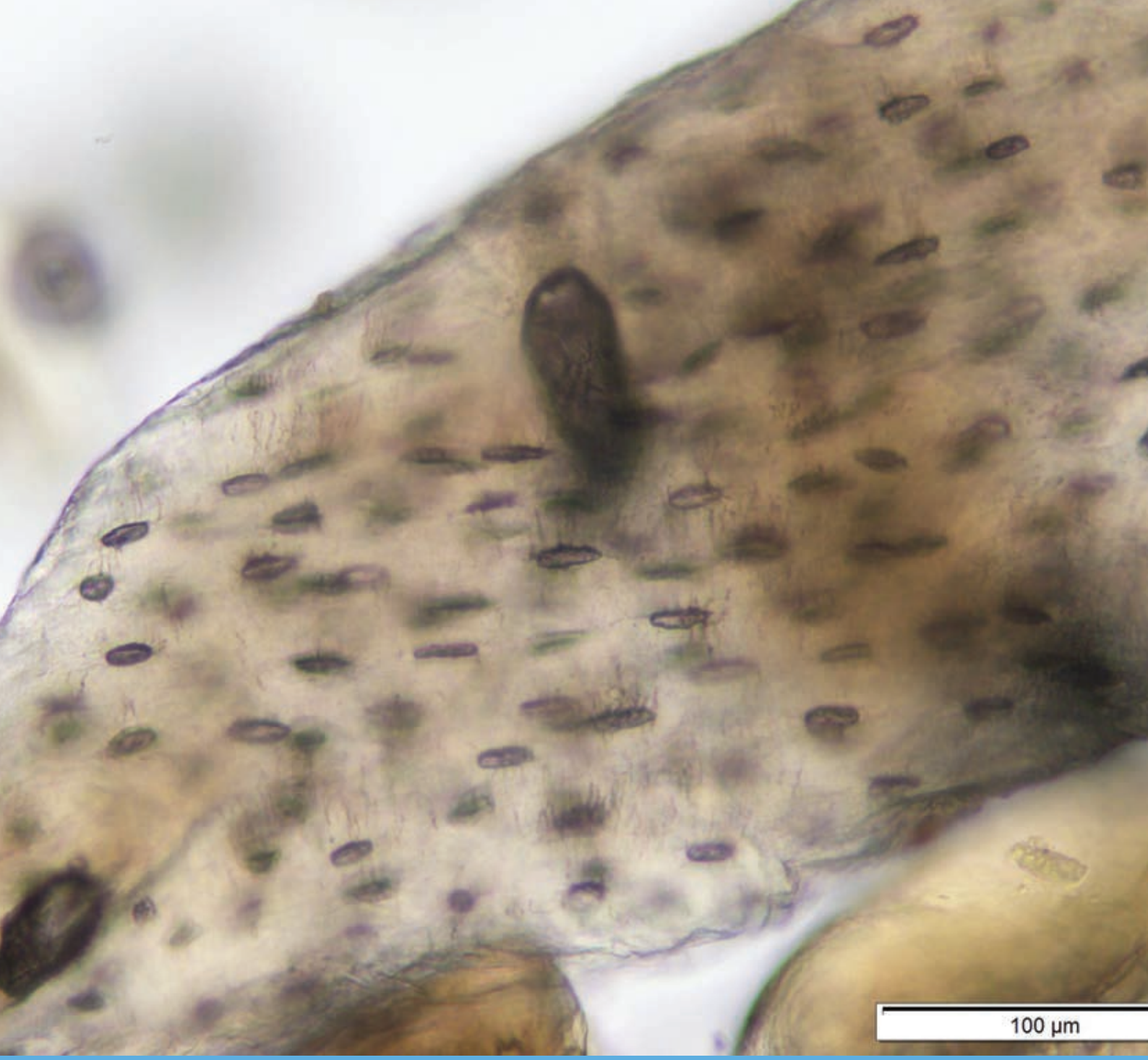

IAG ring test animal proteins 2015

L.W.D. van Raamsdonk, N. van de Rhee, I.M.J. Scholtens, T.W. Prins, J.J.M. Vliege, V.G.Z. Pinckaers

RIKILT
WAGENINGEN UR

IAG ring test animal proteins 2015

L.W.D. van Raamsdonk, N. van de Rhee, I.M.J. Scholtens, T.W. Prins, J.J.M. Vliege, V.G.Z. Pinckaers

RIKILT Wageningen UR
Wageningen, December 2015

RIKILT report 2015.016

RIKILT
WAGENINGEN UR

Van Raamsdonk, L.W.D, N. van de Rhee, I.M. Scholtens, T.W. Prins, J.J.M. Vliege and V.G.Z. Pinckaers, 2015. *IAG ring test animal proteins 2015.* Wageningen, RIKILT Wageningen UR (University & Research centre), RIKILT report 2015.016. 32 pp.; 0 fig.; 10 tab.; 13 ref.

P.O. Box 230, 6700 AA Wageningen, The Netherlands, T +31 (0)317 48 02 56, E info.RIKILT@wur.nl, www.wageningenUR.nl/en/rikilt. RIKILT is part of Wageningen UR (University & Research centre).

RIKILT report 2015.016

Photo cover: A ruminant bone fragment collected from salmon meal

Distribution list:

- International Association for Feeding stuff Analysis (IAG), Section Feeding stuff Microscopy (I. Paradies-Severin, J. Vancutsem, G. Frick, R. Weiss)
- European Commission (EC; K. van Dijck, M. Plantady, L. Carrouee, F. Verstraete)
- European Union Reference Laboratory, Animal Proteins (CRA-W; G. Berben, P. Veys, V. Baeten)
- Joint Research Centre, Geel (IRMM-JRC; C. von Holst, A. Boix-Sanfeliu)
- All participants of the ring test
- European Fat Processing and Renderers Association (EFPRA; M. Alm, S. Woodgate)
- International Fishmeal and Fish oil Organisation (IFFO; A. Jackson, I. Pike)
- Laboratory of the General Chemist (LGC; M. Walker)
- Ministry of Economic Affairs (EZ; E.L.J.M. Pierey, mw. L.A.M. Claassen, E.R. Deckers)
- Netherlands Food and Consumer Product Safety Authority (NVWA; W. Ooms,. R. Theelen, R. Herbes)

Contents

Summary

A ring test was organized for the detection of animal proteins in animal feed by microscopy in the framework of the annual ring tests of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy. 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 2015 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 as published in Regulation (EC) 51/2013 amending Annex VI of Regulation (EC) 152/2009 together with accompanying SOPs.
Two matrices have been used in the design of the study. Samples A and C were based on a pig feed containing whey powder and consequently were positive for ruminant DNA. Samples B and D were based on a cattle feed. Two different types of fish meal were added to samples A and D at a level of 0.1%, and a frequently tested ruminant meat and bone meal (MBM) was added to sample B at a level of 0.1%.
The participants were invited to apply the ruminant PCR as well. Twelve participants submitted both microscopic as well as PCR results, and two participants returned exclusively PCR results. Four out of 42 participants applied the wrong number of microscopic determinations, although the report form was interactive and guided the participant through the process of choosing the right number of repetitions.

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. 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 48 participants 42 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 threshold (between 1 and 5 particles per determination cycle inclusive) have been considered positive and as alternative considered as negative. The choice to consider these numbers 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.
A total of 42 sets of microscopic results were returned.
Most of the specificity and sensitivity scores for microscopy were at good or reasonable levels. In the presence of fish meal originating from Denmark, 10 out 42 participants erroneously recognised some particles of terrestrial animal origin (specificity 0.76). For both samples B and C, not containing fish meal, five out of 42 participants reported the presence of fish meal (specificity 0.88). Considering numbers of particles below threshold as negative, all sensitivity scores were at a level of 0.93 or higher. In contrast, applying a threshold for positive reporting consequently results in lower scores (0.88 or higher). 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
Samples A, B and C were correctly identified as positive for ruminant DNA by all 14 laboratories that performed ruminant PCR (sensitivity 1.0). For sample D false positive results were sent in by 2 of the 14 laboratories (specificity 0.86).

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 (LOD [1]) of five particles was set per determination cycle based on a laboratory sample of 10 grams. As of 1 June 2013 non-ruminant material is allowed as ingredient in aquafeed (Regulation (EC) 56/2013 amending Annex IV of Regulation (EC) 999/2001). Ruminant material remains prohibited, which needs a specified identification method, which was implemented by a PCR method.

The 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 2015 version of the IAG ring test for animal proteins is the first one to combine the method for microscopy with detection of ruminant PCR.

In this report the ring test for animal proteins 2015 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 2015 was chosen to be based on two compound feeds produced in the framework of the EU funded project STRATFEED with a composition intended for cattle and pig, respectively. The cattle feed was based on a formulation with cornglutenfeed (31%), palmkernelmeal (20%), citruspulp (16%), beetpulp (10%), sunflowerseedmeal (5%), coconutmeal (5%), soybeanmeal (2%), mineral mix (1.4%) and microscopically undetectable materials (fat, molasse: 9%). The pig feed was composed of wheat and wheat products (52%), soya products (11%), bakery by-products (8%), sunflowerseedmeal (7%), palm expeller (5%), beet pulp (5%), rapeseedmeal (3%), whey powder (1%), barley (1%), mineral mix (1.5%) and microscopically undetectable materials (fat, molasse: 5%). The shares are given without decimal indication except for the mineral/vitamin mix, which is an indication for the expected amount of sediment.
The IAG ring test for animal proteins 2015 was combined with the IAG ring test for botanic composition (sample 2015-C). The results of this ring test are being published in a separate report (van Raamsdonk *et al.*, 2015).
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 2015.

Label	matrix	Content
2015-A	Pig feed	0.1% fish meal (origin: Denmark)
2015-B	Cattle feed	0.1% ruminant meal (CRA-W DQ-03-0031-01)
2015-C	Pig feed	Blank
2015-D	Cattle feed	0.1% fish meal (origin: Peru)

The fish meals were samples from practice, which were examined in the RIKILT regular control program and found to be negative for land animal material.
The meat and bone meal was produced in the framework of the EU funded project STRATFEED and distributed by the coordinator among the participants.
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: 2015-C - 2015-A - 2015-D - 2015-B. 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 2015-C were filled with 50 grams of the pure pig feed, closed and set aside. The other samples were produced by step-wise dilution of the dedicated contaminants down to a level of 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: 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	Rum
MBM CRA-W		Pos	Neg	Pos
Fish meal Peru		Neg	Pos	Neg
Fish meal Denmark		Neg	Pos	Neg
Cattle feed		Neg	Neg	Neg
Pig feed with whey		Neg	Neg	Pos
2015-A Pig feed with whey + 0.1% fish meal Denmark	53-81 mg/10 g	Neg	Pos	Pos
2015-B Cattle feed + 0.1% MBM CRA-W	76-106 mg/10 g	Pos	Neg	Pos
2015-C Pig feed with whey - blank	48-63 mg/10 g	Neg	Neg	Pos
2015-D Cattle feed + 0.1% fish meal Peru	89-132 mg/10 g	Neg	Pos	Neg

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 2015 by means of an invitations in the IAG Newsletter of 2014 (http://www.iag-micro.org/index.php?article_id=194). Until the beginning of March a total of 48 participants for the ring test animal proteins were listed. The sets of four samples with an accompanying letter (see Annex 1) were sent to all participants on Thursday 5th of March 2015. On Monday March 9th 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 6th. Several requests were received to extent the period for analysis with several 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. 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 the end of April and early May.
Participants outside Europe were informed to be aware of possible problems with custom regulations.

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 June 2nd.

2.5 Analysis of results

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, 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

Forty-eight packages with four samples were sent to all participants. Of the 44 participants in total, which successfully submitted their results, 42 presented results for the microscopic method, in 12 of these reports combined with PCR results. The remaining two participants reported exclusively PCR results. The participants 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 5. Five member states have been involved with three or more participating laboratories: Germany (16 labs), Belgium (4), France (4), the Netherlands (4), and Italy (3). These figures are a bit lower compared to those of the ring test of last year (van Raamsdonk *et al.*, 2014).
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. One participant sent in the results for three samples. Since specific results can deliberately be omitted from the final report, only full reports are considered. 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

Four participants (7, 20, 29, 53) carried out too many determination cycles for one or more samples. Participant 40 should have carried out one extra cycle for one sample.

3.2 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 6 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.
The results as presented in Table 7 generally show a good application of the method, except for the number of determinations applied and for the embedding agent. Differences with previous years will be presented in the next chapter (Discussion).
The amounts of sediment produced ranged for samples A and C (pig feed) between 69 and 177 mg per 10 gram feed, and ranged for samples B and D (cattle feed) between 86 and 240 mg per 10 gram feed. Excessively higher amounts were reported for sample A (1020 mg), for sample B (1230 mg) and for sample D (1694 mg). Most participants applied 12% or more of the collected sediment material on the slides; three participants applied as low as 3-9% of material on the slides.

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	37	
	no; too many determinations	4	
	no; insufficient determinations	1	
Extra milling step (<1.0 mm)	no	30	
	yes	10	
amount of material used for sedimentation of feed	10 grams	42	
	Other amounts	0	
type of glassware	chemical sedimentation funnel	22	
	conical glass with cock	7	
	champagne glass *	5	
	beaker (flat bottom)	4	
	other	4	
sedimentation agent	TCE	42	
	TCE/Petroleumether	0	
use of staining of sediment	no	27	
	yes	15	
use of binocular for examination at lower magnifications	yes	26	
	no	16	
size of cover glass used	small (e.g. 20 x 20 mm)	32	
	medium	6	
	large (e.g. 26 x 50 mm)	3	
share of the total sediment used for examination	minimum		3%
	maximum		100%
embedding agent for the sediment	glycerine / glycerol	17	
	paraffin oil	13	
	immersion oil	8	
	Norland Adhesive	3	
	other (water, glycerol:water mixture, mineral oil)	0	
Use of ARIES	yes	3	
	no	39	

3.3 Microscopic detection

The results of the application of the microscopic detection yielded in general a reasonable result (Table 3; Annex 7). A number of participants reported the presence of terrestrial animal material in sample A (specificity 0.76). although the fish material from Denmark was spiked at a low level (0.1%), still some particles were apparently recognised as terrestrial. The number of particles was in almost all cases below the threshold of 5 particles per determination and the specificity was much higher (0.98) when results below 5 on average were considered negative. This specificity problem was more limited in the presence of fish meal from Peru. The detection of 0.1% MBM of terrestrial animal material is suboptimal (0.93). The sensitivity when considering results below the threshold of 5 particles is lower (0.88), which indicates that in several cases only a few particles were found. 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 3

*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.*

		MBM				Fish			
		A	B	C	D	A	B	C	D
n		0%	0.1%	0%	0%	0.1%	0%	0%	0.1%
42	specificity	0.76		0.95	0.93		0.88	0.88	
		0.98		*1.0*	*0.98*		*0.95*	*0.93*	
	sensitivity		0.93			0.95			1.0
			0.88			*0.88*			*0.95*

A: fish meal from Denmark

D: fish meal from Peru

In seven cases the report of presence/absence was inconsistent with the number of particles found (see Table 4). The alternative scores based on the actual found number of particles is presented in Table 5. In all cases the difference is limited to one or two false positive or false negative findings, since the inconsistencies are well divided over five different scores.

Table 4

Participants' comments on the background of the false positives and negatives reported for the calculations in Table 3. Cells marked in red: inconsistent reporting; asterisk: report based on PCR results.

sample, contaminant	Participant	count	reported as
A: land animal material	11	6 bones, muscles	present
	12 PCR	4 ND	<threshold
	14	1 bone	absent
	17	2 bones	<threshold
	20	7 ND	<threshold
	28	4 bones	<threshold
	35 PCR	10 muscle fibres	<threshold
	36	1 feather	<threshold
	37	5 bones	<threshold
	40 PCR	2 hair	<threshold
	47	8 ND (in 2 cycles)	<threshold
B: land animal material	7	Phosphate from bones	absent
	8	16 TCP bone material	absent
C: land animal material	11	1 ND	absent
	12 PCR	1 ND	<threshold
	35 PCR	0 bones	<threshold *
	47	7 ND	<threshold
D: land animal material	32	2 bones	<threshold
	35 PCR	0 bones	<threshold *
	36	1 feather	<threshold
	47	6 ND	present
A: fish material	35	10 muscle fibres	absent
B: fish material	13	1 fish bone	<threshold
	17	4 bones, tooth	<threshold
	39	> 0.1 % fish bones	present
	47	3 ND	<threshold
	56	16 scales, bones	present
C: fish material	3	1 fish bone	present
	11	2 scales, muscle	<threshold
	12	6 ND	present
	26	14 bones	present
	46	6 bones	<threshold

Table 5

Sensitivity and specificity scores for the detection of animal proteins based on the number of particles as reported by the participants in 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.

		MBM				Fish			
		A	B	C	D	A	B	C	D
n		0%	0.1%	0%	0%	0.1%	0%	0%	0.1%
42	specificity	0.74		0.93	0.93		0.88	0.88	
		0.93		*1.0*	*0.98*		*0.95*	*0.95*	
	sensitivity		0.95			0.98			1.0
			0.90			*0.90*			*0.95*

Nine out of 12 participants, who applied the combination of microscopy and PCR, correctly found the ruminant DNA resulting from whey in sample 2015-A, but still reported correctly the absence of terrestrial animal material. Two participants reported the (erroneous) presence of terrestrial animal material in sample A, and reported this in a correct way.
Participant 35 corrected several findings based on their DNA results.
The results of the ring test based on the full set of microscopic+DNA results is presented in Table 6. There are hardly differences with the microscopic results as presented in Table 3.

Table 6

*Sensitivity and specificity scores for the detection of animal proteins by the **combined** method (microscopy and PCR) of four samples (top row: values below LOD considered positive; bottom row in italics: values below LOD considered negative). Abbreviations: n: number of participants. Capitals A to D: sample indication.*

		MBM				Fish			
		A	B	C	D	A	B	C	D
n		0%	0.1%	0%	0%	0.1%	0%	0%	0.1%
42	specificity	0.76		0.93	0.90		0.88	0.88	
		0.98		*1.0*	*0.98*		*0.95*	*0.93*	
	sensitivity		0.93			0.95			1.0
			0.88			*0.88*			*0.95*

3.4 Detection by other methods

Participants were invited to perform DNA analysis targeted for ruminants (EURL-AP Method) and to submit their results together with the results for microscopy. Fourteen participants submitted results. The results are presented in Table 8 and in Annex 8. Participants 3 and 35 reported positive for the presence of ruminant DNA in sample D. This resulted in one incorrect report for the presence of land animal (<threshold as conclusion for zero particles). One participant assumed the presence of bakery products containing ruminant DNA.

Table 8

*Results for DNA analyses (PCR) for four samples. Target: ruminant. *: presence of ruminant DNA from whey powder.*

		Ruminant			
		A *	B	C *	D
n		0%	0.1%	0%	0%
14	specificity				0.86
	sensitivity	1.0	1.0	1.0	
	Homogeneity study	pos	pos	pos	neg

4 Discussion

The 2015 setup of the IAG ring test for animal proteins was relatively straightforward: in all cases adulteration was achieved at the technical constraint of 0.1% (EC, 2013a), and mixtures were not used. The possibility to use microscopy and PCR for identification in cases of a positive result was facilitated by using a feed containing ruminant DNA for two samples. One of the fish meals appeared to result in quite a number of positives for land animal material. The aspects of DNA detection and of specificity will be discussed in the next paragraphs.

4.1 Combination of microscopy and DNA detection

Regulation (EC) 152/2009 amended by Regulation (EC) 51/2013 (EC, 2013a) allows to combine the detection by microscopy and real-time PCR in specific situations. A binding SOP provides a flow chart for directing the order of the two methods. Only in cases of aqua feeds PCR is allowed to be applied first, otherwise only samples positive for land animal material after microscopy should be subjected to ruminant PCR detection (EURL-AP, 2015). The samples in the current ring test were not indicated to be aqua feed, which meant that microscopy was the preferred method followed by PCR for positive samples.
In all cases where the participants applied PCR, all four samples were subjected to PCR analysis, which is not in compliance with the binding SOP of the EURL AP.
One participant changed the conclusion of the microscopic detection according to the PCR results. Although all other participants applying PCR reported the presence of ruminant DNA in samples A, B and C, their conclusions seem to be based on the microscopic findings.

4.2 Specificity and sensitivity

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 in all cases for sensitivity (Table 3 and 4; van Raamsdonk *et al.*, 2014: Table 4). The number of particles wrongly recognised (specificity) could be expected to be reasonably low and using a threshold would limit the number of false positives. At the same time, very 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, 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.
Suboptimal results were achieved in the current study for the adulteration with MBM at 0.1% (Table 3: 0.93 or 0.88, depending on application of the threshold). These results were the lowest in 13 years of IAG ring tests (Table 9). There is also a difference between the two fish meals used. The fish meal from Peru was slightly better recognisable (1.0 vs. 0.95, or 0.95 vs. 0.88 with results below threshold as negatives; Table 3). The fish meal from Denmark showed clearly more particles which were confused with terrestrial animal particles (0.76 vs. 0.93, or 0.98 vs. 0.98 with results below threshold as negatives; Table 3). It has to be noted that all samples except the blank were adulterated at 0.1% w/w, which is the minimum required performance limit for all methods (EC, 2013a).
The equal score (0.98) for detection of terrestrial animal material in the presence of both types of fish meal (samples A and D) when considering findings below the threshold as negative indicates that a measure for adjusting sensitivity is capable of correcting a specificity problem (incorrect identification of fish bones as terrestrial animal material). This does not imply that specific actions for avoiding incorrect identifications are unnecessary.

Table 9

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 (2008-2015) 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), current results	0.95			0.93			0.88	0.90	

*: 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. Notable developments are the number of participants applying the correct number of determination cycles, and those using less than 10 grams of material for sedimentation (2015: zero). The share of participants which uses a binocular was lower than in previous years.

The amount of sediment collected by the participants is higher than in the homogeneity study (Table 2). The production of a sediment, containing the heavy particles from a sample, e.g. minerals, is aiming at a concentration of the principal target: bone fragments. Considering an average sediment share of 2%, the concentration achieved is a factor of 50x. If the fraction called sediment contains additional sample material and this fraction has a share of e.g. 5%, the concentration factor is limited to 20x. In the case of presence of bone fragments these are "diluted" in that extended fraction compared to an optimally achieved sediment.

Table 10

Comparison between parameters distribution in the IAG ring studies between 2008 and 2015.

parameter correct number of cycles	parameter choice	2008	2009 -2014 2014: 67.3%	2015 88.1%
amount of material used for sedimentation	5 grams	16	5-0	0
	10 grams	26	41-50	42
	other	3	3-1	0
type of glassware	chemical sedimentation funnel	22	28-33	22
	beaker (flat bottom)	11	13-3	4
	champagne glass	6	5-9	5
	conical glass with cock	3	1-9	7
	other	3	2-4	4
use of staining of sediment	no	31	31-36	27
	yes	14	14-22	15
use of binocular for examination at lower magnifications	yes	29	37-45	26
	no	16	9-15	16
size of cover glass used	small (e.g. 20 x 20 mm)	34	27-42	32
	medium	1	4-10	6
	large (e.g. 26 x 50 mm)	9	16-6	3
share of the total sediment used for examination	minimum	4%	0.2%-2%	3%
	maximum	100%	100%	100%
embedding agent for sediment	glycerine / glycerol	8	10-25	17
	immersion oil	8	7-14	8
	paraffin oil	18	12-23	13
	Norland Adhesive	0	2-7	3
	chloral hydrate	3	1-0	0
	other (e.g. Depar 3000, water)	8	5-1	0

5 Conclusions and recommendations

5.1 Conclusions

Certain fish meals might cause erroneous identification of fish particles as terrestrial animal material. Additionally, fish meal material was considered to be present in some samples without fish material. The number of false positives seems to be corrected when all findings below the threshold are considered negative (higher score for specificity). This procedure has a negative effect on the scores for sensitivity. 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. In all cases (14 participants) the procedures were not followed correctly. Nevertheless, in all but two cases correct PCR results were reported for the presence of ruminant DNA. It has to be noted that the current ring test is the first one integrating both microscopy and PCR with reference to the official methods including the SOPs.
The quality of the sediment, expressed by the amount of material collected after the sedimentation procedure, can be expected to influence the performance of the method. The presence of surplus material in a sediment would hamper the detection of fragments originating from animals. In the current ring test there are only two types of information: the sediment amounts as reported by the participants is generally higher than obtained in the homogeneity study, and the variation in the reported sediment amounts is considerable. Any conclusion needs to be based on actual examinations of the sediments as obtained by different laboratories.

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 contents of sediments as obtained by different laboratories is recommended.

Acknowledgements

The board of IAG section Feeding Stuff Microscopy (dr. I. Paradies-Severin (LUFA, Hameln), dr. G. Frick (ALP, Posieux), ir. J. Vancutsem (FAVV, Tervuren) and dr. R. Weiss (AGES, Vienna)) supported this study as advisory board for communication with the scientists and laboratories working in this research field, and in the final report activities. Their contributions are greatly acknowledged. Thanks to colleagues dr. L. van der Geest, dr. J. de Jong and dr. A. Peijnenburg for the internal RIKILT review procedure.

References

EURL-AP Standard Operating Procedure, 2014. Detection of ruminant DNA in feed using real-time PCR, version 1.1, 24-06-2014, http://eurl.craw.eu/img/page/sops/EURL-AP%20SOP%20Ruminant%20PCR%20V1.1.pdf

EURL-AP Standard Operating Procedure, 2014. DNA extraction using the "Wizard® Magnetic DNA purification system for Food" kit, version 1.1. 24-6-2014, http://eurl.craw.eu/img/page/sops/EURL-AP%20SOP%20DNA%20extraction%20V1.1.pdf.

EURL-AP Standard Operating Procedure, 2015. Operational protocols for the combination of light microscopy and PCR. version 3.0, 13-05-2015, http://eurl.craw.eu/img/page/sops/EURL-AP%20SOP%20operational%20schemes%20V3.0.pdf.

European Commission, 2009. Commission Regulation (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed. Official Journal of the European Communities L 54, 26.2.2009, p. 1–130.

European Commission, 2013a. Commission Regulation (EU) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed Text with EEA relevance. Official Journal of the European Communities L 20, 23.1.2013, p. 33–43.

European Commission, 2013b. Corrigendum to Commission Regulation (EU) No 51/2013 of 16 January 2013 amending Regulation (EC) No 152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed (OJ L 20, 23.1.2013). Official Journal of the European Communities L 62, 6.3.2013, p. 36–36.

European Commission, 2013c. Commission Regulation (EU) No 56/2013 of 16 January 2013 amending Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies Text with EEA relevance. Official Journal of the European Communities L 21, 24.1.2013, p. 3–16.

European Union, 2001. Regulation (EC) No. 999/2001 of the European Parliament and of the Council of 22 May 2001 laying down rules for the prevention, control and eradication of certain transmissible spongiform encephalopathies. Official Journal of the European Communities L 147, 1–40.

Lindenmayer, M.A.; Burgman, 2005. Monitoring, assessment and indicators. Practical Conservation Biology (PAP/CDR ed.). Collingwood, Victoria, Australia: CSIRO Publishing. pp. 401–424.

Raamsdonk, L.W.D. van, V. Pinckaers, J.J.M. Vliege, 2015. IAG ring test feed composition 2015. Report 2015.017. RIKILT, Wageningen, pp. 23.

Raamsdonk, L.W.D. van, V.G.Z. Pinckaers, I.M. Scholtens, T.W. Prins, H. van der Voet, J.J.M. Vliege, 2014. IAG ring test animal proteins 2014. Report 2014.011. RIKILT, Wageningen, pp. 39.

Raamsdonk, L.W.D. van, V.G.Z. Pinckaers, J.J.M. Vliege, 2012. Animal proteins in feed. IAG ring test 2012. Report 2012.009, RIKILT, Wageningen, pp. 39.

Veys, P., G. Berben and V. Baeten, 2010. CRL-AP Proficiency Test 2009. Community Reference Laboratory for Animal Proteins in feedingstuffs, Walloon Agricultural Research Centre, Valorization of Agricultural Products Department, Gembloux, Belgium.

Annex 1 Introduction to the test

Test 2015-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, otholiths). If more than 16 fragments per category are found in any cycle, just choose "16" from the drop-down list.

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

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

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

Annex 2 Basic instructions for the test procedure

IAG ring test 2015 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 as well. Take care to homogenise the content of each vial before taking the amount for analysis.
The samples are prepared in such a way that you can start with the procedure in paragraph 2.1.3.4: use 10 grams for sedimentation etc. The sample amount allows you to analyse three determinations of 10 grams as indicated in paragraph 2.1.4.3. The process of analysis as included in this ring test will stop at the beginning of paragraph 2.1.5: the reporting sentences will not be used. Instead, the report form allows you to enter the number of particles per determination cycle and a final conclusion.
Differentiation has to be made between particles of terrestrial animals (bone fragments, hairs, horn, skin, feathers) and those of fish (fish bone fragments, scales, gills, otholiths). If more than 16 particles are found in any category, please enter the value 16.
The report form is interactive: if the results in the first determination cycle make it necessary to perform a second or third analysis according to the requirements of the Regulation, additional cells will turn pink.
The final conclusion, according to Regulation (EC) 152/2009, can be reported in three ways, depending on the average number of particles found per category:
= Zero particles: animal proteins absent. If the first determination reveals no particles in any category, a second determination is not necessary.
= More than 5 particles on average per determination: present.
= Between 1 and 5 particles on average: sample is positive but a risk of a false positive result cannot be excluded. For the sake of the framework of the current report form the term '<LOD' has to be chosen.
Click here for the Regulation and connected SOPs

3 Reporting consists of the following steps:

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

3b Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after your have entered it at the page Procedure. Enter yourself the four unique labels of the vials.

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 April 6th, 2015.

RIKILT Wageningen UR, the Netherlands

Annex 3 Report form for procedure details

Please complete at least all the cells with a drop down list	select your choice from a drop down list	type in your answer if necessary
IAG ring test 2015 animal proteins		
Please select your unique lab number	**-- select --**	
Have you read the ring test instructions?	-- select --	
Did you apply PCR ruminant detection method?:	-- select --	
Did you apply grinding before performing the detection procedure?	-- select --	
Indicate your glassware for sedimentation	-- select --	
if other, please specify		
Describe your sedimentation agent	-- select --	
if other, please specify		
Did you apply staining of the sediment (e.g. alizarin staining) as standard procedure?	-- select --	
Did you examine at lower magnifications (using a binocular)?	-- select --	
Indicate the size of cover glass	-- select --	
Please describe your embedding agent for the sediment material	-- select --	
if other, please specify		
Did you use the expert system ARIES for identification of particles?	-- select --	

Annex 4 Report form: results

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

IAG ring test 2015 animal proteins

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

Annex 5 List of participants

institute	country
Austrian Agency for Health and Food Safety-AGES	Austria
LFSAL	Belgium
FLVVT	Belgium
Laboratorium ECCA nv	Belgium
Oleotest N.V.	Belgium
China Agricultural University (East campus)	China
Croatian Veterinary Institute	Croatia
Danish Veterinary and Food Administration	Denmark
IPL Atlantique	France
S.C.L. Laboratoire de Rennes	France
Inovalys-Nantes	France
AdGène Laboratoire	France
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	Germany
LLFG Landesanstalt für Landwirtschaft	Germany
Universität Hohenheim, LA Chemie (710)	Germany
Futtermittelinstitut Stade (LAVES)	Germany
Veravis GmbH	Germany
CVUA-RRW	Germany
WESSLING GmbH	Germany
LUFA Nord-West	Germany
Thüringer Landesanstalt für Landwirtschaft	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	Germany
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	Germany
LTZ Augustenberg	Germany
SGS Germany GmbH	Germany
Landeslabor Berlin-Brandenburg	Germany
LUFA-Speyer	Germany
LUFA Rostock	Germany
Ministry of reconstuction of production, environment & energy, Feedingstuffs control laboratory of Athens (E.E.KY.Z)	Greece
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
CCL - Nutricontrol	Netherlands
Nutreco Nederland BV - Masterlab	Netherlands
TLR	Netherlands
Eurofins Food Testing Rotterdam BV	Netherlands
Nofima AS	Norway
Certificaciones y Calidad SAC, Laboratorio de Microbiología	Peru
NSF INASSA 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
Trouw nutrition Espana	Spain
Laboratorio Agroalimentario de Cordoba	Spain
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	ARIES
2	yes	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine	yes
3	no	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
4	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
5	no	conical champagne glass	top	TCE	no	yes	medium	glycerine	no
6	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	glycerine	yes
7	yes	beaker (flat bottom)	top	TCE	yes	yes	small (20 x 20 mm)	immersion oil	no
8	no	conical champagne glass	top	TCE	no	yes	small (20 x 20 mm)	glycerine	no
9	no	other		TCE	no	no	large (26 x 50 mm)	paraffin oil	no
10	yes	special conical glass with cock	top/bottom	TCE	no	yes	small (20 x 20 mm)	glycerine	no
11	no	special conical glass with cock	top/bottom	TCE	no	yes	small (20 x 20 mm)	glycerine	no
12	no	special conical glass with cock	top/bottom	TCE	no	no	small (21 x 26 mm)	immersion oil	no
13	no	chemical sedimentation funnel	top/bottom	TCE	yes	no	small (20 x 20 mm)	Norland adhesive 65	no
14	no	special conical glass with cock	top/bottom	TCE	yes	yes	small (21 x 26 mm)	paraffin oil	yes
15	no	other		TCE	no	no	small (20 x 20 mm)	glycerine	no
17	no	beaker (flat bottom)	top	TCE	no	no	medium	paraffin oil	no
20	no	chemical sedimentation funnel	top/bottom	TCE	yes	no	medium	paraffin oil	no
21	no	chemical sedimentation funnel	top/bottom	TCE	no	no	small (20 x 20 mm)	glycerine	no
22	yes	beaker (flat bottom)	top	TCE	no	yes	small (20 x 20 mm)	immersion oil	no
23	no	other		TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
24	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	immersion oil	no
25		conical champagne glass	top	TCE	no	no	small (20 x 20 mm)	immersion oil	no
26	yes	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	Norland adhesive 65	no
27	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
28	yes	other		TCE	no	yes	small (20 x 20 mm)	immersion oil	no
29	no	conical champagne glass	top	TCE	yes	no	small (20 x 20 mm)	immersion oil	no
30	no	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
32	no	chemical sedimentation funnel	top/bottom	TCE	yes	yes	medium	immersion oil	no
33	no	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine	no

lab nr	grinding	glassware		agent	staining	binocular	size	embedding	ARIES
34	yes	chemical sedimentation funnel	top/bottom	TCE	no	no	medium	paraffin oil	no
35	no	chemical sedimentation funnel	top/bottom	TCE	yes	yes	small (20 x 20 mm)	glycerine	no
36	no	beaker (flat bottom)	top	TCE	no	no	small (21 x 26 mm)	glycerine	no
37	no	special conical glass with cock	top/bottom	TCE	yes	yes	large (22 x 50 mm)	paraffin oil	no
39		chemical sedimentation funnel	top/bottom	TCE	yes	no	small (20 x 20 mm)	-- select --	no
40	yes	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	paraffin oil	no
43	no	conical champagne glass	top	TCE	no	yes	small (20 x 20 mm)	glycerine	no
46	no	chemical sedimentation funnel	top/bottom	TCE	no	no	medium	paraffin oil	no
47	no	special conical glass with cock	top/bottom	TCE	no	yes	small (20 x 20 mm)	glycerine	no
51	yes	special conical glass with cock	top/bottom	TCE	yes	no	large (26 x 50 mm)	glycerine	no
52	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (20 x 20 mm)	glycerine	no
53	no	chemical sedimentation funnel	top/bottom	TCE	no	no	small (20 x 20 mm)	paraffin oil	no
54	yes	chemical sedimentation funnel	top/bottom	TCE	no	no	small (21 x 26 mm)	Norland adhesive 65	no
56	no	chemical sedimentation funnel	top/bottom	TCE	no	yes	small (21 x 26 mm)	paraffin oil	no

Annex 7 Results: presence of animal proteins, microscopic detection

lab nr	sample number				Land				Fish			
					0%	0.1%	0%	0%	0.1%	0%	0%	0.1%
					A	B	C	D	A	B	C	D
2	251	237	258	249	absent	present	absent	absent	present	absent	absent	present
3	256	227	248	269	absent	present	absent	absent	present	absent	present	present
4	241	232	263	19	absent	present	absent	absent	present	absent	absent	present
5	246	182	253	254	absent	present	absent	absent	present	absent	absent	present
6	261	272	273	264	absent	present	absent	absent	present	absent	absent	present
7	266	257	218	14	absent	absent	absent	absent	present	absent	absent	present
8	271	247	243	4	absent	absent	absent	absent	present	absent	absent	present
9	161	222	213	274	absent	present	absent	absent	present	absent	absent	present
10	166	162	183	44	absent	present	absent	absent	present	absent	absent	present
11	156	242	188	89	present	present	absent	absent	present	absent	<threshold	present
12	191	77	223	49	<threshold	present	<threshold	absent	present	absent	present	present
13	186	252	153	94	absent	present	absent	absent	present	<threshold	absent	present
14	151	197	33	34	absent	present	absent	absent	present	absent	absent	present
15	181	207	98	39	absent	present	absent	absent	present	absent	absent	present
17	171	152	68	259	<threshold	present	absent	absent	present	<threshold	absent	present
20	201	112	93	79	<threshold	present	absent	absent	present	absent	absent	present
21	1	27	193	154	absent	present	absent	absent	present	absent	absent	present
22	31	262	3	99	absent	present	absent	absent	present	absent	absent	present
23	11	32	163	84	absent	present	absent	absent	present	absent	absent	present
24	176	97	73	109	absent	present	absent	absent	present	absent	absent	present
25	26	52	228	9	absent	present	absent	absent	<threshold	absent	absent	present
26	21	2	43	9	absent	present	absent	absent	present	absent	present	present
27	46	7	198	104	absent	present	absent	absent	present	absent	absent	present
28	221	172	203	184	<threshold	present	absent	absent	present	absent	absent	present
29	41	187	78	194	absent	present	absent	absent	present	absent	absent	present
30	16	67	233	54	absent	present	absent	absent	present	absent	absent	present

lab nr	sample number				Land				Fish			
					0%	0.1%	0%	0%	0.1%	0%	0%	0.1%
					A	B	C	D	A	B	C	D
32	206	212	108	24	absent	present	absent	<threshold	present	absent	absent	present
33	66	267	133	114	absent	present	absent	absent	present	absent	absent	present
34	71	12	138	124	absent	present	absent	absent	present	absent	absent	present
35	36	127	113	244	<threshold	present	absent	absent	absent	absent	absent	present
36	141	22	268	144	<threshold	present	absent	<threshold	present	absent	absent	present
37	146	62	123	199	<threshold	present	absent	absent	present	absent	absent	present
39	136	57	8	64	absent	absent	absent	absent	present	present	absent	present
40	116	177	13	74	<threshold	present	absent	absent	present	absent	absent	present
43	111	157	168	209	absent	present	absent	absent	present	absent	absent	present
46	76	42	83	174	absent	present	absent	absent	present	absent	<threshold	present
47	211	102	178	134	<threshold	present	<threshold	present	absent	<threshold	absent	present
51	86	107	148	139	absent	present	absent	absent	present	absent	absent	present
52	106	137	238	219	absent	<threshold	absent	absent	<threshold	absent	absent	<threshold
53	51	132	143	59	absent	present	absent	absent	present	absent	absent	present
54	126	87	173	149	absent	<threshold	absent	absent	<threshold	absent	absent	<threshold
56	56	217	28	224	absent	present	absent	absent	present	present	absent	present

Annex 8 Results: PCR ruminant

Participant	A whey	B 0.1% MBM	C whey	D -
3	pos	pos	pos	pos
4	pos	pos	pos	neg
6	pos	pos	pos	neg
9	pos	pos	pos	neg
12	pos	pos	pos	neg
13	pos	pos	pos	neg
15	pos	pos	pos	neg
26	pos	pos	pos	neg
31 *	pos	pos	pos	neg
35	pos	pos	pos	pos
38 *	pos	pos	pos	neg
40	pos	pos	pos	neg
52	pos	pos	pos	neg
54	pos	pos	pos	neg

*: exclusively PCR; no microscopic results.

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

RIKILT report 2015.016

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.

To explore the potential of nature to improve the quality of life

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

RIKILT report 2015.016

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.