimum	4%	0.2%-3%	2%
	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-25	20
	paraffin oil	18	11-23	10
	immersion oil	8	7-14	9
	Norland Adhesive	0	2-7	6
	chloral hydrate	3	0-1	0
	other (e.g. Depar 3000, water)	8	0-5	0

4.4 PCR

The presence of both 0.1% of ruminant MBM and 3% milk powder was detected correctly by all participants (n=20). One false positives occurred. One participant reported a positive result in the presence of fish meal. Further conclusions cannot be drawn in the absence of the Cq values.

5 Conclusions and recommendations

5.1 Conclusions

Excellent to acceptable results were achieved across the board when considering the PAPs in its strict definition. There did not seem to be any obvious confounding factors for the detection of PAPs. Notwithstanding this general conclusion, several issues were addressed in this version of the IAG PT for animal proteins.

Milk powder, when brought to attention through a label, is found in a majority of cases (sensitivity 0.67). This is not at the level generally described as acceptable (threshold in this PT: 0.95), yet it is still 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). Due to the labelling, lack of awareness of the probable presence of these ingredients has been removed as an obfuscating factor. As such, it is likely that the difficulty to recognise these materials cause the low performance of their 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.

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. As such we chose to merely indicate the presence of a particle for which inattentiveness is a known obfuscating factor, but did not add any further information for other ingredients in order to have an open PT as in other years. This approach was an implementation of one of the suggestions in the report of the 2021 edition.

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, and remove types of animal products for which the microscopic method is not validated.
- Operating procedures should be read carefully and discussed with the microscopists.

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Annex 1 Introduction to the test

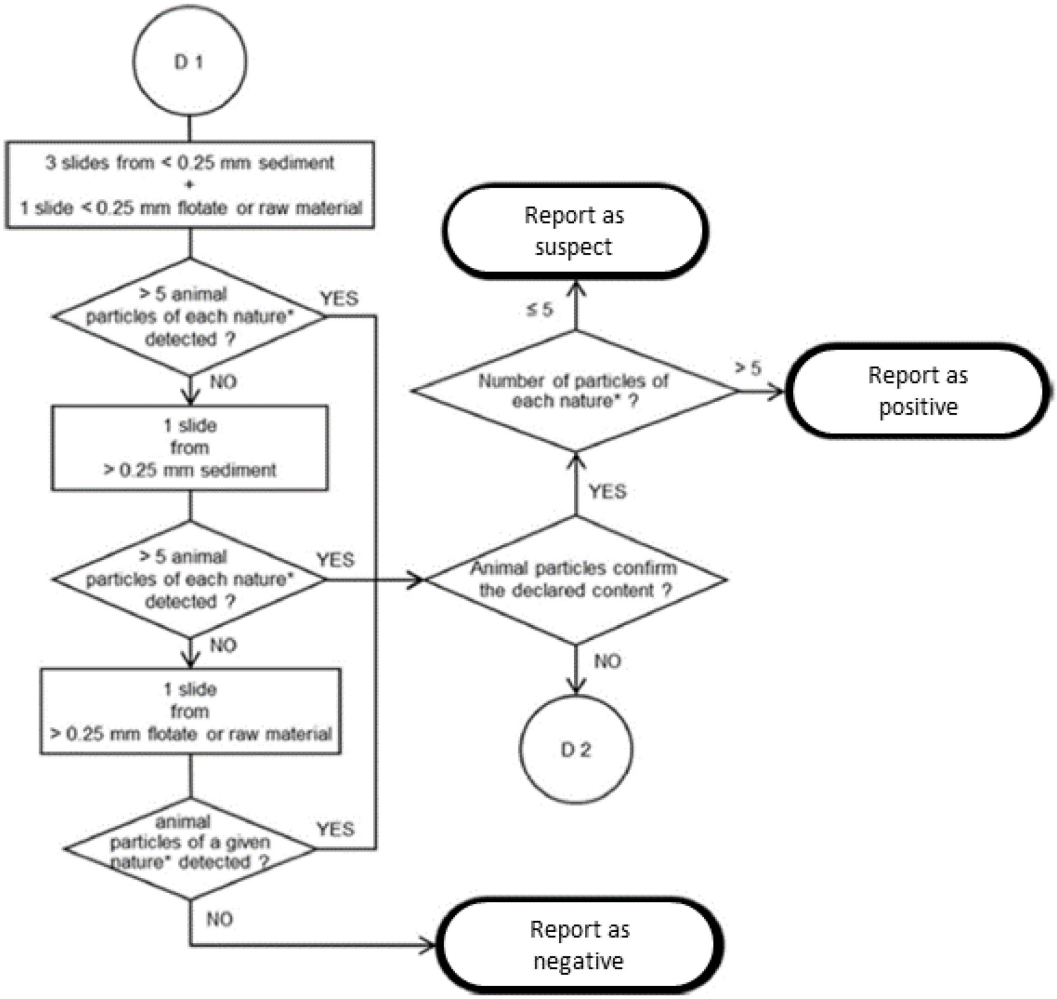
Test 2022-A: animal proteins in feed
The IAG proficiency test (PT) animal proteins in feeds is designed to apply both the microscopic method and the PCR ruminant method. The procedures to be followed are described in Annex VI of Regulation (EC) 152/2009 from the European Union, amended by Commission Implementing Regulation (EU) 2020/1560, and the related SOPs. All the documentation can be found on the website of the EURL AP: https://www.eurl.craw.eu/legal-sources-and-sops/method-of-reference-and-sops/. PLEASE NOTE: this new version implies to apply a maximum of two cycles. The control of the label declaration is now part of the method.

The jars contain 50 gram of feed, which is sufficient for carrying out two cycles of the microscopic method and/or for carrying out the PCR 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 as described in paragraph 2.1.3.3.4: use a portion of 10 g for sedimentation etc. Differentiation has to be made between particles of terrestrial vertebrate animals (bone fragments, hairs, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). 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 enter a final conclusion whether each of the two types is absent in a sample (zero particles), below the decision limit (between 1 and 5 particles) or present (6 or more particles).

In addition to the work flow 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 determinations.

The procedure for the IAG PT as explained in the previous paragraphs is presented in the flow diagrams as included in Regulation (EC) 152/2009 amended by Commission Implementing Regulation (EU) 2020/1560. In the absence of a declaration, as usual in most PTs, any finding of a particle in a sample should be followed by a second determination. This could imply up to eight analyses for four samples. In order to optimise the work load, the following choices has been made.

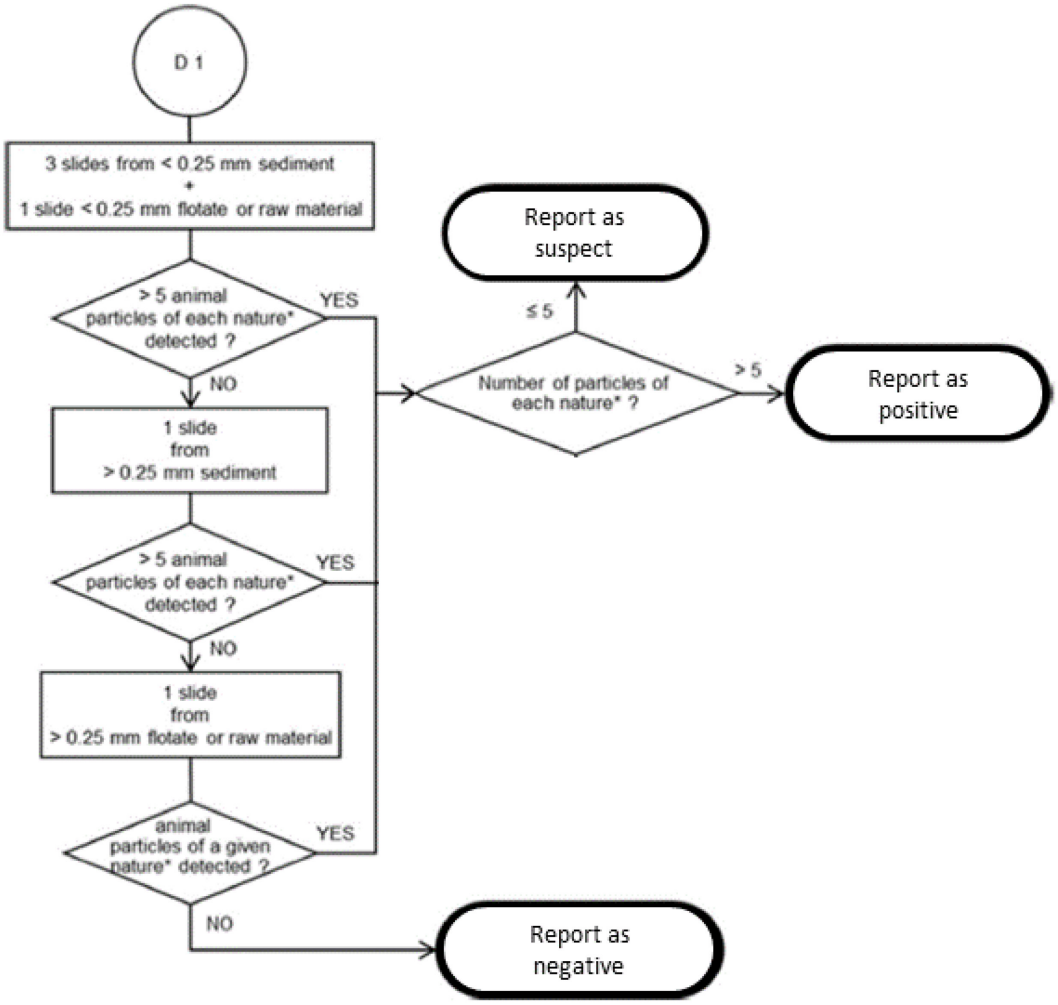
A. Two samples have a label declaration indicating the presence of milk powder
In the presence of a label declaration the flow diagrams of the official protocol can be followed. The flow diagram of the first determination cycle is reproduced in these instructions below. The report options are modified in order to show the choices of the report form.



If milk powder globules are found in one of the two labelled samples, this would confirm the label declaration of that one sample and the result can be reported.
If milk powder globules are not found in one of the two labelled samples, this would not confirm the label declaration of that one sample and a second determination has to be carried out.
This procedure has to be repeated for the second labelled sample with respect to milk powder globules.

B. Any other ingredient (bone fragments, fish bone fragments, gills, scales, cartilage etc.) not declared in any of the samples

For any other ingredient in any of the four samples the absence of a label will be ignored. This is expressed in the modified flow diagram below. If one or more particles is found (bone fragments, fish bone fragments, gills, scales, cartilage etc.) the result can be reported without a second determination cycle.



Reporting need to be combined for all possible findings:

- Labelled samples: the sum of the numbers of milk powder globules, bone fragments and other particles originating from terrestrial vertebrate animals will be reported as one total count for that category. The number of particles originating from fish can be reported as such.
- Unlabelled samples: the number of particles either originating from terrestrial vertebrate animals or from fish can be reported as such.

All results can be entered in the report form with "animal proteins" in the name, which will be send to you separately.

PLEASE BE AWARE THAT THIS STRATEGY IS ONLY VALID FOR THE DESIGN OF THIS PROFICIENCY TEST. IN ALL OTHER SITUATIONS THE PROCEDURE OF REGULATION (EC) 152/2009 AMENDED BY COMMISSION IMPLEMENTING REGULATION (EU) 2020/1560 INCLUDING THE SUPPORTING SOPS WILL APPLY.

General instructions for reporting

All report files will be send to you by mail. The report files consists of:

- A tab with specific instructions.
- A tab with an inquiry for a specification of the procedure.
- A tab for entering the results.

All pink cells have to be filled. In case a second determination is not necessary for the analysis of animal proteins, all these cells can be left empty.

After completing the two forms "Procedure" and "Results", they have to be saved as 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 send to the organisers in two ways. Every form has to be sent by E-mail as Excel file and as a signed scan (preferably *.PDF), and send the two files to microscopie.wfsr@wur.nl. IF EITHER THE EXCEL FILE OR THE PDF FILE IS NOT SUBMITTED RESULTS WILL BE IGNORED. Results will be included in the final analyses and report only if the reporting forms are send in by electronic mail.

Annex 2 Basic instructions for the test procedure

IAG proficiency test 2022 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 of the Regulation and the SOPs can be found on the EURL website. The sample design allows for 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 2.1.3.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse two 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 NOT interactive, as it was in previous years. Please follow the flow charts in the Instruction Letter enclosed in the package.
The final conclusion, according to Regulation (EC) 152/2009 (latest amended version !), 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: a low level presence below the decision limit of the method.
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 your have entered it at the page Procedure. Enter the four unique labels of the vials yourself. 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.rikilt@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 Thursday April 14th, 2022.

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 2022 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the ring test instructions?	-- select --	
Do you apply PCR ruminant detection method?	-- select --	
Do you apply grinding before performing the detection procedure?	-- select --	
Indicate your glassware for sedimentation	-- select --	
Describe your sedimentation agent	-- select --	
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 --	
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 20 particles were found in any category, please enter the value 20.

IAG proficiency test 2022 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	land	fish	land	fish
Result of first determination cycle	- select -	- select -	- select -	- select -	- select -	- select -	- select -	- select -
Second determination								
weight of sediment before analyses (in mg)								
weight of sediment after analyses (in mg)								
sediment % used for analyses	-		-		-		-	
	land	fish	land	fish	land	fish	land	fish
Result of second determination cycle	- select -	- select -	- select -	- select -	- select -	- select -	- select -	- select -
Total number of particles per category	0	0	0	0	0	0	0	0
	land	fish	land	fish	land	fish	land	fish
Final conclusion microscopy	- select -	- select -	- select -	- select -	- select -	- select -	- select -	- select -
Type of particles								
Comment, if necessary								
PCR results								
Ruminant (EURL method)								

Signature:

Comment, if necessary:

Date: 23-5-2022

Annex 5 List of participants

Austrian Agency for Health and Food Safety-AGES	Austria
Laboratorium ECCA nv	Belgium
FLVVT	Belgium
LFSAL	Belgium
CRA-W	Belgium
China Agricultural University (East campus)	China
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
Estonian Veterinary and Food Laboratory	Estonia
Laboratoire Départemental d'Analyse & de Recherche	France
Inovalys-Nantes	France
S.C.L. Laboratoire de Rennes	France
SGS Germany GmbH	Germany
LUFA-Speyer	Germany
Landeslabor Berlin-Brandenburg	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
LTZ Augustenberg	Germany
CVUA-RRW	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
Landesuntersuchungsamt f. Chemie, Hygiene und Veterinärmedizin	Germany
LUFA Rostock	Germany
SGS Analytics Germany GmbH, Jena	Germany
Futtermittelinstitut Stade (LAVES)	Germany
LUFA Nord-West	Germany
Thüringer Landesanstalt für Landwirtschaft Jena	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
WESSLING GmbH	Germany
Equine Centre	Ireland
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Ministero delle politiche agricole alimentari e forestali, Laboratorio di Modena	Italy
BIOCHEMIE LAB S.r.l.	Italy
CCL - Nutricontrol	Netherlands
Nutrilab BV	Netherlands
Labora	Netherlands
ForFarmers	Netherlands
Nutreco Nederland BV - Masterlab	Netherlands
TLR	Netherlands
Eurofins Food Testing Rotterdam	Netherlands
SGS Analytics Norway AS	Norway
World Survey Services SAC	Peru
SGS del Perú S.A.C.	Peru
NSF INASSA S.A.C.	Peru
Pacific Control CMA SAC	Peru
Cargill Poland	Poland
Lab. Regional de Veterinária	Portugal
Scientific Veterinary Institute "Novi Sad	Serbia
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
APHA	United Kingdom

Annex 6 Details of procedures applied, microscopic method

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

Lab nr	prior grinding	Glassware		agent	staining	binocular	size	embedding
34	no	chemical sedimentation funnel	bottom	TCE	yes	no	medium	immersion oil
35	no	special conical glass with cock	bottom	TCE	no	no	small (20 x 20 mm)	paraffin oil
36	no	special conical glass with cock	bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine
37	no	conical champagne glass	top	TCE	no	no	small (20 x 20 mm)	immersion oil
38	no	special conical glass with cock	bottom	TCE	no	yes	medium	glycerine
39	no	chemical sedimentation funnel	bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine
40	yes	chemical sedimentation funnel	bottom	TCE	yes	no	small (20 x 20 mm)	Norland adhesive 65
41	no	chemical sedimentation funnel	bottom	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65
42	no	conical champagne glass	top	TCE	no	no	medium	-- select --
43	no	conical champagne glass	top	TCE	yes	yes	small (20 x 20 mm)	glycerine
44	no	other	other	TCE	no	no	small (20 x 20 mm)	paraffin oil
45	no	chemical sedimentation funnel	bottom	TCE	yes	no	small (20 x 20 mm)	Norland adhesive 65
46	no	special conical glass with cock	bottom	TCE	no	yes	small (21 x 26 mm)	glycerine
47	no	chemical sedimentation funnel	bottom	TCE	no	yes	small (20 x 20 mm)	glycerine
49	no	chemical sedimentation funnel	bottom	TCE	no	no	small (21 x 26 mm)	paraffin oil
50	no	chemical sedimentation funnel	bottom	TCE	yes	yes	large (22 x 50 mm)	glycerine

Annex 7 Results: presence of animal proteins, microscopic detection

lab nr		sample number				land				milk powder				fish			
	PCR					A	B	C	D	A	B	C	D	A	B	C	D
1	yes, only																
2	no	122	382	320	454	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
3	yes	164	172	201	405	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
4	no	171	165	411	139	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
6	yes	192	438	488	125	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
7	no	199	228	376	118	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
8	no	206	242	362	160	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
9	no	185	263	369	216	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
10	no	241	452	432	440	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
11	no	248	347	236	167	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
12	yes	262	277	138	335	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
13	yes	283	340	250	223	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
14	yes	290	354	355	181	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
15	yes	304	319	159	230	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
16	no	318	466	229	265	suspect	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
17	no	276	312	208	244	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
18	no	549	403	509	251	absent	present	present	absent	absent	absent	absent	present	absent	absent	present	suspect
19	no	563	368	173	202	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
20	yes	423	550	124	384	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
21	yes	437	123	425	314	absent	present	absent	suspect	absent	absent	absent	absent	absent	absent	present	absent
23	yes	514	326	495	349	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
24	no	374	361	145	426	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
25	yes	521	200	411	391	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
26	no	570	270	502	321	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
27	yes	577	235	222	363	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
28	yes	325	333	264	398	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
29	no	409	249	215	433	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
30	no	346	144	306	447	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
31	no	332	480	404	356	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent

lab nr		sample number				land				milk powder				fish			
	PCR					A	B	C	D	A	B	C	D	A	B	C	D
32	yes	402	424	467	419	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
33	no	430	417	481	370	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
34	yes	367	410	131	300	suspect	present	absent	suspect	absent	absent	absent	absent	absent	absent	present	suspect
35	no	556	473	299	293	absent	suspect	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
36	yes	255	221	474	328	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
37	no	535	305	285	377	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
38	no	528	459	341	258	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
39	no	542	291	117	209	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
40	no	227	137	313	286	absent	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
41	yes	220	298	446	272	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
42	yes	311	158	243	237	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
43	no	269	130	516	503	absent	suspect	suspect	absent	absent	absent	suspect	absent	absent	absent	present	absent
44	no	129	445	327	188	absent	present	present	present	absent	absent	absent	absent	absent	absent	suspect	absent
45	yes	507	186	334	146	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
46	no	178	375	152	153	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
47	no	297	557	460	195	absent	present	absent	absent	absent	absent	absent	present	absent	absent	present	absent
49	no	213	396	187	307	absent	suspect	absent	absent	absent	absent	absent	absent	absent	absent	present	absent
50	no	353	256	257	496	suspect	present	absent	absent	absent	absent	absent	absent	absent	absent	present	absent

Annex 8 Results: PCR ruminant

lab nr	sample number				land			
	A	B	C	D	A	B	C	D
1	115	487	278	132	absent	present	absent	present
3	164	172	201	405	absent	present	absent	present
6	192	438	488	125	absent	present	absent	present
12	262	277	138	335	absent	present	absent	present
13	283	340	250	223	absent	present	absent	present
14	290	354	355	181	absent	present	absent	present
15	304	319	159	230	absent	present	absent	present
20	423	550	124	384	absent	present	absent	present
21	437	123	425	314	absent	present	absent	present
23	514	326	495	349	absent	present	absent	present
25	521	200	411	391	absent	present	present	present
27	577	235	222	363	absent	present	absent	present
28	325	333	264	398	absent	present	absent	present
32	402	424	467	419	absent	present	absent	present
34	367	410	131	300	absent	present	absent	present
36	255	221	474	328	absent	present	absent	present
40	227	137	313	286	absent	present	absent	present
41	220	298	446	272	absent	present	absent	present
42	311	158	243	237	absent	present	absent	present
45	507	186	334	146	absent	present	absent	present

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