IAG ring test animal proteins 2016

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Contents

	Summary	5
1	Introduction	7
2	Methods	8
	2.1 Materials	8
	2.2 Procedure for production	8
	2.3 Homogeneity study	9
	2.4 Organization of the ring trial	9
	2.5 Analysis of results	10
3	Results	12
	3.1 Application of the method	12
	3.2 Microscopic procedure	12
	3.3 Microscopic detection	13
	3.4 Detection by PCR	14
	3.5 Combined method	14
4	Discussion	15
	4.1 Combination of microscopy and DNA detection	15
	4.2 Specificity and sensitivity	15
	4.3 Method parameters	16
5	Conclusions and recommendations	18
	5.1 Conclusions	18
	5.2 Recommendations	18
	Acknowledgements	19
	References	20
	Annex 1 Introduction to the test	21
	Annex 2 Basic instructions for the test procedure	22
	Annex 3 Report form for procedure details	24
	Annex 4 Report form: results	25
	Annex 5 List of participants	26
	Annex 6 Details of procedures applied, microscopic method	27
	Annex 7 Results: presence of animal proteins, microscopic detection	29
	Annex 8 Results: PCR ruminant	31

Summary

The annual ring test for the detection of animal proteins in animal feed of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy was organized by RIKILT - Wageningen UR, The Netherlands. The aim of the ring study was to provide the participants information on the performance of the local implementation of the detection method for their local quality systems. A further aim was to gather information about the application of the microscopic method. The current 2016 version of the IAG ring test for animal proteins facilitated the full scenario with the methods for microscopy and PCR as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs.

All four samples were based on an artificial feed mimicking a formulation for ruminant feed. Two samples were labelled as fish feed (B and D), which was effectuated by adding 2% of a general fish meal. Adulteration was achieved by adding 0.1% pig MBM (B), 0.1% ruminant MBM (D) and a combination of 0.1% ruminant MBM and 0.1% fish meal (C). This combination of different spikes allowed the diverse application of the detection methods.

Forty eight participants enrolled for the ring test, of which 45 submitted microscopic results. Of these, 20 participants applied the combination of microscopic and PCR analysis. Three participants submitted exclusively PCR results.

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 the method used.

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 and as alternative considered as negative. By comparing both ways of analysis it is possible on one hand to compare the results with those from previous ring trials (there these numbers were considered positive based on the legal principle of zero tolerance), and on the other hand to compare it to the official method (where numbers between 1 and 5 are considered negative).

Most of the specificity and sensitivity scores for microscopy were at good or reasonable levels. In the combination of fish meal (0.1%) and ruminant MBM (0.1%) the detection of fish material was sub optimal. Considering numbers of particles below threshold as negative, the sensitivity scores show a considerable drop. The results indicate that the overall performance of the microscopic method is satisfactory, but applicants of the microscopic method could benefit from good and effective training and documentation in order to achieve a higher reliability in identifying particles.

PCR

The specificity (samples A and B) and sensitivity (samples C and D) scores for PCR were between 0.87 and 0.91.

Combined scenarios for microscopy and PCR.

Several participants applied incorrect numbers of determination cycles, either too many or too less. The scenarios for correct combination of microscopy and/or PCR are published in an accompanying SOP. Several deviations from these scenarios were applied, such as examination by PCR of the terrestrial animal feeds, reporting the presence of fish in the fish feeds, and the absence of a final conclusion combining the results of both methods where appropriate. It is, however, a very good situation to have all (intermediate) results reported for each of the two methods in order to have a good documentation in the framework of a ring test.

1 Introduction

The monitoring of the presence of animal proteins in feed for prevention of mad cow disease is an important part of the required active monitoring by member states of the European Union. A range of official control methods was combined in 2009 in Regulation (EC) 152/2009. A new method for microscopic detection of animal proteins is effective from 12 February 2013 (Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009: EC, 2013a, and its corrigendum EC, 2013b). In addition an official method for DNA identification of ruminant material by means of PCR was published as well.

The changes in the microscopic method implement a more detailed procedure. The modifications were directed by the desire to gain in reproducibility and in harmonization (e.g. Veys et al., 2010). A Limit of Detection (LOD¹) of five particles was set per determination cycle based on a laboratory sample of 10 grams. As of 1 June 2013 non-ruminant material is allowed as ingredient in aquafeed (Regulation (EC) 56/2013 amending Annex IV of Regulation (EC) 999/2001). Ruminant material remains prohibited, which needs a specified identification method, which was implemented by a PCR method.

The European Commission stimulates official reference laboratories to include a lot of procedural details in Standard Operational Procedures (SOPs) instead of a full methodological description in Regulations in order to enhance flexibility. In the area of the monitoring of animal proteins the European Union Reference Laboratory (EURL) is responsible for the development of methods and for the public availability of these SOPs. SOPs supporting the new method include details of the microscopic and PCR procedures, and the strategy for the combination of these two methods.

The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy organises annually a ring test for animal proteins in feeds for all their members. RIKILT – Wageningen UR organises this ring test on behalf of the IAG section Microscopy. The current 2016 version of the IAG ring test for animal proteins is intended, as in previous years, to provide the participants data on the performance of their own way of implementation, and to document the application of the two enforcement methods.

In this report the ring test for animal proteins 2016 is presented.

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

2 Methods

2.1 Materials

The IAG ring test for animal proteins 2016 was chosen to be based on an artificially produced compound feed; the formulation was taken from a ruminant feed. The feed was based on a formulation with cornglutenfeed (30%), citrulpulp (15%), beetpulp (15%), palmkernelmeal (15%), rapeseedmeal (8.5%), wheat (5%), wheat semolina (5%), soybeanmeal (5%) and mineral mix (1.4%). All ingredients were bought as pure materials and tested. In two samples 2% of fish meal was added in order to mimic a fish feed (2016-B and 2016-D).

The IAG ring test for animal proteins 2016 was combined with the IAG ring test for botanic composition (sample 2016-A). The results of this ring test are being published in a separate report (van Raamsdonk et al., 2016).

The design of the ring test animal proteins allowed to apply the full method for the detection of animal proteins as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 (EC, 2013a), its corrigendum (EC, 2013b) and the accompanying SOPs. The samples were chosen to be fit for detection by both microscopy and PCR. The choice and order of the methods was part of the study. The composition of the four samples is listed in Table 1.

Table 1

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

Label	Sample type	Content
2016-A	ruminant feed	blank
2016-B	fish feed	0.1% pig MBM (2% fish)
2016-C	ruminant feed	0.1% ruminant MBM + 0.1% fish
2016-D	fish feed	0.1% ruminant MBM (2% fish)

The fish meals used for spiking were samples from practice, which were examined in the RIKILT regular control program and found to be negative for land animal material.

The ruminant meat and bone meal was produced in the framework of the EU funded project STRATFEED and distributed by the coordinator among the participants. The pig meat and bone meal was obtained from a Danish supplier, which is used for years in tests and appeared to be free of ruminant DNA.

All materials were checked on purity (absence of any contamination) and identity, and were all found to be fit for application.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order: 2016-A - 2016-B - 2016-D - 2016-C. All samples were prepared in a laboratory that is located at a distance from the RIKILT microscopy laboratory. A sample size of 50 grams was chosen which was sufficient for applying all three determinations as mentioned for the full method in Regulation (EC) 152/2009. Jars for sample 2016-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%.

2.3 Homogeneity study

Two RIKILT microscopists examined independently all basic materials and five jars of all four samples according to the procedure of Regulation (EC) 152/2009. PCR was carried out according to the EURL-AP protocol and SOPs for ruminant with IRMM plasmids. Cut-off: C_q = 33.93.

Table 2

Results of the homogeneity study. Sediment amounts are based on 10 grams. Microscopy: five replicates. PCR: four replicates for ruminant, two replicates for pig and fish. Green cells indicate the correct positive findings. Rum: ruminant target.

Sample	Sediment amount	Microscopy		PCR	
		MBM	fish	Rum	Pig
2016-A blank	183-221 mg/10 g	absent	absent	absent	absent
2016-B 0.1% pig MBM (2% fish)	204-245 mg/10 g	present	present	absent	present
2016-C 0.1% ruminant MBM + 0.1% fish	181-213 mg/10 g	present	present	present	absent
2016-D 0.1% ruminant MBM (2% fish)	202-244 mg/10 g	present	present	present	absent

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

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

2.4 Organization of the ring trial

All IAG members, all NRLs, participants of former ring tests and a series of putative interesting laboratories were informed about the ring test for 2016 by means of an invitations in the IAG Newsletter of 2015.

(http://www.iag-micro.org/files/newsletter_2015_iag_section_feed_microscopy_final.pdf) Until the beginning of March a total of 48 participants for the ring test animal proteins were listed. Participants outside Europe were informed to be aware of possible problems with custom regulations. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on Monday 7th of March 2016. On Friday March 18th an E-mail message was sent to all participants, together with a file containing a sheet with instructions (see Annex 2) and the electronic report forms (see Annex 3 and 4), and the request to confirm the receipt of the package.

The closing date for reporting results was fixed at April 8th. Several requests were received to extend the period for analysis with several weeks. Results received after the date at which the evaluation of the results was started were ignored. The analysis of the results was carried out at early May.

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

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

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

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

The decision rule for the third determination was based on the sum after two determinations. The text of the new method requires to perform an extra determination when the number of particles is between 1 and 5, which interpreted as directing a second (or third) analysis when ONLY one of the types of material was found to be within this range.
The draft report was finalised at May 20th.

2.5 Analysis of results

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

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

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

The results are analysed in two ways: numbers below threshold (between 1 and 5 inclusive) have been considered 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:

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

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

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

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics are presented as fractions. Accuracy (specificity or sensitivity) has been calculated for each sample type.

As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

3 Results

All 48 participants subscribed for the IAG ring test animal proteins returned their results. Of these, 45 sets were received for microscopy and 23 for PCR; three participants submitted only PCR results. The participants originated from 19 countries: 15 member states of the European Union, and five other countries (China, Norway, Peru, Serbia 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 (5), France (4), Belgium (3) and Italy (3).

All results were received by E-mail, in most cases by means of a scan and the original report file. Not in all cases a scan as pdf-file was submitted although clearly requested. Two participants were asked to clarify their results in the view of the PCR results. In all those cases that a participant sent in several versions of the report sheet the most recent version was used. All full reports were included. The full results are presented in the tables of Annex VI, VII and VIII.

3.1 Application of the method

Five participants (2, 8, 16, 42, 51) carried out too many determination cycles for one or more samples. Participants 27 and 50 should have carried out one extra cycle for one or two samples.

3.2 Microscopic procedure

An inventory of nine different parameters was added to the report sheet of the actual results of the four samples. These results are shown in Annex 6 and summarised in Table 3. The main purpose of this inventory was to provide benchmark information for the individual participants for comparison with the general application of the method. Although this has to be considered additional information only, a ring test with a random set of participants provides a good opportunity to collect meta-data on the application of the method. The current results provide the opportunity to discuss some parameters of the microscopic method.

The results as presented in Table 3 generally show a good application of the method, except for the number of determinations applied and for the embedding agent. Differences with previous years will be presented in the next chapter (Discussion).

Eleven participants used between 4 and 15% of the sediment as obtained from the samples. All other participants usually used a much higher share of the sediment, up to 100%.

Table 3

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

Parameter	parameter state	number of participants	amount
Correct application of the number of determinations	yes	39	
	no; too many determinations	4	
	no; insufficient determinations	2	
Extra milling step (<1.0 mm)	no	38	
	yes	5	
type of glassware	chemical sedimentation funnel	22	
	conical glass with cock	8	
	champagne glass *	5	
	beaker (flat bottom)	4	
	other	4	
sedimentation agent	TCE	45	
	TCE/Petroleumether	0	
use of staining of sediment	no	31	
	yes	14	
use of binocular for examination at lower magnifications	yes	30	
	no	15	
size of cover glass used	small (e.g. 20 x 20 mm)	38	
	medium	4	
	large (e.g. 26 x 50 mm)	3	
share of the total sediment used for examination	minimum		4%
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	16	
	paraffin oil	12	
	immersion oil	10	
	Norland Adhesive	6	
	other (water, glycerol:water mixture, mineral oil)	0	

3.3 Microscopic detection

After analysis of the results it appeared that all participants correctly interpreted their own encountered numbers in terms of absent, below threshold or positive. This situation implies that the results in terms of numbers (Figure 1 index 'b') and in terms of the final conclusion (Figure 1 index 'c') are identical. Therefore, the overall microscopic results are presented based on the raw numbers (Figure 1 index 'b' and 'e'). The results of the application of the microscopic detection yielded in general a reasonable result (Table 4; Annex 7). A number of participants reported the absence of fish material in sample C (sensitivity 0.84). The finding of pig material (sample 2016-B) and of ruminant material (sample 2016-D) is suboptimal, especially when amounts below threshold are considered negative (0.91 and 0.78, respectively). This evaluation of amounts below threshold is also an issue for the detection of fish meal at a level of 0.1% (sample 2016-C). By principle, the establishment of a threshold for positive findings results in a lower score in cases of sensitivity and in a higher score in cases of specificity.

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.*

n		MBM				Fish			
		A	B	C	D	A	B	C	D
		0%	0.1%	0.1%	0.1%	0%	2%	0.1%	2%
45	specificity	0.96				0.98			
		<i>1.0</i>				<i>1.0</i>			
	sensitivity		0.96	0.98	0.91		0.98	0.84	1.0
			<i>0.91</i>	<i>0.87</i>	<i>0.78</i>		<i>0.98</i>	<i>0.78</i>	<i>1.0</i>

The results were stratified according to several of the parameters as presented in Table 3. No significant differences were found among the different states for each of these parameters.

3.4 Detection by PCR

Participants were invited to perform DNA analysis targeted for ruminants (EURL-AP Method) and to submit their results together with the results for microscopy. The PCR results are presented in Table 5 and in Annex 8. Seven participants (six for sample 2016-C) correctly submitted no results for the two animal feeds, since SOP 'operational schemes v3.0' figure 1 does not include the PCR method. The results for the PCR method are suboptimal.

Table 5

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

	Ruminant			
	A	B	C	D
	0%	0%	0.1%	0.1%
specificity	0.88	0.87		
sensitivity			0.88	0.91
n	16	23	17	23
Homogeneity study	absent	absent	present	<i>present</i>

3.5 Combined method

In the situation of sample 2016-B, where terrestrial animal material is found in a fish feed and the subsequent PCR analysis does not reveal the presence of ruminant material, absence of prohibited materials should have been reported according to the SOP. Only participant 1 adjusted its initial positive result for terrestrial animal material in that sample to 'absent' based on the PCR result. Participant mentioned that they performed PCR on all four samples, but according to the SOP only the necessary results for the two fish feeds have been reported.

4 Discussion

4.1 Combination of microscopy and DNA detection

The current ring test, which includes animal feed and fish feed samples, allows to test two different scenarios combining light microscopy and PCR as methods (Figure 1).

Several deviations from the official scenarios were found among the current results:

- Twenty-five participants (56%) did not apply PCR on the two fish feeds, although terrestrial animal material was found.
- Out of 23 sets of PCR results, 16 participants applied the PCR method on the animal feed as well.
- All participants reported on the presence or absence of fish in the fish feed.
- Besides one participant, the findings of non-ruminant MBM in a fish feed were not adjusted to 'absence of prohibited material'.

The extent of the relevance of these statistics can be argued. At first it can be assumed that private laboratories are free, after consultation of their clients, to apply modified versions of the official method. Secondly, the history of the IAG ring tests for animal proteins is aimed at the exclusive application of the microscopic method. Finally, the framework of a ring test or proficiency test could be considered to have the primary aim to collect documentation on the performance of the tests rather than on making the necessary choices. Although the instructions stated to apply the full procedures as published in Regulation (EC) 152/2009 together with the connected binding SOPs, the value of the current set of data is higher compared to the situation where all PCR results had been lacking for samples 2016-A and C, and fish meal presence had been ignored for samples 2016-B and D (see Figure 1 for further references for these situations).

4.2 Specificity and sensitivity

Apparently, an amount of 0.1% of fish meal in a compound feed (2016-C: sensitivity 0.84; Table 4) can occasionally offer problems for full detection. This result is lower than those obtained in the IAG ring test of 2015 (0.95 and 1.0 for 0.1% of fish meal from Denmark and Peru, respectively). In the large study of the EURL for quantification of fish meal, one out of 22 participants missed the fish meal at a level of 0.15% (Veys and Baeten, 2007). The IAG ring test scores were lower after considering findings below threshold as negative, indicating several reports of low numbers of fish particles at that level of 0.1% (van Raamsdonk et al., 2015: Table 3). It is known that a lot of teleost fish show acellular bones, i.e. bones without (visible) lacunae, which complicates the recognition (Kranenbarg et al., 2005; Cohen et al., 2012).

In general, the application of a threshold and considering results below that threshold as negative will result in all cases in higher scores for specificity and in lower scores for sensitivity (Table 4; van Raamsdonk et al., 2014: Table 4; van Raamsdonk et al., 2015: Tables 3 and 5). One or a few wrongly recognised particles results in false positives, which is a specificity problem and using a threshold would give the positive effect of limiting this number of false positives. At the same time, low numbers of correctly recognised particles are considered negative as well, and this would introduce false negatives, especially at low adulteration levels. In this principal relationship between false positives and negatives (Lindenmayer and Brugman, 2005) an optimal level for the threshold needs to be established for optimising both scores for sensitivity and specificity. The effect of a threshold depends on the level of adulteration at which the data is collected. The current results show that even at levels of 0.1% adulteration undesirable effects on the sensitivity scores can occur (Table 4).

The results of the current ring test are in line with those of previous years (Table 6).

Table 6

Results for detection of material of terrestrial animals and of fish in feed samples based on sediments of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008-2016) on behalf of the IAG section Microscopy. Results have been communicated in the framework of this Section. Results indicate specificity in the case of the blank, and sensitivity in the case of the other sample types.

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

*: TCP used as contaminant for land animal material

4.3 Method parameters

The way the microscopic method is implemented and the development of several parameters during the years is shown in Table 10. For most parameters there seems to be a stabilizing situation. Solvents other than TCE are no longer in use in the population of the participants. Other embedding agents than glycerine remain popular. The effect of an embedding agent with a high viscosity (glycerine) compared to one with a lower viscosity (e.g. paraffin oil) depends on the applied strategy: examination of each slide directly after preparation, or preparing a set of slides with examination after a period of hours. Both strategies are allowed.

Table 7

Comparison between parameters distribution in the IAG ring studies between 2008 and 2016. Number of cycles since 2014.

parameter	parameter choice	2008	2009 –2015	2016
correct number of cycles			67.3% - 88.1%	86.7%
amount of material used for sedimentation	5 grams	16	5-0	
	10 grams	26	41-50	
	other	3	3-0	
type of glassware	chemical sedimentation funnel	22	22-33	22
	beaker (flat bottom)	11	13-4	4
	champagne glass	6	5-9	5
	conical glass with cock	3	1-9	8
	other	3	2-4	6
use of staining of sediment	no	31	27-36	31
	yes	14	14-22	14
use of binocular for examination at lower magnifications	yes	29	26-45	30
	no	16	9-16	15
size of cover glass used	small (e.g. 20 x 20 mm)	34	27-42	38
	medium	1	4-10	4
	large (e.g. 26 x 50 mm)	9	16-3	3
share of the total sediment used for examination	minimum	4%	0.2%-3%	4%
	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-25	16
	immersion oil	8	7-14	10
	paraffin oil	18	12-23	12
	Norland Adhesive	0	2-7	6
	chloral hydrate	3	1-0	0
	other (e.g. Depar 3000, water)	8	5-0	0

5 Conclusions and recommendations

5.1 Conclusions

Most results are within acceptable limits and in concordance with the results of the IAG ring test animal proteins of previous years. The detection of 0.1% fish meal in the presence of 0.1% of land animal MBM appears to be difficult. A fraction of the false positive results seems to be corrected when all findings below the threshold are considered negative (i.e. a higher score for specificity). This procedure, however, has a negative effect on the scores for sensitivity. The sensitivity for the detection of 0.1% land animal MBM in the presence of 2% fish meal decreased from 0.91 (results below threshold below positive) to 0.78 (results below threshold negative). Other strategies, such as documentation and training, although already part of the efforts of expertise centres, might contribute to a decrease in false identifications.

The combination of microscopy and PCR is directed by a binding SOP published by the EURL AP. Several types of deviations from the strict application of the procedures were encountered in this ring test. It can be argued whether there is any issue with these practices in the framework of a ring test; results are generally submitted as extended as possible and this implies the availability of an almost complete set of data for evaluation.

There is limited development in the choice for several parameters. The lowest portion of sediment used for examination is still 4%. The use of a higher share of sediment material could implicate that higher numbers of particles (i.e. above the threshold) could be encountered, with higher scores for sensitivity as effect. The application of embedding agents with moderate viscosity (e.g. immersion oil, paraffin oil) could be reasonable in the view of specific circumstances for the examination of the slides.

5.2 Recommendations

- A further investment is necessary in documentation and training for correct identification of particles of animal origin.
- Attention for a proper implementation of the combination of microscopy and PCR would be desirable.
- A further investigation into the effects of certain parameters of the microscopic method on the performance could be advantageous.

Acknowledgements

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Annex 1 Introduction to the test

Test 2016-A: animal proteins in feed

The IAG ring test animal proteins in feeds is designed to apply both the microscopic method and the PCR ruminant method. The procedures to be followed are described in Annex VI of Regulation (EC) 152/2009 from the European Union, amended by (EC) 51/2013, and the related SOPs. All the documentation can be found on the website of the EURL AP: <http://eurl.craw.eu/index.php?page=187>. The jars contain 50 gram of feed, which is sufficient for carrying out three cycles of the microscopic method and/or for carrying out the PCR analysis. Take care to homogenise the content of each vial before taking the amount for analysis. The samples are prepared in such a way that you can start with the procedure as described in 'EURL-AP SOP operational schemes', followed by the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle.

Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, feathers) and those of fish (fish bone fragments, scales, gills, otoliths). If more than 16 fragments per category are found in any cycle, just choose '16' from the drop-down list.

Based on the average number of particles found, you have to make the decision whether each of the two types is absent in a sample (zero particles on average), below LOD (between 1 and 5 particles on average) or present (6 or more particles on average).

In addition to the work flow as presented in the paragraphs 2.1.3.4 until and including 2.1.4.3, it is mandatory to weight the sediment BEFORE and AFTER the analysis as performed in every determination.

All results can be entered in the report form with 'animal proteins' in the name.

Annex 2 Basic instructions for the test procedure

IAG ring test 2016 animal proteins

Instructions for the IAG ring test

- 1 You have received a box with an introduction letter and four vials containing 50 grams of possibly contaminated animal feed. Please report the receipt of your package as soon as possible by E-mail to the address mentioned below.
- 2 The samples have to be analysed according to Annex VI of Regulation (EC) 152/2009 from the European Union, modified by (EC) 51/2013. The consolidated version and the SOPs can be found on the EURL website. The sample design allows to carry out the PCR ruminant analysis, but follow the SOPs carefully!. **Take care to homogenise the content of each vial before taking the amount for analysis.**
The samples are prepared in such a way that you can start with the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse three determinations of 10 grams as indicated in paragraph 2.1.4.3. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle and a final conclusion.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, horn, skin, feathers) and those of fish (fish bone fragments, scales, gills, otoliths). If more than 16 particles are found in any category, please enter the value 16.
The report form is interactive: if the results in the first determination cycle make it necessary to perform a second or third analysis according to the requirements of the Regulation, additional cells will turn pink.
The final conclusion, according to Regulation (EC) 152/2009, can be reported in three ways, depending on the average number of particles found per category:
= Zero particles: animal proteins **absent**. If the first determination reveals no particles in any category, a second determination is not necessary.
= More than 5 particles on average per determination: **present**.
= Between 1 and 5 particles on average: sample is positive but a risk of a false positive result cannot be excluded. For the sake of the framework of the current report form the term '<LOD' has to be chosen.

[Click here for the Regulation and connected SOPs](#)
- 3 Reporting consists of the following steps:
 - 3a Please fill in the questionnaire on the page 'Procedure'.
Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.
Your unique lab number is mentioned in the introduction letter, enclosed in the box.
All the fields with a drop-down list have to be completed.
 - 3b Please enter your results in the fields at page 'Results'. Your unique lab number automatically shows up after 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 Nastasja.vanderhee@wur.nl.
- 4b Results will be included in the final analyses and report only if both forms are send in by electronic mail, and after the proper receipt of the requested fee.
- 5 Direct any questions to leo.vanraamsdonk@wur.nl
- 6 Closing date is Friday April 8th, 2016.

RIKILT Wageningen UR, the Netherlands

Annex 3 Report form for procedure details

Please complete at least all the cells with a drop down list	select your choice from a drop down list	type in your answer if necessary
<div> <div> IAG ring test 2016 animal proteins </div> <div> </div> </div>		
Please select your unique lab number	-- select --	
Have you read the ring test instructions?	-- select --	
Did you apply PCR ruminant detection method?:	-- select --	
Did you apply grinding before performing the detection procedure?	-- select --	
Indicate your glassware for sedimentation if other, please specify	-- select --	
Describe your sedimentation agent if other, please specify	-- 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 if other, please specify	-- 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 16 particles were found in any category, please enter the value 16.

IAG ring test 2016 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

-	-
---	---

Result of first determination cycle

land	fish	land	fish
- select -	- select -	- select -	- select -

Second determination

-	-
land	fish

Third determination

-	-
land	fish

Total number of particles per category

0	0	0	0
---	---	---	---

PCR results

Ruminant (EURL method)

--	--

Final conclusion

Type of particles

land	fish	land	fish
- select -	- select -	- select -	- select -

Comment, if necessary

Annex 5 List of participants

institute	country
Austrian Agency for Health and Food Safety-AGES	Austria
Laboratorium ECCA nv	Belgium
LFSAL	Belgium
FLVVT	Belgium
China Agricultural University (East campus)	China
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
AdGène Laboratoire	France
IPL Atlantique	France
Inovalys-Nantes	France
S.C.L. Laboratoire de Rennes	France
CVUA-RRW	Germany
SGS Germany GmbH	Germany
Landeslabor Berlin-Brandenburg	Germany
LUFA Nord-West	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Veravis GmbH	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
WESSLING GmbH	Germany
LTZ Augustenberg	Germany
LUFA-Speyer	Germany
Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
LUFA Rostock	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Thüringer Landesanstalt für Landwirtschaft Jena	Germany
Equine Centre	Ireland
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
MIPAAF – ICQRF – LABORATORIO DI MODENA	Italy
Istituto Zooprofilattico della Sicilia	Italy
Inst. Zooprofilattico Sperimentale della Sardegna	Italy
Nutreco Nederland BV - Masterlab	Netherlands
Eurofins Food Testing Rotterdam BV	Netherlands
CCL - Nutricontrol	Netherlands
TLR	Netherlands
Nutrilab BV	Netherlands
Nofima AS	Norway
World Survey Services SAC	Peru
NSF INASSA S.A.C.	Peru
Cargill Poland	Poland
Instytut Zootechniki PIB, Pracownia w Szczecinie	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
Trouw nutrition Espana	Spain
Departamento de Calidad, Laboratorio de Diagnóstico General,S.L.	Spain
National Veterinary Institute, SVA	Sweden
Agroscope (ALP), Swiss Research Station	Switzerland

Annex 6 Details of procedures applied, microscopic method

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

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

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	226	242	53	199	absent	present	present	present	absent	present	present	present
2	no	216	117	38	204	absent	present	present	present	absent	present	present	present
3	no	251	187	133	29	absent	present	present	present	absent	present	present	present
4	no	256	127	128	14	absent	present	<threshold	absent	absent	present	<threshold	present
5	no	261	107	28	269	absent	<threshold	absent	<threshold	absent	present	present	present
6	no	266	192	243	19	absent	present	present	present	absent	present	present	present
7	no	211	142	23	24	absent	present	present	present	absent	present	present	present
8	no	236	27	218	59	absent	present	present	present	absent	present	present	present
10	yes	241	177	33	239	absent	present	present	present	absent	present	present	present
11	yes	221	62	208	94	absent	present	<threshold	present	absent	present	absent	present
12	yes	231	2	123	89								
13	no	201	182	3	144	absent	present	present	present	absent	present	present	present
14	yes	196	172	13	224	absent	present	present	present	absent	present	present	present
15	yes	171	92	213	64	absent	present	present	present	absent	present	present	present
16	no	186	197	18	209	absent	present	present	present	absent	present	present	present
17	no	181	102	143	244	absent	present	present	present	absent	present	absent	present
18	no	206	22	158	9	absent	present	present	present	absent	present	absent	present
19	yes	131	217	73	124	absent	present	present	present	<threshold	present	present	present
20	no	111	12	48	114	absent	present	present	present	absent	present	present	present
21	yes	141	202	58	229	absent	present	present	present	absent	present	present	present
22	yes	191	152	248	139	absent	present	present	present	absent	present	present	present
23	no	176	252	148	214	absent	present	present	absent	absent	present	present	present
24	yes	166	32	103	159	absent	present	present	present	absent	present	present	present
25	no	156	7	108	249	absent	absent	present	<threshold	absent	absent	present	present

lab nr		sample number				land			fish				
26	yes	151	257	78	129								
27	no	146	122	83	219	absent	<threshold	present	<threshold	absent	present	present	present
28	yes	161	137	238	179	absent	present	present	present	absent	present	present	present
29	yes	101	147	193	79	absent	present	present	present	absent	present	present	present
31	yes	86	212	43	259	absent	present	present	present	absent	present	absent	present
32	yes	96	232	188	134	absent	present	present	present	absent	present	present	present
33	no	121	42	163	169	absent	present	present	present	absent	present	present	present
34	no	126	157	178	99	absent	present	present	present	absent	present	absent	present
35	no	91	162	233	194	absent	present	present	present	absent	present	<threshold	present
36	no	276	267	113	189	absent	present	<threshold	absent	absent	present	present	present
37	yes	76	47	228	104	absent	present	present	present	absent	present	present	present
38	no	71	87	258	54	absent	present	present	present	absent	present	present	present
39	no	61	247	8	164	<threshold	absent	present	present	absent	present	absent	present
40	no	31	37	118	264	absent	present	present	present	absent	present	present	present
41	yes	46	222	203	119	<threshold	present	present	present	absent	present	present	present
42	no	56	132	88	184	absent	present	present	<threshold	absent	present	present	present
43	no	51	57	168	154	absent	present	present	present	absent	present	present	present
44	yes	41	207	253	49	absent	present	present	present	absent	present	present	present
46	yes	26	67	153	109	absent	present	present	present	absent	present	present	present
47	yes	16	227	98	39	absent	present	present	present	absent	present	present	present
48	yes	21	17	68	254								
49	yes	6	82	63	84	absent	present	present	<threshold	absent	present	present	present
50	no	271	262	198	74	absent	present	<threshold	<threshold	absent	present	absent	present
51	yes	11	237	268	69	absent	present	<threshold	absent	absent	present	<threshold	present

Annex 8 Results: PCR ruminant

lab nr	ruminant			
	A	B	C	D
1	absent	absent	present	present
10	- select -	absent	- select -	present
11	absent	absent	present	present
12 *	present	present	absent	absent
14	absent	absent	present	present
15	absent	absent	present	present
19	absent	absent	present	present
21	- select -	absent	- select -	present
22	absent	absent	present	present
24	- select -	absent	- select -	present
26 *	absent	absent	present	present
28	- select -	absent	- select -	present
29	absent	absent	present	present
31	- select -	absent	- select -	present
32	- select -	present	- select -	present
37	- select -	absent	present	present
41	absent	present	present	present
44	absent	absent	present	present
46	absent	absent	present	present
47	present	absent	present	present
48 *	absent	absent	absent	absent
49	absent	absent	present	present
51	absent	absent	present	present

*: exclusively PCR; no microscopic results.

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RIKILT report 2016.008

RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and reliability of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

The mission of Wageningen UR (University & Research centre) is 'To explore the potential of nature to improve the quality of life'. Within Wageningen UR, nine specialised research institutes of the DLO Foundation have joined forces with Wageningen University to help answer the most important questions in the domain of healthy food and living environment. With approximately 30 locations, 6,000 members of staff and 9,000 students, Wageningen UR is one of the leading organisations in its domain worldwide. The integral approach to problems and the cooperation between the various disciplines are at the heart of the unique Wageningen Approach.

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