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* updated version

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RIKILT
WAGENINGEN UR

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Summary

A ring test was organized for the detection of animal proteins in animal feed in the framework of the annual ring tests of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy. Although microscopic analyses were the primary target of this ring test, the possibility to apply PCR analyses was included as well. The organizer of the ring test was 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 2014 version of the IAG ring test for animal proteins is the first one in the IAG series of ring tests applying the full new method for detection of animal proteins in feed as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009.

Three of the four samples used in the ring test were based on an artificial feed with a formulation comparable to that of an average cattle feed. A mix of minerals was included at a level of 1%. The fourth sample was based on a chicken feed produced at a pilot plant dedicated to produce animal protein free test feeds. Three samples were designed for a proficiency testing: no animal proteins (blank), 2% of fish meal and 0.1% of land animal material, and 1% of insect meal (*Locusta*). The fourth sample was especially designed for gathering more information of the performance at low adulteration levels: this sample contained 30 bone fragments per jar (1 bone fragment per 1 gram of sample). All participants were requested to determine the presence or absence of land animal and/or fish and/or protein material of other animal sources (including unidentified muscle fibres and arthropods), and to indicate the type of material found. The participants were asked to report the amount of sediment found (the fraction containing minerals and bones, if present) before and after applying the actual analyses and to answer questions on a series of parameters of the microscopic method. Of the 56 participants 52 sets of results were returned with results using the microscopic method.

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 the 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, and it allows a way to compare the present results with those of previous years. About one-third (16 out of 52) of the participants applied the wrong number of determinations, although the report form was interactive and guided the participant through the process of choosing the right number of repetitions. Most of the specificity and sensitivity scores were at good levels. The specificity score for incorrect detection of meat and bone meal (MBM) in the blank is good (0.96). The detection of 0.1% of MBM in the presence of 2% fish material appeared to be acceptable (0.94). The detection of animal material of any kind in the sample contaminated with insect meal was insufficient (0.69), and the detection of insect fragments and of relatives (arthropods) as such was very low (0.19). The method performance at a contamination level of 1 bone fragment per gram material showed a sensitivity of 0.92, but in the situation that results below the threshold were considered negative the sensitivity was very low (0.44). A significant relationship was found between the amount of sediment used for observations and the number of particles found.

The way in which the new method should be implemented in the IAG ring test for animal proteins in 2014 needs further discussion.

The results as obtained for PCR are difficult to interpret. The sensitivity for a level of 0.1% ruminant (sample 2014-B) was as low as 0.12 whereas the same MBM at a level of 0.05% was correctly detected in the ring test of 2013. A chicken feed as produced in the framework of the EU project STRATFEED appeared to be positive for ruminant. The positive findings of 94% of the participants can be indicated as "*agrees with consensus*" as is applied in the FAPAS proficiency tests (www.fapas.com). A positive result for ruminant was achieved as well in the homogeneity study.

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 in 2009 combined 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). The changes imply a more detailed procedure for the microscopic detection. In addition an official method for DNA identification of ruminant material by means of PCR was published as well. The modification of the microscopic method is due to the situation that the reproducibility was considered to be insufficient at low contamination levels (e.g. Veys *et al.*, 2010). Therefore, a threshold indicated as Limit of Detection (LOD)[1] of five particles in a portion (laboratory sample for a single analysis) is set. 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 more specific monitoring in the view of this relaxation of the ban.

The European Commission stimulates testing 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 2014 version of the IAG ring test for animal proteins is the first one in the IAG series of ring tests applying the full new method for microscopy. Major changes compared to the old method are the repetition of the analysis in more than one and up to three subsequent determinations depending on the number of particles found, a fixed amount of slides per determination, and three instead of two sets of reporting sentences. A dedicated report form as developed by RIKILT to guide the user through the procedure of requested repetitions was used by all participants for reporting their results. The current ring test consists of three samples for proficiency testing of the participants and one sample for monitoring the performance of the microscopic method at low contamination levels.

In this report the ring test for animal proteins 2014 is presented, which was organised by RIKILT on behalf of the IAG Section Feeding stuff Microscopy. The study was designed to fit the requirements of the new method.

This report has been rearranged to allow a more clear distinction between two underlying goals being addressed in the ring trial. The ring test results have been separated into two sections, one with results of regular ring test samples (the actual ring test, samples A-C), and one section describing the results from a more challenging sample (sample D) that was added in order to investigate the limits of the microscopic method. The results as presented in the Annexes and the evaluation of samples A to C remained unchanged. Further documentation on the background of sample D and the analysis of its results are presented in Annex 10. An error in the conclusions of the PCR method that unfortunately

[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 are 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.

had been overlooked in the previous version has been corrected as well. This new version was published in November 2015 and will replace the previous version. A reference to the original version (2014.011) will be regarded to be a reference to the current (final) version of the report (2014-011).*

2 Methods

2.1 Materials

The ring test 2014 was chosen to be based on a compound feed completely produced by RIKILT, in a composition that mimics an average cattle feed. The feed was composed of citrus (20%), wheat (20%), maize (30%), beet pulp (10%), rapeseed (9.5%), palm expeller (9.5%), mineral mix (1%). 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.
Three samples were produced based on the artificially produced feed. A fourth sample was based on a chicken feed produced in the framework of the European project STRATFEED. The reason was the combination of the current ring test for animal proteins with the IAG ring test for botanic composition. The results of this ring test are being published in a separate report (van Raamsdonk *et al.*, 2014). The chicken feed consisted of wheat meal (46%), soybean products, partly extracted (28%), corn meal (11%), rapeseed and rapeseed meal (7%), vegetable fat (5%), mix of minerals and vitamins (3%).
The composition of the four samples is listed in Table 1.

Table 1
Composition of the samples in the NRL-IAG ring trial 2014.

Label	matrix	Content	Purpose
2014-A	Artificial cattle feed	blank	Regular proficiency testing
2014-B	Artificial cattle feed	2% fish meal, 0.1% ruminant meal	
2014-C	Chicken feed	1.0% insect meal (*Locusta*)	
2014-D	Artificial cattle feed	1 ruminant bone fragment per gram matrix (estimated at 0.005% w/w)	Method performance test

The fish meal was a sample from practice (Peru) which was examined in the RIKILT regular control program and found to be negative for land animal material.
The meat and bone meal was produced in Uruguay and collected after export to China. It was declared as ruminant MBM. The bone fragments used to spike sample 2014-D were taken from the sediment of the same MBM.
The insect meal (grasshoppers; *Locusta*) was bought on the internet as entire (dried) animals. This material was ground and degreased before being used as animal protein.
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: 2014-A - 2014-C - 2014-B - 2014-D. All samples were prepared in a laboratory which is located at a distance from the RIKILT microscopy laboratory. A sample size of 30 grams was chosen in order to be sure that all particles in sample 2014-D could be found when applying all three determinations as mentioned for the full method in Regulation (EC) 152/2009.
The production scheme is presented in Figure 1.
Jars for sample 2014-A and for sample 2014-D were filled with 30 grams of the pure feed, closed and set aside. Sample 2014-B was produced by thoroughly mixing 50 g of fish meal in 2.45 kg of feed.

This resulted in a concentration of approximately 2% fish meal. The material for samples 2014-B and 2014-C were produced by step-wise dilution of the dedicated contaminants. The jars of samples 2014-B and 2014-C were set aside before the portions of counted particles of ruminant sediment entered the laboratory.

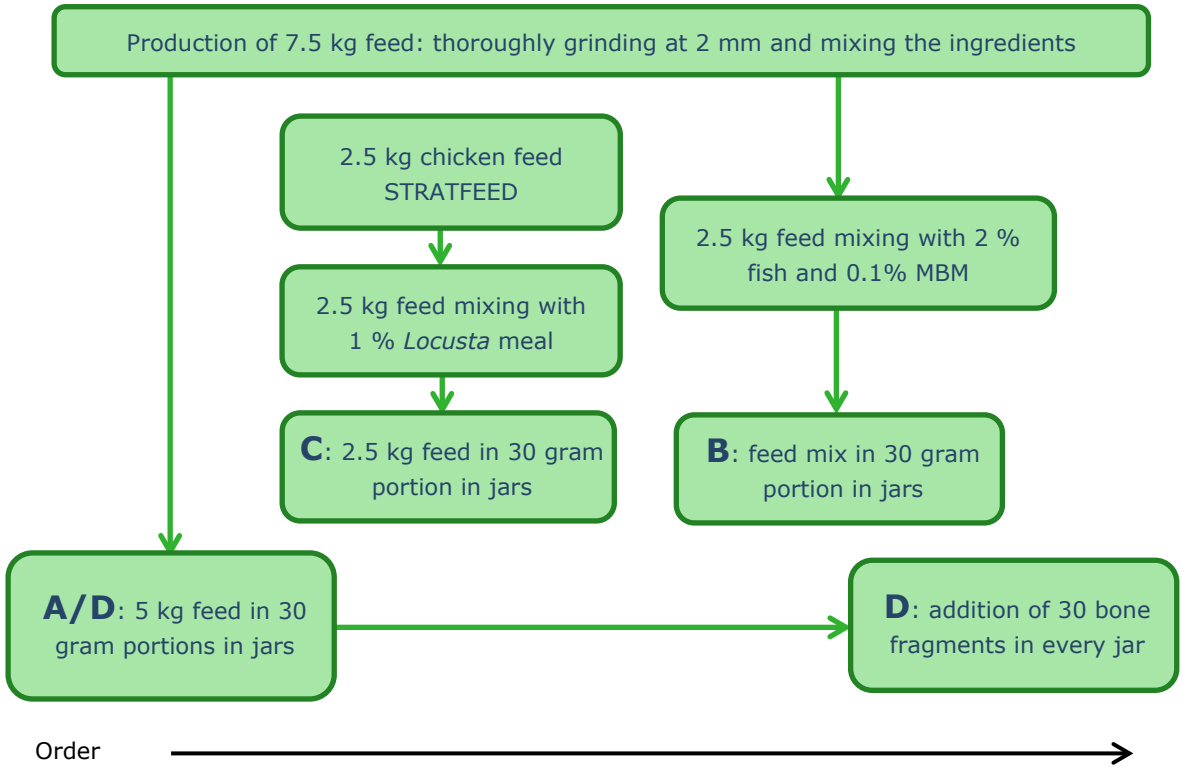

Figure 1 *Overview of the production scheme for the four samples of the IAG ring test animal proteins 2014.*

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: Cq = 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	insect	Rum
MBM Uruguay		Pos	Neg	Neg	Pos
Fish meal Peru		Neg	Pos	Neg	Neg
Locusta meal		Neg	Neg	Pos	Neg
Locusta meal, degreased		Neg	Neg	Pos	Neg
Chicken feed		Neg	Neg	Neg	Pos
2014-A blank	11.0 – 12.4 mg/g	Neg	Neg	Neg	Neg
2014-B 2% fish / 0.1% MBM	13.1 – 15.3 mg/g	Pos	Pos	Neg	Neg
2014-C 1.0% insect	18.2 – 19.8 mg/g	Neg	Neg	Pos	Pos
2014-D 1 fragment/g	10.6 – 13.2 mg/g	Pos	Neg	Neg	--

The microscopic results were correct in all cases (Table 2). The results for PCR are inconclusive in some cases, as listed in Table 2. Sample 2014-D was not tested by PCR for homogeneity, because the jars were individually adulterated and the MBM used was identical to the one use for adulterating sample 2014-B. The results of the homogeneity study will be discussed further in the chapter Results and Discussion.
The microscopy research group and the PCR research group of RIKILT did not participate in the further laboratory analysis of this ring trial.

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 2014. In all cases an invitation letter, a participation form and an invoice were distributed. Until the beginning of March a total of 56 participants for the microscopic method were listed. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on the Tuesday 4th of March 2014. On Wednesday March 5th 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 1st . Several requests were received to extent the period for analysis with two weeks. This request was granted and the closing date was set at April 15th. In several cases participants appeared not to be able to submit their results even within the extended period. A total of 52 sets of results were received late March or during April. Since the analysis of the results was carried out at the end of April and early May, all these results were considered valid and taken into consideration. All sets received after May 1st were neglected. Participants outside Europe were informed to be aware of possible problems with custom regulations. In one occasion the package with samples was kept by customs. Finally a second package arrived safely at the participant's laboratory.

Since the new Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 is fully operational, the reporting form got a fully new design. Number of particles for land animal and for fish, and a decision on absence or presence could be entered for every sample. The choice "suspect" was added for expressing a number below the threshold. In addition a third category was included in the framework of this particular ring test for those types of particles that cannot be assigned to any of the two categories (muscle fibres, cartilage). This third category was also necessary to get a report on the presence of insect meal. The report form was interactive. Only the results for one determination could be entered. 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 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] OR [#other 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, but it is not stated if this second determination should include all contaminants (land animal AND fish) or should only be subjected to the contaminant found (land animal OR fish). Therefore, in this ring study it was chosen to do a FULL second (or third) determination if only ONE category got a result between 1 and 5.
Further instructions to the participants were enclosed in the box with samples, which are reproduced in Annex 5.

Participants were invited to perform analysis by other methods such as PCR and immunoassay. Since the sets for microscopy were fixed at 30 grams for having a precise amount for the microscopic analysis, additional sets of 15 grams of the same sample material were planned to be prepared. RIKILT fixed a date and time for submission in order to be able to prepare these extra samples. 18

participants applied for additional PCR analysis, and two participants applied for immunoassay analysis. One participant managed to use the primary sample set for PCR as well. Sixteen participants returned their results for PCR and two participants reported for immunoassays.
The draft report was finalised at May 16th 2014. In order to achieve a more detailed analysis of sample D and to present a clear difference between the proper proficiency test (samples A to C) and the sample for method performance testing, a new version of the report was published in October 2015. This report replaces the former version. The results as presented in the Appendices and the evaluation of samples A to C remained unchanged. A reference to the original version (2014.011) will be regarded to be a reference to the current (final) version of the report (2014-011*).

2.5 Participants

The 52 participants, which successfully submitted their microscopic results, originated from 19 countries: 15 member states of the European Union, and four other countries (China, Norway, Peru and Switzerland). The list of participants is presented in Annex 6. Five member states have been involved with three or more participating laboratories: Germany (17 labs), Italy (5), Belgium (5), the Netherlands (3) and France (3). These figures are slightly lower compared to those of the ring test of last year (van Raamsdonk *et al.*, 2013a).

2.6 Analysis of results

2.6.1 Statistical analysis of proficiency test results (samples A, B and C)

The results are analysed in two ways: numbers below the 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, and it allows a way to compare the present results with those of previous years. Several assumptions restrict this strategy: (a) the lack of lab contamination, (b) the approved skill to identify all particles correctly, and (c) a sufficient reproducibility of the results. Assumption (a) is an inherent part of a proficiency test as currently reported, and assumption (b) and (c) will be analysed further using sample D. In any way an integral comparison of both interpretations will be given.

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.

2.6.2 Statistical analysis of method performance test results (sample D)

The applied approach of analysis and the detailed results for sample D are presented in Annex 10.

3 Results

Fifty-six packages with four samples were sent to all participants, of which 54 were dedicated for microscopic analysis. A total of 52 participants returned results for the microscopic method, 16 sets of results were received for PCR analysis, and two sets were submitted for immunoassay analysis. 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. One participant switched the results for two samples, and two participants submitted very incomplete results. In all three cases this could be corrected within the reporting period; otherwise these reports would have been omitted. In all those cases that a participant send in several versions of the report sheet the most recent version was used. All reports were included.
The full results are presented in the tables of Annex VI, VII and VIII.

3.1 Application of the method

The procedure to follow according to Regulation (EC) 152/2009 as amended by Regulation (EC) 51/2013 (to be referred to as "new method") is more complicated than the previous one. Therefore, very detailed instructions had been included in the documentation for the participants. Furthermore, the report sheet was designed to be interactive: the sheet provided guidance for the number of determination to be carried out based on the already entered results. Nevertheless, approximately one-third of the participant applied the wrong number of determinations, as shown in Table 3. A share of 35.9% (14 out of 39) of the non-NRL participants choose perform another number of repetitions than requested by the Regulation, notwithstanding the situation that the reporting form guided the participants through the procedure. This choice can be due to a legitimate choice of non-NRLs to apply their own method. Nevertheless, any indication that a participant deliberately deviated from the official method was not given in any report form.

Table 3
Number of participants and percentage applying the wrong number of determinations. The percentage is calculated based on the total number of labs per category (NRL, other).

Incorrect number of determinations	Total number of labs	NRL network	Other
Total	16 (30.7%)	2 (15.4%)	14 (35.9%)
Insufficient number	3 (5.8%)	0 (0.0%)	3 (7.7%)
Too many	13 (25.0%)	2 (15.4%)	11 (28.2%)
Number of labs	**52**	**13**	**39**

3.2 Proficiency test results

Most of the specificity and sensitivity scores for MBM or fish were at good levels (Table 4; Annex 8). Results for the situation that numbers below the threshold were considered positive are at top row; the figures indicating numbers below the threshold as negative at the second row in italics. In several samples some specific situations occur. For sample 2014-C (insect meal) five participants reported land animal (0.90), three of them higher than the threshold (0.94 with <threshold=negative). Six participants reported fish material for sample 2014-D (1 bone fragment per gram sample; 0.88), one of them with a higher number than the threshold (0.98).
The third category (muscle fibres or other animal material) showed interesting results (Table 5). Only 10 participants (19.2%) reported correctly the presence of insects, parts thereof (chitin), or a type of

particles which is highly comparable (krill) in sample 2014-C. Sixteen participants (30.7%) reported muscle material. In the presence of fish or land animal these muscle fibres would have been assigned to any of these two "official" categories. Besides these results for sample 2014-C, the results should be evaluated in the view of the results as shown in Table 4. Especially for sample 2014-B (land animal and fish) the number of muscle fibres was reported separately. For sample 2014-A and sample 2014-D muscles should be absent.

Table 4

*Sensitivity and specificity scores for the detection of animal proteins in the **sediments** of three 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 C: sample indication.*

		MBM			Fish		
		A	B	C	A	B	C
n		0%	0.1%	0%	0%	2%	0%
52	specificity	0.96		0.90	0.96		0.92
		1.0		*0.94*	*0.98*		*0.96*
	sensitivity		0.94			1.0	
			0.94			*1.0*	

Table 5

*Sensitivity and specificity scores for the detection of animal proteins in the **flotate or raw material** of three 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 C: sample indication. *: the presence of muscle fibres in sample B is related to the content of fish and terrestrial animals as indicated in Table 4; for sample C exclusively the concentration of insects is given.*

		Muscle fibres or insects *		
		A	B	C
n		0%		1.0%
52	specificity	0.92		
		1.00		
	sensitivity		0.67	0.69
			0.60	*0.63*

A total of ten participants out of 52 (19.2%) correctly found material of arthropods in sample 2014-C. Although five participants specified this as insect material, furthermore indications of krill, crustaceans or chitin are accepted as positive since discrimination between these categories is assumed to be virtually impossible. In the current situation any indication of muscle material would point to muscle fibres of the insect material included, since other animal material was absent from the sample. Other positive results include undefined reports, by-catch (undefined) and hair/skin. Thirteen participants did not find any animal material in this sample. The compiled results are presented in Table 6.

Table 6

The different types of particles found in sample 2014-C (contamination with insect meal), with the number of participants and the way these results are counted.

Type of particles found in sample 2014-C (insect meal)	Number of reporting participants	Assigned as
Insect, krill, Crustacea or chitin	10	Positive
Muscle fibres	16	
Other positive	10	
Land animal, including TCP	2	Negative
Fish	1	
None (negative)	13	

The difference between the two ways of evaluating the results, i.e. below the threshold considered positive or below the threshold considered negative, has two distinct effects. Considering the results below the threshold as negative will always result in a higher specificity and in a lower sensitivity. The minor error of identifying one or a few particles in a wrong way is nicely corrected when the numbers below the threshold are considered negative (Table 2: MBM in the blank 2014-A, fish in 2014-D).

3.3 Microscopic procedure

An inventory of ten different parameters was added to the report sheet of the actual results of the four samples. These results are shown in Annex 7 and summarised in Table 7. 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.

Table 7

*Inventory of parameters for microscopic detection and their application. Pink cells indicate deviations from the new 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	35	
	no; too many determinations	13	
	no; insufficient determinations	4	
Extra milling step (<1.0 mm)	no	47	
	yes	5	
amount of material used for sedimentation of feed	10 grams	51	
	30 grams	1	
type of glassware	chemical sedimentation funnel	30	
	conical glass with cock	8	
	champagne glass *	9	
	beaker (flat bottom)	3	
	other	2	
sedimentation agent	TCE	51	
	TCE/Petroleumether	1	
use of staining of sediment	no	29	
	yes	23	
use of binocular for examination at lower magnifications	yes	37	
	no	15	
size of cover glass used	small (e.g. 20 x 20 mm)	42	
	medium	4	
	large (e.g. 26 x 50 mm)	6	
share of the total sediment used for examination	minimum		8%
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	25	
	paraffin oil	12	
	immersion oil	7	
	Norland Adhesive	5	
	other (water, glycerol:water mixture, mineral oil)	0	
Use of ARIES	yes	4	
	no	47	

The results as presented in Table 9 show generally 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).

3.4 Detection by PCR of proficiency samples

Participations were invited to perform DNA analysis and to submit their results, separated for every single target. Sixteen participants submitted results covering a total of seven different targets. These targets are classified at three hierarchical levels: class (fish, insect), order (ruminants), genus/species (bovine, sheep, pig, chicken). The results are presented in Table 8 and in Annex 9.

Table 8a

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

n		Ruminant A 0%	B 0.1%	C 0%
17	specificity	1.0		0.06
	sensitivity		0.12	
	Homogeneity study	neg	neg	pos

Table 8b

Results for DNA analyses (PCR) for three samples. Target: cattle.

n		Cattle A 0%	B 0.1%	C 0%
4	specificity	1.0		0.75
	sensitivity		0.0	

Table 8c

Results for DNA analyses (PCR) for three samples. Target: pig.

n		Pig A 0%	B 0 %	C 0%
6	specificity	1.0	0.17	1.0
	sensitivity			

Table 8d

Results for DNA analyses (PCR) for three samples. Target: insect.

n		Insect A 0%	B 0 %	C 1%
1	specificity	pos	neg	
	sensitivity			pos

The ruminant PCR results can be seen in Table 8a. All participants reported the blank sample 2014-A as not detected. Most participants (88%) did not detect the 0.1% ruminant MBM in sample 2014-B. Sample 2014-C, chicken feed adulterated with 1% of insect meal, gave predominantly positive results (94% detected).

One out of four participants detected cattle DNA in sample 2014-C (Table 8b), but none of the four participants were able to detect the 0,1% ruminant MBM in sample 2014-B with the cattle PCR. Moreover, four out of five participants detected pig DNA in sample 2014-B (Table 8c).

The one report for insect DNA showed a positive result for the blank sample 2014-A and for the 1% insect sample 2014-C.(Table 8d). In addition to the results shown in Table 8a, 8b, 8c and 8d three participants reported negative results for chicken DNA for all three samples, two participants reported negative results for sheep DNA in all three samples and one participant detected fish DNA in sample 2014-B and not in 2014-A and 2014-C (see Annex 9).

3.5 Performance of the microscopic method at low levels of contamination

Sample 2014-D (1 bone fragment per 1 gram of sample) was analysed in more detail in the homogeneity study. Five samples have been analysed in three determinations of 10 gram each, meaning that the entire amount of 30 grams per sample had been used. In all cases the entire sediment (100%) has been examined in order to be able to recover all the included fragments. The results are presented in Table 9. The recovered number of bone fragments ranged from 24 to 29 particles, which would result in an R-score between 0.80 and 0.97. For four out of five samples one determination would have been sufficient to reach a positive conclusion. In one sample a second determination was necessary. Nevertheless, for the framework of this ring study the full material was analysed.

Table 9

Results of the homogeneity study for the particles count in sample 2014-D.

	Determination			total
Sample 2014-D	first	second	third	
1	8	10	7	25
2	10	13	6	29
3	8	9	7	24
4	10	8	7	25
5	5	12	11	28

The results of the participants have been analysed based on the correct number of determinations in order to get comparable results according to a correct application of the new method. One participant used 30 grams for one determination and several participants did not indicate the starting and finishing amount of sediment in a correct way. These results were neglected as well, leaving 44 sets of results.
The presence of only 1 bone fragment per gram sample material (MBM in sample 2014-D) results in low numbers of reported particles. At these low levels, resulting in a relatively low sensitivity (0.92), considering numbers below the threshold as negative results in a very low performance (0.44).
The R-scores of the 44 results are shown in Figure 2.

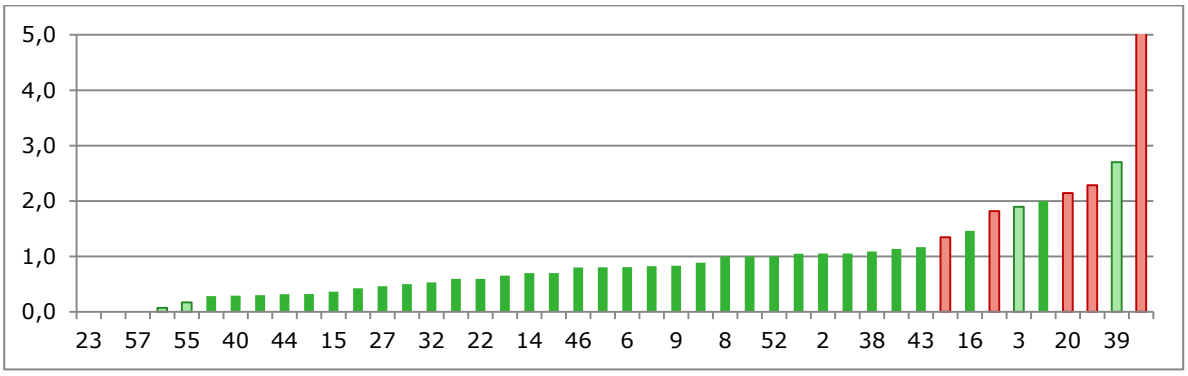

Figure 2 *R-scores for number of particles found in sample 2014-D. Optimal value is 1. Red bars: pre-treated material (ground). Light green bars: outliers. Green bars: results used for evaluation.*

Two deviations seemed to occur:

- Very high R-scores, up to 14.5. Ten participants were inquired to indicate a possible pre-treatment of the sample. Five participants declared to have applied grinding at 0.5 mm prior to analysis in

order to enhance homogeneity (participants 5, 10, 20, 41 and 49). This pre-treatment obviously resulted in a higher number of bone fragments. These results have been ignored for further analysis.
- Iterative outlier detection based on minimization of the standardized residuals (see Annex 10) revealed another five results as outliers (participants 3, 21, 39, 55 and 57). These results have been ignored for further analysis as well.

These deviations are indicated in Figure 2.
The number of bone fragments related to the amount of sediment used is plotted in Figure 3. The dotted line in Figure 3 indicates all hypothetical results with an R-score of 1. Figure 3 shows a relations between the amount of sediment used and the number of particles found (straight line). The correlation was R = 0.741.

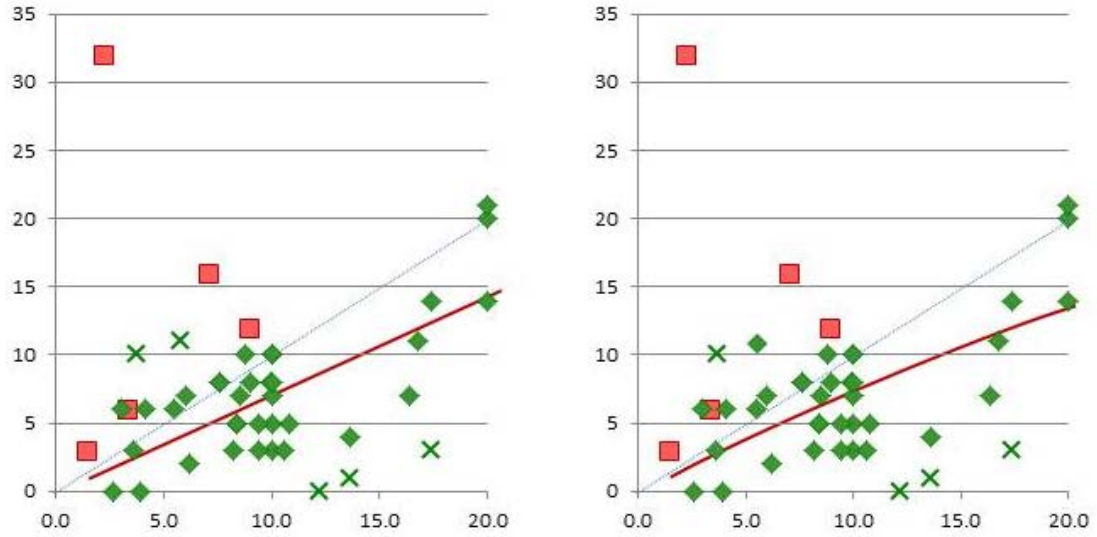

Figure 3 *Plot of number of bone fragments found (y-axis) related to the represented amount of sample material (x-axis). Dotted line: hypothetical R-scores equalling 1. Straight line left figure: linear model fitted according to quasi-Poisson model, Straight line right figure: non-linear model fitted according to quasi-Poisson model. Red squares: pre-treated material (ground). Green crosses: outliers. Green diamonds: results used for evaluation.*

The null hypothesis to be tested is the significance of the relationship between the amount of material examined and the number of recovered bone fragments (Annex 10). Fitting the quasi-Poisson model (model 1) to the remaining data gives an estimate of RF equal to 0.74. The fit (Figure 3 left) shows a significant amount of over-dispersion (k=1.59, deviance 52.44 with 33 df, $p<0.02$) although outliers have been removed. In this analysis there is a strongly significant relationship between the amount of material examined and the observed number of fragments.
When fitting the quasi-Poisson model with allowing non-proportionality (model 2) four outliers have been identified (21, 39, 55 and 57). After excluding these outliers a β value of 0.90 with standard error 0.18 was estimated. A value of $\beta = 0.90$ indicates a slightly non-linear relationship (Figure 3 right). This is strongly significantly different ($p<0.001$) from the alternative hypothesis where no relation is assumed between the amount of material analysed and the number of fragments recovered ($\beta = 0$)).
The robustness of this approach and an alternative strategy as an approval is presented in Annex 10.
The detection of ruminant DNA in sample 2014-D resulted in a sensitivity of 0.82 (see raw data in Annex 9). Sample D was not tested by PCR in the Homogeneity study.

4 Discussion

4.1 Method application

The new microscopic method as included in the current version of Regulation (EC) 152/2009 requires to perform a second determination when the encountered number of fragments is between 1 and 5 inclusive. The text does not discriminate between land animals and fish for this repetition. The second determination can be caused by one of these categories and then it can include a search for both categories. The strategy followed in this ring test was to request results for both categories in all cases that a second determination was necessary. In addition, a third category "undefined" was included for two reasons: 1) muscle fibres cannot be identified and assigned to any of the two categories in the absence of any bone material, hairs, feather etc., and 2) participants should be able to report the presence of insects separately. For the second reason this category was not named "insects" or "arthropods" for avoiding any indication of the material that could be expected. The participants were clearly instructed how to report the different types of particles among the categories (see Annex 2). In this framework several situations can exist for deciding on a second determination. Some of these situations are illustrated in Figure 4. A second determination was applied to hypothetical sample 37 because the value for land animals was higher than zero but below the threshold. The value for land animals in sample 168 was higher than the threshold but the result for the category undefined was below the threshold, making a second determination necessary, at least in the framework of this ring test. The number of particles for land animals in sample 371 was higher than the threshold and it was not accompanied with any other result higher than zero and below the threshold, which makes it sufficient to take the final decision after one determination.
The number of determinations to be applied is apparently difficult to find out (Table 3). Any further documentation (format for reporting, interactive report form, flow chart of the method) would be helpful.

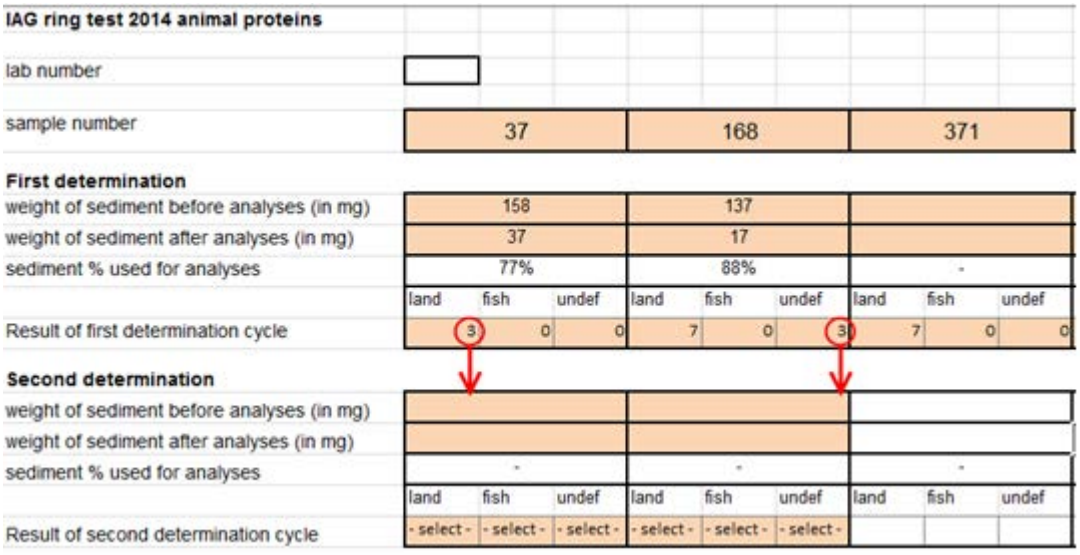

Figure 4 *Part of the report sheet for microscopy with three hypothetical samples. Red arrows indicate the reason to apply a second determination.*

The strategy as followed in this ring test for reporting could be modified in the next version of the IAG ring test for animal proteins. An assignment model for handling specific particles impossible to identify (muscle fibres, cartilage) can be established. Since insects and other arthropods are assumed to be increasingly important as alternative source of proteins (Van Huis *et al.*, 2013), and since the use of

insects is currently considered as prohibited in animal feed (Spiegel *et al.*, 2013), a third category of animals seems unavoidable to be included in the Regulation.

4.2 Method proficiency

The results as obtained in this most recent version of the annual IAG ring tests for microscopic detection of animal proteins in feed is comparable to the historic record of previous years (Table 10). In several occasions the accuracy was comparable to the level that is usually considered as limit (0.95). The sensitivity of the detection at the level of 0.1% MBM or below could be considered as good to very good (Regulation (EC) 152/2009: detection limit at or below 0.1% w/w), also in the view of earlier results (Table 10). In this year, as in previous years, the low number of wrong reports for any animal material in blank samples is sufficient, but almost always just below 1.0.

Table 10

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 (2008-2014) 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)	current study	0.96		0.94				0.96		

*: TCP used as contaminant for land animal material

The identification of insect material appeared to be difficult (sensitivity: 0.19). Several other types of animal ingredients also show less optimal or worrying levels of sensitivity. In feed with feather meal most participants reported bone fragments (0.98), but a minority reported the feather meal itself (0.33; van Raamsdonk *et al.*, 2011). The same problem was found in the 2013 proficiency test of the EURL (Veys *et al.*, 2014). A majority of participants found erroneously land animal material in a feed sample exclusively spiked with a fish meal fortified with salmon meal (0.70; van Raamsdonk *et al.*, 2012a). Improvement of the performance of the microscopic method with special attention to ingredients such as feather meal, salmon meal and insect meal could be achieved by investing in the skills for identification of several types of particles.

4.3 Method performance

The results for sample 2014-D are not included in Table 10 because of the special way of adulteration. The number of particles found in sample 2014-D (1 bone fragment per 1 gram of sample material) shows interesting results (Annex 10). There are several aspects which can have their effect on the expected number of bone particles to be detected:

- The samples in this ring test were ground in a Retsch mill type ZM with a mesh size of 2 mm and thoroughly mixed before adulteration. Five participants applied a pre-treatment procedure for homogenization of the sample material. These pre-treatments usually involve procedures such as crushing or grinding using a mortar or a mill. The obvious side effect is that the size of the particles is decreased and, hence, the number of particles is increased. This side effect is illustrated in Figures 2 and 3. Earlier reports of the relationship between grinding and the number of particles is given by van Raamsdonk *et al.* (2005: Figure 2; 2012b: Figure 5.3) and by the Irish NRL in its presentation at the IAG annual meeting 2013 in Vienna, which indicate the same effect.
- The amount of sediment used for performing the analyses is a major factor influencing the number of encountered bone fragments. A Poisson model (model 1) indicating a linear relationship between the amount of sediment used and the reported number of particles, is fully significant to describe the results, and the robustness of the current statistical approach is demonstrated (Annex 10). Considering bone fragment numbers below the threshold as negative, the difference between using less than 50% of the sediment versus 50% or more is significant ($p=0.031$). With a limit of 60% this is even more significant ($p=0.003$). A fixed number of slides (3 for the fine sediment fraction) as stated in the official method might well be accompanied by setting a minimum amount of sediment per slide.
- The particles added to the sample 2014-D were relatively large. The examination of the coarse fraction of the sediment is requested in Regulation (EC) 152/2009, although the use of a binocular for examination is neither recommended nor prohibited. The use of a binocular facilitates a rather quick and efficient examination of the full amount of sediment, both the coarse and the fine fractions.
- The principle choice whether to consider values below the threshold (between 1 and 5 particles inclusive) as positive or as negative has a large effect on the sensitivity at low contamination levels. Considering numbers below the threshold as negative, using either less than 50% or 60% of the amount of sediment has a significant effect on the sensitivity (See Annex 10). The results obtained in the 2013 version of this ring test for sample 2013-D (consisting of approximately 0.01% recognisable bone fragments in TCP as contaminant) showed the same effect. The difference between using less than 50% of the sediment (sensitivity: 0.81) and using 50% or more of the sediment (sensitivity: 1.0) was near to being significant ($p=0.053$; van Raamsdonk *et al.*, 2013).
- Last but not least the skill to recognise the bone fragments as such has its effect on the number of bone fragments found. With a level of 1 bone fragment per gram sample material, the average R-score of recovered fragments is 0.743, with a median of 0.8. Four out of 52 participants were not able to find any bone fragments at all (sensitivity: 0.92 when taking values below the threshold as positive).

The results obtained after analysis of sample D indicate an effect of grinding circumstances and of the amount of material used for evaluation. A robustness analysis (Annex 10) showed that including possibly misinterpreted particles would hardly influence the performance of the method. At the low level of contamination of one particle per gram feed (estimated as 0.005%) the reproducibility seems to be sufficient: 48 out of 52 participants appeared to be able to recover at least one bone fragment, provided that results below the threshold were considered positive.

4.4 Method parameters

The way the microscopic method is implemented and the development of several parameters is shown in Table 11. Notable developments are the number of participants using less than 10 grams of material for sedimentation (2014: zero), increase in the application of Alizarin staining (2008: 31%, 2014: 44%), use of the binocular (2008: 64%, 2014: 71%), and use of glycerol (2008: 18%, 2014: 50%). It might be useful to further analyse the effect of using an embedding agent with a much higher viscosity than paraffin oil and using Alizarin staining instead of the plain sediment material on the sensitivity and specificity of the microscopic method.

Table 11

Comparison between parameters distribution in the IAG ring studies between 2008 and 2014.

parameter	parameter choice	2008	2009 -2013	2014
amount of material used for sedimentation	5 grams	16	5-2	0
	10 grams	26	41-50	51
	other	3	3-1	1
type of glassware	chemical sedimentation funnel	22	28-33	30
	beaker (flat bottom)	11	13-5	3
	champagne glass	6	5-8	9
	conical glass with cock	3	1-9	8
	other	3	2-4	2
use of staining of sediment	no	31	31-36	29
	yes	14	14-22	23
use of binocular for examination at lower magnifications	yes	29	40-45	37
	no	16	9-12	15
size of cover glass used	small (e.g. 20 x 20 mm)	34	27-38	42
	medium	1	5-10	4
	large (e.g. 26 x 50 mm)	9	16-9	6
share of the total sediment used for examination	minimum	4%	0.2%-2%	8%
	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-19	25
	immersion oil	8	8-14	7
	paraffin oil	18	12-23	12
	Norland Adhesive	0	2-7	5
	chloral hydrate	3	1-0	0
	other (e.g. Depar 3000, water)	8	5-1	0

4.5 PCR

The results as obtained for PCR are difficult to interpret. The sensitivity for ruminant in sample 2014-B (0.1% MBM) was as low as 0.12 whereas the same MBM in sample 2014-D resulted in a sensitivity of 0.82, although this artificial cattle feed contained only one ruminant bone fragment per gram matrix (estimated at 0.005% MBM). The same MBM at a level of 0.05% was correctly detected in the ring test of 2013 (van Raamsdonk *et al.*, 2013). Since most participants reported a positive result for pig in sample 2014-B, the actual contamination level could have been below 0.1%. The negative findings for ruminant in sample 2014-B as reported by the participants is in concordance with the results of the homogeneity study.

Although the positive signal for ruminant in sample 2014-C (1% of insect meal) of the homogeneity study (Table 2) is at the cut-off level, the participants' results are in concordance with this result of the homogeneity study (Table 8a: specificity 0.06). The results would suggest a source of ruminant DNA in the STRATFEED chicken feed which is not visible by microscopic research (Table 4: specificity 0.90). This feed was produced in 2003 in the framework of the project STRATFEED. Although a dedicated pilot plant was used, emphasis was on preventing carry-over which could hamper visual and

near infrared detection instead of focusing on possible DNA carry-over. The positive findings of 94% of the participants can be indicated as "*agrees with consensus*" as is applied in the FAPAS proficiency tests (www.fapas.com).

5 Conclusions and recommendations

5.1 Conclusions

The new method as published in Regulation (EC) 152/2009, amended by Regulation (EC) 51/2013, intends to harmonise the application of the microscopic method and tries to avoid the problem of repeatability at low contamination levels. Despite its detailed character, the combination of counting particles related to the application of a threshold, and the absence of a specified grinding protocol and of setting a minimum requirement for the amount of sediment to be used, would hamper the initial goals. The chance to get a result below the threshold (interpreted as negative) will increase when less sediment material is applied. The detailed character appears to be difficult for a straight implementation. Although launched only one year ago, and with an expectation of getting more familiar through time, some more documentation might be welcome.
Besides the choice whether counts below the threshold are considered positive or negative, several assumptions should be met when interpreting results of the microscopic method. Lab contamination needs to be avoided in all cases. Results of proficiency tests as presented in this report could assist in detecting possible lab contamination. Specificity will be supported by training for proper identification. This is profitable in the view of having an average R-score for recognising bone fragments of 0.743 for sample 2014-D, where the homogeneity study provided R-scores between 0.80 and 0.97. Training will also support to enhance the specificity in blank samples. The availability of expert systems could be helpful.
The PCR results were at some points inconclusive. The issues found could be attributed to incomplete characterization of the materials used upon production in the past. The nature and exact composition of the materials as used in this study need to be a primary target for further evaluation.

5.2 Recommendations

- Provision of more documentation on implementation of the new microscopic method as published in Regulation (EC) 152/2009, such as a format for reporting, interactive report form, or a flow chart of the method.
- Further harmonisation of the new microscopic method with respect to grinding of the sample material, minimum amount or percentage of sediment to be used within reasonable limits, use of a binocular and an assignment model for particles that cannot be identified by itself (muscle fibres, cartilage).
- Improvement of skills by means of training and by using expert systems.
- Further evaluation of standard materials for future use.

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Annex 1 Invitation letter

Dear colleague, Dear IAG member,

The IAG section Feeding stuff Microscopy organizes annually several ring tests for the evaluation of composition or detection of prohibited constituents in animal feed. The presidium of the IAG section Feeding stuff Microscopy and RIKILT have agreed to organize together the 2014 ring test for the following situations:

- Test IAG-2014-A. Detection of the presence of animal proteins in a set of four samples. This test was organised by RIKILT in previous years. Targeted protocol: Regulation (EC) 152/2009, consolidated version of February 12, 2013.
- Test IAG-2014-B. Declaration of the composition of a compound feed (one sample). This test was organised in previous years by a colleague institute. RIKILT will take over the organisation for the year 2014. Targeted protocol: IAG method A2.
- Test IAG-2014-C. Detection of undesired botanical substances in two samples of bird feed. Seeds of *Ambrosia* will be part of the test, combined with one other botanical substance as listed in Directive 2002/32/EC. Targeted protocol: IAG method A5.

The costs for participating in the animal protein test will be €220, and for the undesired botanical substances test will be €100. The composition test is free of charge. The single sample for the composition test will be part of the animal protein test. RIKILT will encourage you to subscribe to both these tests (A and B), although this is not mandatory. On behalf of the IAG section Feeding stuff Microscopy, RIKILT will invite you for participation in these ring tests.

The samples for test IAG-2014-A and IAG-2014-B will be sent around late February or early March 2014. Also a questionnaire will be sent by E-mail, together with instructions and relevant documentation on protocols. A time slot of four weeks is planned for the analyses of the samples by every participant. This means that late March or early April all results are expected to be returned to RIKILT. The samples of test IAG-2014-C will be sent mid-March and results needs to be reported mid-April. All results are intended to be reported at the annual meeting of the IAG working group Microscopy in Posieux (Switzerland) in June 2014. The final reports will be published later in 2014. All communications of the evaluation will be fully anonymous.

If you are interested to participate in one or more ring tests, please return the application form and make a payment of the appropriate amount to RIKILT. You will receive an invoice after receipt of your application form. Make sure that the reference number, your name and your institute's name is mentioned. This information is necessary to avoid loss of payments that cannot be linked to participating institutes.

We are looking forward to have a nice cooperation for the next ring tests and to have results which will support your laboratory quality system.
On behalf of the IAG section Microscopy and the RIKILT organizing team,

L. van Raamsdonk

Annex 2 Basic instructions for the test procedure

IAG ring test 2014 animal proteins

Instructions for the IAG ring test

1 You have received a box with an introduction letter and four vials containing 30 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 can be found on the EUR-LEX website. Take care to homogenise the content of each vial before taking the amount for analysis.
The samples are prepared in such a way that you can start with the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse three determinations of 10 grams as indicated in paragraph 2.1.4.3. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle and a final conclusion.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, horn, skin, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). Since not all particles found can be assigned to one of these categories, a third category is included for muscle fibres, cartilage and possibly other animal fragments.
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 '**suspect**' has to be chosen.

Click here for the Regulation

3 Reporting consists of the following steps:

3a	Please fill in the questionnaire on the page "Procedure". Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list. Your unique lab number is mentioned in the introduction letter, enclosed in the box. All the fields with a drop-down list have to be completed.
3b	Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after your have entered it at the page Procedure. Enter yourself the four unique labels of the vials. 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 (preferably *.PDF) to leo.vanraamsdonk@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 April 1st, 2014.
	RIKILT Institute of food safety, Wageningen, the Netherlands

Annex 3 Report form for procedure details

Please complete at least all the cells with a drop down list	select your choice from a drop down list	type in your answer if necessary
IAG ring test 2014 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the ring test instructions?	-- select --	
Detection method:	Microscopy	
Please indicate your starting amount of material for sedimentation of FEED material	-- select --	
if other, please specify		
Indicate your glassware for sedimentation	-- select --	
if other, please specify		
Describe your sedimentation agent	-- select --	
if other, please specify		
Did you apply staining of the sediment (e.g. alizarin staining) as standard procedure?	-- select --	
Did you examine at lower magnifications (using a binocular)?	-- select --	
Indicate the size of cover glass	-- select --	
Please describe your embedding agent for the sediment material	-- select --	
if other, please specify		
Did you use the expert system ARIES for identification of particles?	-- select --	

Annex 4 Report form

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 2014 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	undef	land	fish	undef
Result of first determination cycle	- select -	- select -	- select -	- select -	- select -	- select -
Second determination						
	-			-		
	land	fish	undef	land	fish	undef
Third determination						
	-			-		
	land	fish	undef	land	fish	undef

Total number of particles per category	0	0	0	0	0	0
Final conclusion	- select -	- select -	- select -	- select -	- select -	- select -
Type of particles						

Annex 5 Additional instructions

Relevant part of the letter included in the box with the samples

In the case that you will apply the microscopic method as well any other method (PCR and/or immunoassay), the JARS are meant for microscopic analysis. These jars contain 30 gram of feed, which is just sufficient for carrying out three cycles of the microscopic method according to Annex VI of Regulation (EC) 152/2009 from the European Union, modified by (EC) 51/2013, part microscopy. The consolidated version can be found on the EUR-LEX website. 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.

Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). Since not all particles found can be assigned to one of these categories, a third category is included for muscle fibres, cartilage and possibly other animal fragments. 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 sample is negative (zero particles on average), "suspect" (between 1 and 5 particles on average) or positive (6 or more particles on average) for each of the three categories.

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 "microscopy" in the name.

Annex 6 List of participants

Institute	Country
Austrian Agency for Health and Food Safety-AGES	Austria
LFSAL	Belgium
Laboratorium ECCA nv	Belgium
Oleotest N.V.	Belgium
CRA-W	Belgium
FLVVT	Belgium
China Agricultural University	China
Chinese Academy of Inspection and Quarantine	China
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
IPL Atlantique	France
S.C.L. Laboratoire de Rennes	France
IDAC	France
CVUA-RRW	Germany
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	Germany
LTZ Augustenberg	Germany
SGS Germany GmbH	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
Agri Q-service GmbH	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Landeslabor Berlin-Brandenburg	Germany
LUFA-Speyer	Germany
LUFA Nord-West	Germany
WESSLING GmbH	Germany
Thüringer Landesanstalt für Landwirtschaft	Germany
Inst. Fur Veterinar-Pharmakologie und Toxicologie	Germany
Universität Hohenheim, LA Chemie (710)	Germany
Landesuntersuchungsamt für Chemie, Hygiene und Veterinärmedizin	Germany
LUFA Rostock	Germany
MGSZH ÉTBI TAKARMÁNYVIZSGÁLĆ NEMZETI LABORATÓRIUM	Hungary
Equine Centre	Ireland
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Ireland
Inst. Zooprofilattico Sperimentale delle Venezie	Italy
Inst. Zooprofilattico Sperimentale della Sardegna	Italy
Istituto Zooprofilattico Sperimentale Abruzzo & Molise "G. Caporale"	Italy
IZS PLV Torino - CReAA	Italy
Istituto Zooprofilattico della Sicilia	Italy
MasterlabBV	Netherlands
CCL - Nutricontrol	Netherlands
Nutrilab	Netherlands
Eurofins Food Testing	Netherlands
TLR	Netherlands
Nofima Ingredients	Norway
SGS del Perú S.A.C.	Peru
Laboratorio de Microbiología	Peru

Institute	Country
Inspectorate Services Perú S.A.C.	Peru
Cargill Poland	Poland
Lab. Regional de Veterinária	Portugal
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	Slovenia
Laboratorio de Diagnóstico General, S.L.	Spain
Trouw nutrition Espana	Spain
SVA	Sweden
Agroscope (ALP), Swiss Research Station	Switzerland
ELISA Technologies	Unites States

Annex 7 Details of procedures applied, microscopic method

lab nr	amount							
	feed	glassware	agent	staining	binocular	size	embedding	ARIES
1	10	chem.sed.funnel	TCE	no	yes	small	glycerin	yes
2	10	conical glass with cock	TCE	no	no	medium	mineral oil	no
3	10	chem.sed.funnel	TCE	no	yes	small	paraffin oil	no
4	10	chem.sed.funnel	TCE	no	yes	small	paraffin oil	no
5	10	chem.sed.funnel	TCE	no	yes	small	immersion oil	no
6	10	chem.sed.funnel	TCE	yes	no	small	glycerin	yes
7	10	chem.sed.funnel	TCE	no	yes	small	glycerin	no
8	10	chem.sed.funnel	TCE	yes	yes	small	Norland	no
9	10	conical champagne glass	TCE	no	yes	small	glycerin	no
10	10	conical glass with cock	TCE	no	yes	small	glycerin	no
11	10	conical champagne glass	TCE	no	yes	small	immersion oil	no
12	10	chem.sed.funnel	TCE	no	yes	large	paraffin oil	no
13	10	chem.sed.funnel	TCE	no	no	small	vaseline oil	no
14	10	chem.sed.funnel	TCE	yes	no	small	Norland	no
15	10	chem.sed.funnel	TCE	yes	yes	small	glycerin	no
16	10	chem.sed.funnel	TCE	no	yes	small	paraffin oil	no
17	10	conical champagne glass	TCE	no	yes	medium	glycerin	no
18	10	beaker (flat bottom)	TCE	no	yes	small	immersion oil	no
19	10	chem.sed.funnel	TCE	no	yes	small	iso-paraffin	no
20	10	conical champagne glass	TCE	yes	no	large	glycerin	no
21	10	chem.sed.funnel	TCE	yes	yes	small	paraffin oil	no
22	10	conical glass with cock	TCE	no	yes	small	glycerin	no
23	10	chem.sed.funnel	TCE	yes	yes	small	glycerin	no
25	10	chem.sed.funnel	TCE	no	yes	small	paraffin oil	no
26	10	mensur	TCE	no	no	large	paraffin oil	no
27	10	chem.sed.funnel	TCE	yes	yes	medium	immersion oil	no
29	10	conical champagne glass	TCE	yes	yes	small	glycerin	no

lab nr	amount							
30	10	beaker (flat bottom)	TCE	no	no	small	glycerin	no
31	10	conical champagne glass	TCE	no	no	small	glycerin	no
32	10	conical glass with cock	TCE	yes	yes	small	paraffin oil	yes
33	10	chem.sed.funnel	TCE	no	yes	small	glycerin	no
35	10	chem.sed.funnel	TCE	yes	no	small	Norland	no
36	10	chem.sed.funnel	TCE	yes	yes	small	glycerin	no
37	10	chem.sed.funnel	TCE	yes	no	small	glycerin	no
38	10	chem.sed.funnel	TCE	yes	no	small	immersion oil	no
39	10	chem.sed.funnel	TCE	yes	yes	medium	glycerin	no
40	10	chem.sed.funnel	TCE	no	no	small	glycerin	no
41	10	chem.sed.funnel	TCE	yes	yes	small	Norland	no
42	10	conical champagne glass	TCE	no	yes	small	glycerin	yes
43	10	other	TCE	no	yes	small	paraffin oil	no
44	10	chem.sed.funnel	TCE	yes	yes	small	glycerin	no
45	10	conical champagne glass	TCE	no	no	small	glycerin	no
46	10	chem.sed.funnel	TCE	yes	no	small	glycerin	no
47	30	conical glass with cock	TCE	no	yes	small	paraffin oil	no
48	10	conical glass with cock	TCE	yes	yes	large	paraffin oil	no
49	10	beaker (flat bottom)	TCE	yes	yes	small	immersion oil	no
52	10	conical glass with cock	TCE	yes	yes	small	Norland	no
53	10	chem.sed.funnel	TCE	yes	yes	small	glycerin	no
54	10	chem.sed.funnel	TCE	no	yes	small	immersion oil	no
55	10	chem.sed.funnel	TCE	no	yes	large	glycerin	no
56	10	conical glass with cock	TCE	yes	no	large	paraffin oil	no
57	10	conical champagne glass	TCE/PE	no	yes	small	glycerin	no

Annex 8 Results: presence of animal proteins, microscopic detection

lab nr	sample number				land				fish				other			
					A	B	C	D	A	B	C	D	A	B	C	D
1	171	287	33	269	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
2	191	12	188	154	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	present	absent
3	6	272	198	249	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
4	336	52	218	89	absent	present	<trshd	present	absent	present	absent	<trshd	absent	absent	absent	absent
5	156	22	53	279	absent	present	absent	present	absent	present	absent	absent	absent	present	present	present
6	231	87	148	264	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
7	86	217	173	59	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	absent	absent
8	161	292	28	179	<trshd	present	absent	present	absent	present	absent	<trshd	absent	present	present	absent
9	96	277	223	184	absent	present	absent	<trshd	absent	present	absent	absent	<trshd	present	present	<trshd
10	221	202	38	149	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
11	196	27	303	209	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	present	absent
12	1	222	278	109	absent	present	absent	<trshd	absent	present	present	absent	absent	present	present	<trshd
13	186	17	203	114	<trshd	present	absent	<trshd	<trshd	present	absent	<trshd	absent	absent	absent	absent
14	66	282	233	139	NA	absent	absent	present		present	absent	absent		present	present	absent
15	31	192	88	219	absent	present	<trshd	<trshd	absent	present	absent	absent	absent	present	present	absent
16	241	117	58	124	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
17	126	247	23	174	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
18	61	252	153	299	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
19	116	57	308	144	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
20	131	82	248	189	absent	present	absent	<trshd	absent	present	absent	<trshd	absent	<trshd	<trshd	absent
21	201	47	243	194	absent	present	absent	<trshd	absent	present	absent	<trshd	<trshd	<trshd	<trshd	absent
22	136	187	43	259	absent	present	present	<trshd	absent	present	absent	present	absent	absent	absent	absent
23	166	212	13	159	absent	present	absent	absent	absent	present	absent	absent	absent	present	present	absent
25	291	2	168	99	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
26	36	157	313	164	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	absent	absent
27	286	127	133	24	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	present	absent
29	106	147	63	294	absent	present	absent	present	absent	present	<trshd	absent	absent	present	absent	absent

lab nr	sample number				land				fish				other			
					A	B	C	D	A	B	C	D	A	B	C	D
30	111	77	273	199	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
31	141	232	258	9	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
32	46	132	263	19	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	present	absent
33	146	72	213	39	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
35	261	107	183	74	absent	present	absent	present	absent	present	absent	absent	absent	<trshd	absent	absent
36	331	102	68	229	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
37	76	112	238	54	absent	present	absent	absent	absent	present	absent	absent	absent	present	present	absent
38	176	137	93	234	absent	present	absent	present	absent	present	absent	absent	absent	absent	present	absent
39	101	227	48	14	absent	present	absent	present	absent	present	<trshd	absent	absent	present	<trshd	<trshd
40	81	142	268	69	absent	present	absent	<trshd	absent	present	absent	absent	absent	absent	absent	absent
41	71	257	163	29	absent	present	absent	present	absent	present	absent	absent	absent	present	present	<trshd
42	51	167	193	254	absent	present	absent	present	absent	present	absent	absent	absent	absent	present	absent
43	211	267	123	104	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
44	256	172	78	239	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	absent	absent
45	226	42	83	274	absent	absent	absent	<trshd	absent	present	absent	absent	<trshd	absent	absent	absent
46	181	97	208	134	absent	present	absent	present	absent	present	absent	absent	absent	present	present	absent
47	206	242	103	64	absent	present	absent	absent	absent	present	absent	absent	absent	present	present	absent
48	246	197	113	94	absent	present	absent	present	absent	present	absent	absent	absent	present	absent	absent
49	151	122	8	284	absent	present	absent	present	present	present	absent	absent	absent	absent	absent	absent
52	91	177	108	224	absent	present	absent	present	absent	present	absent	absent	absent	absent	present	absent
53	21	92	118	4	absent	present	absent	<trshd	absent	present	absent	absent	absent	present	present	absent
54	56	182	143	129	absent	present	absent	present	absent	present	absent	absent	absent	absent	absent	absent
55	251	62	128	214	absent	absent	present	<trshd	absent	present	absent	absent	absent	absent	absent	absent
56	41	207	138	244	absent	present	absent	present	absent	present	absent	absent	absent	absent	absent	absent
57	26	262	98	169	absent	present	present	absent	absent	present	present	absent	<trshd	<trshd	absent	<trshd

Annex 9 Results: PCR

A: blank	rumin.	bovine	sheep	pig	chicken	fish	insect
1	neg						
2	neg					neg	
3	neg						
7	neg						
8	neg	neg	neg	neg	neg		
9	neg						
12	neg						
23	neg						
25	neg						
35	neg			neg	neg	neg	
37	neg						
41 sample	neg	neg		neg			
41 sediment	neg	neg		neg			
44	neg						
49	neg						
53	neg						
54	neg	neg		neg	neg		
58	neg		neg	neg	neg		pos

B: 2% fish, 0.1% MBM	rumin.	bovine	sheep	pig	chicken	fish	insect
1	neg						
2	neg					pos	
3	neg						
7	neg						
8	neg	neg	neg	pos	neg		
9	pos						
12	neg						
23	neg						
25	neg						
35	neg			pos	neg	pos	
37	pos						
41 sample	neg	neg		pos			
41 sediment	neg	neg		pos			
44	neg						
49	neg						
53	neg						
54	neg	neg		pos	neg		
58	neg		neg	neg	neg		pos

C: 1% insect		rumin.	bovine	sheep	pig	chicken	fish	insect
1		pos						
2		pos					neg	
3		pos						
7		pos						
8		pos	neg	neg	neg	neg		
9		pos						
12		pos						
23		pos						
25		pos						
35		pos			neg	neg	neg	
37		pos						
41 sample		pos	neg		neg			
41 sediment		neg	neg		neg			
44		pos						
49		pos						
53		pos						
54		neg	pos		neg	neg		
58		neg		neg	neg	neg		pos

D: 1 fragment MBM / gram		rumin.	bovine	sheep	pig	chicken	fish	insect
1		pos						
2		pos					neg	
3		pos						
7		pos						
8		neg	neg	neg	neg	neg		
9		pos						
12		pos						
23		pos						
25		pos						
35		pos			neg	neg	neg	
37		pos						
41 sample		pos	pos		neg			
41 sediment		pos	pos		neg			
44		pos						
49		pos						
53		pos						
54		neg	pos		neg	neg		
58		neg		neg	neg	neg		pos

Annex 10 Analysis of method performance (sample D)

Principle

The basic principle for sample D is that the entire sediment (100%) extracted from 10 grams of sample material is a representation of that whole sample. If this sediment is fully analysed, the resulting number of fragments y_i is a representation of the entire sample, assuming that no fragments are overlooked nor that bone fragments remained hidden in the flotation. If less than 100% of the sediment is analysed (e.g. 37%), than the result represents only 3.7 grams of sample material.
In this framework, sample D was designed to analyse the relationship between the share of material actually examined and the expected number of particles recovered.

Method

The theoretical average number of particles per gram of sample material in sample 2014-D equals 1. For each result as obtained by the participants a standardised count *R* is calculated and presented in a histogram, as follows:

$$R_i = \frac{number\ of\ fragments\ (y_i)}{represented\ amount of\ sample\ material\ x_i\ (gram)}$$

A statistical analysis of the data was carried out in three steps:

1. An iterative exclusion of outliers.
2. Fitting a model under the assumption of proportionality, i.e. a linear relationship between the amount of material examined and the expected number of particles recovered.
3. Fitting a model with a non-linear relationship between the amount of material and the expected number of particles found, and testing the significance of this model.

1. An iterative method was adopted to identify potential outliers. For this, the data point with the largest residual in the fitted model was identified and the model was refitted to the data minus this potential outlier. This was continued until all (standardized) residuals were lower than 2. This identification of the outliers is based on a well-recognised and widely applied statistical principle: minimisation of the standardised residuals. Robust statistics are being developed for situations were a standard normal distribution can be applied and this is not the case in the current dataset.
2. Every estimation needs to be based on the assumption that not all bone fragments are properly recognised. The overall recovery rate appears to be 74%, which means that approx. one out of four fragments is not detected. This framework has been used to calculate the relationship between the amount of material used and the number of recovered bone fragments. The proportionality model for the expected number of observed fragments is

$$E(y_i) = x_i \cdot RF \cdot \mu \qquad \text{model 1}$$

where μ is the theoretical number of bone fragments per gram (1 in our case), y_i is the observed number of particles representative for x_i gram of material as reported by participant *i*, and where *RF* is the overall recovery factor accounting for the fact that there is a general under-estimation. Under the assumption of a homogeneous distribution of the particles a Poisson distribution is applied. In a Poisson distribution the variance V_i is equal to $\mathrm{E(y_i) = x_i \cdot RF \cdot \mu}$. If the material is not completely homogeneous, the variance will be larger (over-dispersion). A quasi-Poisson method is applied which assumes that the variance is inflated by a fixed factor k, so $\mathrm{V_i = k \cdot x_i \cdot RF \cdot \mu}$. The significance of k > 1 can be tested using a chi-square distribution with as many degrees of freedom as there are residual degrees of freedom in the model, here n-1, because there is only one parameter estimated. Both the Poisson and the quasi-Poisson are fitted in the framework of Generalized Linear Models (McCullagh and Nelder, 1989).

1. In the models in step 1 and 2 proportionality was taken for granted and not tested. An alternative model:

$$E(y_i) = x_i^{\beta} \cdot RF \cdot \mu \qquad \text{model 2}$$

is applied for testing this proportionality by including a factor β, i.e. a possible non-linear relationship between the amount of material x_i and the expected number of particles y_i to be found. With $\beta = 1$ model 2 equals model 1.
Note that β is a slope for $\ln(x_i)$ on the log-link scale of the analysis

$$\ln\big(E(y_i)\big) = \beta \ln(x_i) + \ln(RF) + \ln(\mu)$$

Due to the non-linearity on the original scale, the parameter RF no longer has the interpretation of a recovery factor.
The null hypothesis to be tested is the proportionality in the relationship between the amount of material actually investigated and the number of recovered bone fragments ($\beta_o = 1$). Based on this, the alternative hypothesis is that the slope of model 2 (β_t) differs significantly from $\beta_o = 1$.
Several scenario's has been tested:

- Exclusion/inclusion of assumed misinterpretation (specificity issues). Particles reported as muscle fibres or fish bones/scales could represent correct positives. These finding are included in an alternative statistical analysis.
- Exclusion/inclusion of outliers. The inclusion of outliers will result in a higher over-dispersion, which might influence the results of the model fitting.
- Statistical analysis of the effect of Alizarin staining on the number of particles to be recovered.

Results

Specificity
A total of twelve participants reported the presence of either fish bone particles or muscle fibres. All cases except two concern reports below the threshold. A further analysis of these specific cases reveal the situation as presented in Table A.

Table A
Reported particles for the categories fish and muscle fibres in sample 2014-D.

participant	Reported deviation	# of determinations
4	1 fish bone	2
5	13 muscle fibres [1) 2)]	2 [3)]
8	1 fish bone	2 [3)]
9	1 muscle fibre	2
12	1 undefined	2
13 [4)]	1 fish bone	1
20	2 fish scales [2)]	1
21	2 fish scales	2
22	11 fish bones [1)]	2
39	2 undefined	2 [3)]
41	3 muscle fibres [2)]	2 [3)]
57	1 undefined	2 [3)]

1): results higher than the threshold

2): excluded participants because of grinding prior to sedimentation

3): extra determination applied because of findings of fish or undetermined particles below the threshold

4): excluded because of incomplete reporting of the sediment weights

The results of participant 5 are excluded because of the pre-treatment by grinding. It is highly unlikely that the 11 fish bones as found by participant 22 can be attributed exclusively to the category of

terrestrial animals. The results for participant 22 are excluded as well, since scales and muscles fibres can be clearly discriminated from bones in general. In a worst case scenario 2 fish bones (participants 4 and 8) and 4 undefined particles (participants 12, 39 and 57) could have been misidentified particles of terrestrial origin and could therefore be added to the dataset. In total, all participants reported 268 fragments for sample D. This total is excluding the fragments as reported by the five participants that ground the sample. Six fragments means an extra 2.2% of that total. On the other hand, some reported bone fragments might erroneously be identified as terrestrial, which means that a simple addition of 6 particles in this worst case scenario is likely to be an overestimation.

Robustness
The alternative scenarios have been tested, of which the results are shown in Table B.

Table B
Dispersion after applying a proportional (Model 1) and a non-proportional model (Model 2) in five scenarios. The factor β of model 2 with its 95% confidence interval is given.

scenario	Dispersion model 1 / 2	Slope: β	95% confidence interval of β
all data (n=39) [1)]	3.12 / 3.10	0.75	0.30 – 1.20
mis-identified observations added (n=39)	3.08 / 3.07	0.76	0.31 – 1.20
excluding outliers (n=35)	1.87 / 1.92	0.94	0.57 – 1.30
labs with no staining (n=21)	3.12 / 2.99	0.50	-0.15 – 1.13
labs using staining (n=18)	3.00 / 3.19	1.03	0.35 – 1.70

1): scenario as presented in the IAG / RIKILT report. One participant (22) removed; see Table A.

In all cases the value β_o = 1 fits in the 95% confidence interval for β_t and, hence, the null hypothesis is accepted in all cases. A proportional relationship between the amount of material actually investigated and the number of recovered bone fragments is therefore a reasonable model. Most scenarios point to the same conclusion: the amount of material effectively investigated influences the expected number of particles to be recovered. It is possible to choose a low amount of material for examination in such a way that the threshold of five particles is not likely exceeded at low levels of contamination. This influencing of the result is allowed in a legal sense.

Alternative strategy
Besides the statistical analysis of the relationship between the amount used and the number of recovered particles (Figure 3 of the report), the data of sample D has been used as well to evaluate the effect on the sensitivity. Considering the situation that 10 gram of material of sample D would consist 10 bone fragments on average, the examination of 50% of the sediment would reveal 5 bone fragments on average assuming a full recovery. This value is identical to the threshold, which was the reason to choose the contamination level of 1 fragment per gram material. Taking this 50% as limit between two groups (a group of participants which applied 50% or more of the sediment and a group of participants which applied less than 50% of the sediment), the null hypothesis to be tested was the absence of a significant difference between the sensitivity for the two groups. The situation that the actual recovery is less than 100% applies equally to the two groups, which makes the results mutual comparable among these groups. Alternatively a limit of 60% was tested, which allowed a recovery of 84% for recognising 5 fragments out of the 6 available on average. An important effect of these two limits was the size of the two groups, which should be comparable: with a limit of 50% the groups were 18 vs. 22 participants (lower vs. higher) and with a limit of 60% the groups were 24 vs. 16 participants (lower vs. higher). Together with the other parameter in this analysis (values below the threshold of five particles considered as positive vs. negative) a large negative effect appeared in all scenarios for using less material than the chosen limit. Considering values below the threshold as negative, the null hypothesis was rejected (Table C). This supports the overall value of 0.44 for the sensitivity for sample D considering values below threshold as negative. All results indicate that the new microscopic method is by no means comparable to the previous version, primarily due to the decision to treat results below threshold as negative.

Table C

Evaluation of the reported number of bone fragments in sample 2014-D in four different scenarios: values below threshold considered negative versus positive, and all values based on less or more than 50% versus 60% of sediment used.

2014-D; 1 fragment / gram		Sensitivity		
	Percentage of sediment used	< limit	≥ limit	Fisher p
≤ 5 = positive	Limit: 50%	0.833	0.955	0.196
	Limit: 60%	0.875	0.938	0.354
≤ 5 = negative	Limit: 50%	0.222	0.545	*0.031*
	Limit: 60%	0.208	0.688	*0.003*

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