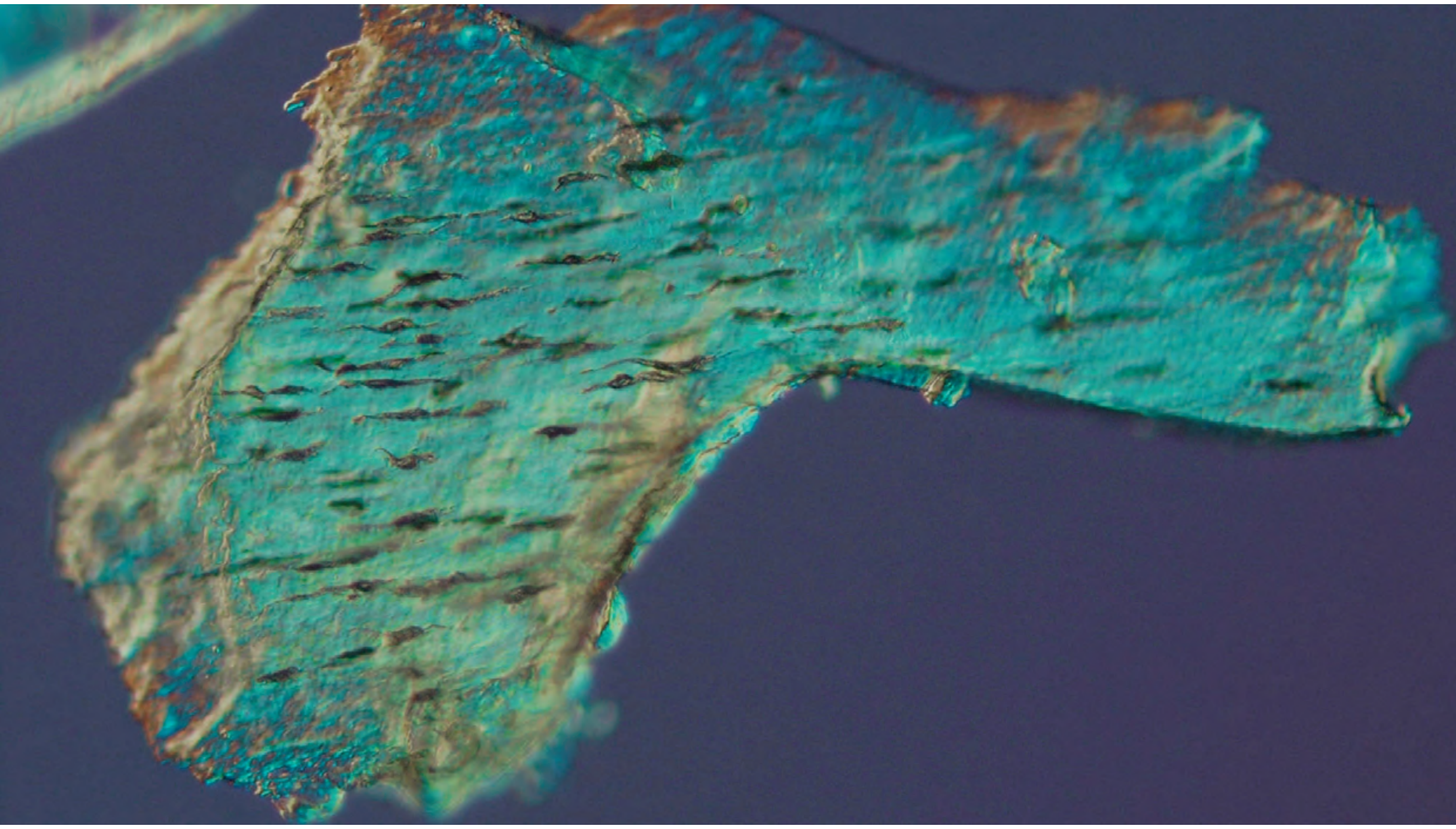

Animal proteins in feed

IAG ring test 2012

RIKILT Report 2012.009

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Cover photo: salmon bone fragment in differential phase contrast.

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 - Institute of food safety, Wageningen University and Research Centre, The Netherlands. The aim of the ring study was to provide the participants information on the local implementation of the detection method for their individual quality systems. A further aim was to gather information about the application of the microscopic method.

Of the four samples prepared three were based on a ruminant feed as matrix: one containing no animal proteins (blank), one with 0.1% of land animal material, and one with 0.02% of land animal material. The fourth sample consisted of a fish meal fortified with 10% of salmon material. All participants were requested to determine the presence or absence of land animal and/or fish protein material and to indicate the type of material found. The participants were also asked to report the amount of sediment found (the fraction containing minerals and bones, if present) and to answer questions on a series of parameters of the microscopic method. Reporting the estimated amount of land animal or fish protein was optional for all participants. 53 Participants returned results using the microscopic method. The three feed samples were evaluated as a proficiency test in a strict sense, whereas the fortified fish meal was considered a challenge sample.

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. Specificity scores for the feed samples were all slightly below 0.95. The detection of the materials of land animals was good in all cases. The presence of material of land animals in the fortified fish meal was indicated in a series of occasions (16 false positives out of 53 observations). Although it can not be ruled out that an occasional trace of avian material is present in a fish meal, this specificity score of 0.70 can be considered to be mainly the effect of the difficult discrimination between salmon bone fragments and land animal material. The staining of the sediment with Alizarin and the use of a binocular appear to influence notable or even considerable the results in terms of specificity and sensitivity. A further harmonization is still possible.

The amount of land animal proteins in the feed was overestimated. All estimations appeared to be significantly deviating from the actual amount below a level of p = 0.5%.

The results give a good overview of the proficiency of the labs performing the microscopic method, although further improvement is desired.

Contents

1 Introduction

Member states of the European Union are requested by EU legislation to maintain an active monitoring program for the safety of feed. The monitoring of the presence of animal proteins in the framework of eradication of mad cow disease is an important part of it. A range of official control methods was in 2009 combined in one Regulation (152/2009/EC). With respect to animal proteins, the microscopic detection method is the only official control method until now. Much effort was put into the further development of identification methods such as PCR and immunoassays (van Raamsdonk et al., 2007; Woodgate et al., 2009; Fumiere et al., 2009; Liu et al., 2010). In the view of the relaxation of the ban on animal proteins, with the authorization of non-ruminant processed animal proteins for aquafeed as first step, PCR will be included in Annex VI of Regulation (EC) 152/2009.

The level of contamination of 0.1%, as stated as performance parameter for official control methods in Annex VI of Regulation 152/2009/EC, forms the basis of most proficiency tests and collaborative studies to establish lab performance and to validate new methods. It is nevertheless obvious that several methods, microscopy a.o., can detect contaminations at lower concentration levels (e.g. Veys et al., 2010).

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. In this report the ring test for animal proteins is presented, which was organised by RIKILT in 2012 on behalf of the IAG Section Feeding stuff Microscopy. A contamination level below 0.1%, i.e. 0.02% of animal proteins from terrestrial animals is also part of the design of this ring test. The possibility that salmon material can confuse the proper detection of material of land animal was used as reason to include a fish meal mixture fortified with 10% of salmon meal.

The indication “ring test” fits in the history of annual proficiency tests for animal proteins carried out under the responsibility of the IAG. The main purpose of the ring test is to monitor the performance of the participating laboratories (internal quality assurance). The main part of this report presents and discusses the results in terms of sensitivity and specificity scores. For a further documentation of laboratory results each participant answered questions on details of the application of the method. These results can be used to interpret the effectiveness of some method parameters.

2 Methods

2.1 Materials

Four samples were produced, based on a cattle feed that was commercially produced, called Prima Biks Ambitie Z25-55873 (samples A, B, D), and a fish meal (sample C). This feed was also used in 2011 (van Raamsdonk et al., 2011).

The ring trial consisted of four samples with a composition as listed in Table 1.

Table 1. Composition of the samples in the NRL-IAG ring trial 2012.

Label	Content
2012-A	Feed with 0.1% MBM
2012-B	Blank feed
2012-C	Fish meal fortified with 10% of salmon meal
2012-D	Feed with 0.02% MBM

The feed material contained the following major ingredients in order of decreasing share: wheat gluten feed, sugar beet pulp, palm kernel flakes, rape seed meal, beet vinasse, beet molasses, maize, wheat, semolina, citrus pulp, vegetal fatty acids, calcium carbonate, and magnesium oxyde. The ingredients were glued together with molasse to form larger particles. Therefore, the feed material was sieved at 2 mm in order to exclude these particles from the samples, avoiding the possibility of animal proteins adhering to them. The composition might be modified as a result of this procedure. Five samples of this feed have been tested microscopically and by means of PCR at RIKILT for the presence of animal proteins (ruminant, pig). No material of the species mentioned was found.

The fish material consisted of a mixture of five different samples obtained from the RIKILT regular monitoring program, all reported negative for land animal material. Three different portions of this mixture were tested by microscopy for other animal proteins than fish and found negative. It was chosen to test the possibility that the presence of salmon material could interfere with a proper identification of terrestrial animal material. Seven samples of salmon meal were tested for the presence of animal material, since the presence of material of sea mammals can never be ruled out in fish meal samples from practice. In four samples bone particles were found for which an undisputable fish origin could not be established. These samples have been rejected because an incorrect identification of fish material might not be violated by a possible presence of sea mammal material. The three remaining samples were mixed and added to the fish meal at a level of 10%.

The meat and bone meal (MBM) used was obtained from a Dutch proficiency test. It is a lamb meal with a bone fragment share of 41%. Microscopic tests in the framework of that proficiency test program and at RIKILT prove the purity of the MBM. No material of animal origin other than from land animals was found. After PCR a positive signal might be expectecd form a ruminant assay, while a cattle assay should result in a negative result.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order: 2012-B - 2012-D - 2012-A - 2012-C. All samples were prepared in a laboratory which is located at a distance from the RIKILT microscopy laboratory.

Jars for sample 2012-B and for sample 2012-D were filled with 40 grams of the pure feed, closed and set aside. Every jar for sample 2012-D was individually spiked with 8 mg of MBM.

Sample 2012-A was produced according to the method of stepwise dilution. 2.8 g of MBM was used to prepare (finally) 2.8 kg of contaminated feed as follows. The initial 2.8 g of MBM was mixed in 2.8 g of feed and stirred for one minute. In nine subsequent steps the remaining amount of feed was added stepwise by mixing according to a fixed scheme.

The samples 2012-A, 2012-B and 2012-D were set aside in order to avoid any contamination with fish meal.

Finally sample 2012-C was prepared by mixing thoroughly 175 g of salmon meal in 1575 g of fish meal. The final jars for sample 2012-C were filled with 40 – 45 grams of material.

2.3 Homogeneity study

Two RIKILT microscopists examined independently three jars of all four samples. In all cases but two a correct result was obtained (Table 2). In two out of the three jars of sample 2012-D traces of fish material (one fragment each) were found. Three new jars of samples 2012-B and of sample 2012-D were examined in addition. In all cases correct results for these jars were obtained.

The finding of two false positives was a major concern, which would lead to postponement the ring study. Several considerations were made before taking the final decision on this. The feed used as matrix was identical to that of 2011 (van Raamsdonk et al., 2011). In total, 26 samples of this feed (not intended to be aldulterated with fish material) investigated by RIKILT in 2011 and 2012 showed correct negative results for fish in 24 of these samples. The remaining two samples are the two samples in the current homogeniety testing. All participants in the 2011 ring test (van Raamsdonk et al., 2011: n=56) reported correct negative results for fish in the blank sample. Moreover, the samples were all produced in an animal proteins free laboratory in a specific order; fish material entered the contained room only after the production of the fish-free samples. Based on these circumstances, the finding of traces of fish material in the first set of jars of sample 2012-D was considered as laboratory contamination. The decision was taken that it was justified to send the sets of four samples around to all participants, with one stipulation. The samples were blindly put in the regular control of RIKILT after the closing date and prior to the final evaluation (April 2012). Sample 2012-A was excluded from this trial, since it would have been suspicious to offer a sample with 0.1% MBM in a regular control programme. The results of this blind trial were evaluated in this study, but not included in the general overview.

Measures have been taken to further minimise the possibility of laboratory contamination. The microscopy research group of RIKILT did not participate in the further laboratory analysis of this ring trial.

Table 2. Results of the homogeneity study. Sediment amounts are based on 10 grams, or 2 grams for the fish meal. The number of portions is indicated for two microscopists independently.

Sample			Sediment amount	Fish	MBM
2012-A	0.1% MBM	(n= 3)	4.2 - 5.4%	3 x negative	3 x positive
2012-B	blank	(n= 6)	4.2 - 5.8%	6 x negative	6 x negative
2012-C	fish meal fortified with salmon meal	(n= 3)	16.3 - 17.3%	3 x positive	3 x negative
2012-D	0.02% MBM	(n= 6)	4.8 - 6.4%	4 x negative*	6 x positive

* *See explanation in text.*

2.4 Organization of the ring trial

All IAG members, all NRLs, and a series of putative interesting laboratories were informed about the ring test for 2012. In all cases an invitation letter, a participation form and an invoice were distributed. Until the beginning of March a total of 54 participants were listed. The sets of four samples with an accompanying letter (see Annex I) were sent to all participants on the 29th of February 2012. On Thursday March 1st an E-mail message was sent around to all participants, together with an electronic report form (see Annex II and III) and the request to confirm the receipt of the package. The report form also contained a sheet with instructions (see Annex IV).

The closing date for reporting results was fixed at April 2nd. In one occasion the package with samples was lost by the courier. A second package was send, and a later deadline was agreed. Besides that, only one participant reported one day too late. All results were included in the final analysis. One participant did not submit any result at all. Therefore, a total of 53 sets of microscopic results could reliably be considered in the final evaluation. In addition to the 53 sets of microscopic results, three participants reported their results of PCR analysis, and one participant of immunoassay analysis. The report was finalised at May 10.

2.5 Participants

The 54 participants originated from 23 countries: 18 member states of the European Union, and five other countries (China, Norway, Peru, Serbia and Switzerland). The list of participants is presented in Annex V. Five member states have been involved with three or more participating laboratories: Germany (16 labs), Belgium (5), Italy (5), France (3) and the Netherlands (3). These figures are comparable to those of the ring test of last year (van Raamsdonk et al., 2011).

2.6 Analysis of results

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.

Significancy of quantitative results were tested by using Student's t-test statistics; see, for example, Hand (2009). Grubbs' outlier test was used to identify outliers in the data on sediment amounts, which were removed prior to further analysis. It was explicitly asked to report the amount of sediment obtained before any staining was applied.

Differences in the results after applying different parameters were analysed using Fisher's exact test (Fisher, 1945).

3 Results

Fifty-four packages with four samples were sent to all participants. Fifty-three participants returned results for the microscopic method, three sets of results were received for PCR analysis, and one set from one participant for protein detection. All results were received by E-mail, and in most cases a FAX message was sent as well. The procedure for FAX handling at RIKILT was changed during the reporting period, which makes it currently impossible to provide a figure for FAX response. One participant submitted a report sheet with the wrong participants number, which was corrected upon request. The link with the original E-mail message and sender could be established beyond doubt in all cases; otherwise these reports would have been omitted. All reports were included. The report sheet was produced in Office 2010 for the first time, which now included selection lists for all results, and lower and upper limit for quantified results. This strategy was succesful to avoid inconclusive results. The report sheet was transferred to Office 2003 format, which finally appeared to have resulted in some errors violating a smooth application. A second version was sent around with bug fixes. In all cases where a participant submitted results for the second time using the corrected report sheet, the latest version was used.

The full results are presented in the tables of Annex VI, VII and VIII. Sample 2012-C was a challenge to examine the possible confusion between salmon bone fragments and those of terrestrial animals. Therefore, the proficicency part of ring test consists of the samples 2012-A, 2012-B and 2012-D. The result for the salmon containing fishmeal will be treated separately.

3.1 Microscopic detection

The blind test trial of samples 2012-B, 2012-C and 2012-D carried out by RIKILT gave correct results in all cases.

Most of the specificity and sensitivity scores were at good or reasonable levels considering the samples for the proficiency test (Table 3; Annex VII). The scores for specificity are just below the level of 0.95.

Table 3. Sensitivity and specificity scores for the detection of animal proteins in four samples. Abbreviations: n: number of participants per group. Capitals A to D: sample indication.

		Fish				MBM			
		A	B	D	*C*	A	B	D	*C*
n		0	0	0	*100%*	0.1%	0	0.02%	*0*
53	Specificity	0.94	0.94	0.92			0.94		*0.70*
	Sensitivity				*1.0*	0.98		0.98	

With respect to the challenge to discriminate between fish material and terrestrial animal material, the presence of material of land animals in sample C was indicated in a series of occasions (16 false positives out of 53 observations).

The comments of the participants for documentation of the erroneous positive findings are presented in Table 4. For several observations only traces were indicated, for others higher amounts are reported, up to 1.93% for the share of land animal material in sample C (fishmeal fortified with 10% of salmon meal). The indication "bone" (singular) or "bones" (plural) is an unsecure indication, since the amounts of 1.95 and 0.96% are documented with the singular indication.

Factors such as laboratory skills, glassware used, and lab procedures on e.g. cleaning to avoid sample pollution (in the case of false positives) might influence the finding of false positives. In the paticular case of sample C misidentifications might be caused by the confusing bone fragments of salmon.

Table 4. Participants' comments on the background of the false positives reported for the calculations in Table 3.

Sample, contaminant	Participant	Comment
A: fish material	17	Bones, muscles, scales
	45	Bones
	54	-
B: fish material	12	0.05%: bone
	46	2.3%: bone, scale
	54	-
B: land animal material	28	Bone and feather material
	43	-
	46	0.6%: bone, hair
C: land animal material	7	Very few poultry bones: bones, feathers, muscles
	9	Bone
	17	0.5-1.5% MBM: bones, muscles
	22	Bone
	32	0.1% MBM: 5 bones in 4 slides = ?
	34	0.01% MBM: bone, cartilage
	36	One bone
	38	0.028% MBM: bones, muscles
	39	Bone, muscle
	41	0.15% MBM: bones
	43	1.93% MBM: bone
	44	0.26% MBM: bone
	48	0.02%: bone and muscle
	49	-
	50	0.1% MBM
	54	-
D: fish material	5	0.06%: bones and scales
	7	Bones, only traces of muscles
	22	0.015%: bone, scale
	46	0.96%: bone

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 VI and summarised in Table 5. The main purpose of this inventory was to provide 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 frequencies of application of choices for several method parameters are presented in Table 5.

Table 5. Inventory of parameters for microscopic detection and their application.

Parameter	Parameter state	Number of participants	Amount
Amount of material used for sedimentation of feed	5 grams	2	
	10 grams	50	
	other	1	
Type of glassware	chemical sedimentation funnel	28	
	beaker (flat bottom)	7	
	champagne glass	7	
	conical glass with cock	6	
	other	4	
Sedimentation agent	TCE	53	
	TCE/Petroleumether	0	
	other	0	
Use of staining of sediment	no	31	
	yes	22	
Use of binocular for examination at lower magnifications	yes	42	
	no	11	
Size of cover glass used	small (e.g. 20 x 20 mm)	32	
	medium	7	
	large (e.g. 26 x 50 mm)	13	
Share of the total sediment used for examination	minimum		2%
	maximum		100%
Embedding agent	paraffin oil	12	
	immersion oil	12	
	glycerine / glycerol	16	
	Norland Adhesive	7	
	other (water, glycerol:water mixture, mineral oil)	4	
Use of ARIES	yes	3	
	no	48	
f-factor for MBM	minimum		15%
	maximum		60%
	none estimated	19	

The results as presented in Table 5 show generally a good application of the method. Differences with previous years will be presented in the next chapter (Discussion).

Only a very low share of the participants used the knowledge system ARIES (van Raamsdonk et al., 2004, 2010). The information in this system could support the discrimination between confusing particles of land animals and fish.

Correlations between specificity and method parameters are relevant only if some sort of causal relationship exists in order to avoid the analysis of random fluctuations of results. In the process of further harmonisation of the microscopic method, almost all participants made the same choice for the application of several parameters: amount of material used for sedimentation (10 grams), sedimentation agent (TCE), non-suited embedding agent: only one participant. As far as substantial numbers among the participants have applied different parameters of the method (see Table 5), actually some differences were found between the results. These situations are linked to the use of alizarin staining and the use of a binocular. Details will be presented in the next paragraphs.

3.2.1 Staining of sediment

Staining of the sediment material with Alizarin Red is applied by 41.5% of the participants, with the goal to facilitate an initial and/or better recognition of bone particles. The results with respect to specificity and sensitivity scores are presented in Table 6. The differences between the results after staining compared to the results after not staining the sediment indicated less optimal results for fish detection after staining. Although none of the differences is statistically significant, especially in the blank (2012-B) all false positives for both fish and land animal material were reported after staining (p=0.066). It could be the situation that non-animal particles show a staining response in such a way that recognition as animal material could not be ruled out.

Table 6. Sensitivity/specificity scores for the detection of animal proteins in four samples, separate for analyses based on a stained or an unstained sediment. Abbreviations: n: number of participants per group. Capitals A to D: sample indication. P: probability of a significant difference according to Fisher's exact test.

Staining of sediment	n		Fish				MBM			
			A	B	D	*C*	A	B	D	*C*
			0	0	0	*100%*	0.1%	0	0.02%	*0*
Stained with alizarin	22	specificity	0.91	0.86	0.91			0.86		*0.73*
		sensitivity				*1.00*	0.95		1.00	
Unstained	31	specificity	0.97	1.00	0.94			1.00		*0.68*
		sensitivity				*1.00*	1.00		0.97	
p			0.306	0.066	0.367	*1.0*	0.415	0.066	0.585	*0.223*

3.2.2 Binocular

A vast majority of the participants used a binocular for examination at low magnification (Table 7: 79.2%). The differences between the two groups, whether or not using a binocular, are minor and evenly in favour of either group considering the three samples of the proficiency test. However, all false positive results for the detection of land animals in fish meal (2012-C) were obtained in combination with examination with a binocular. This difference appeared to be statistically significant (p=0.011).

Table 7. Sensitivity/specificity scores for the detection of animal proteins in four samples, separate for analyses based on the use of a binocular. Abbreviations: n: number of participants per group. Capitals A to D: sample indication. P: probability of a significant difference according to Fisher's exact test.

Use of binocular	n		Fish				MBM			
			A	B	D	*C*	A	B	D	*C*
			0	0	0	*100%*	0.1%	0	0.02%	*0*
Binocular used	42	specificity	0.93	0.98	0.93			0.95		*0.62*
		sensitivity				*1.00*	0.98		0.98	
Binocular not used	11	specificity	1.00	0.82	0.91			0.91		*1.00*
		sensitivity				*1.00*	1.00		1.00	
p			0.490	0.099	0.431	*1.0*	0.792	0.404	0.792	*0.011*

3.3 Quantification

The starting amount of material for sedimentation will obviously influence the results of quantification. Contrary to the previous years, the amount of sediment in the current study was calculated per gram of material used, and presented as mg/g in Table 8. In this way there is no influence of the amount of material used as reported by the participants nor of the application of Alizarin staining. In the current set of results a number of very high sediment amounts was reported, especially by participant 26 and 28 (see Annex VIII; the values in the Annex are shown as reported). Values of 19 mg/g and higher (eight values) appeared to be outliers after iterative application of Grubbs' test and were removed prior to analysis. There is also a large variation in the sediment amounts of the fish meal (2012-C). These results, however, are not analysed further since no comparison can be made to other results for the uniqueness of the material used.

Table 8. Resulting amounts of sediment (in mg/g) separate for the application of staining of the sediment. For every result the average (in normal) and standard deviation (in italics) is given. Calculations were based on data after removal of outliers. Ten participants did not report results for quantification.

	n	amount of sediment (mg/g)		
		A	B	D
total	43	8.189 (2.882)	7.454 (2.701)	8.232 (3.207)

The estimated amounts of MBM in three samples showed a very large variance (Table 9). The average estimates of MBM in both samples A and D were significantly higher than the actual amount.

Table 9. Estimations (in %) for the amount of MBM in three samples. For every result the average (in normal) and standard deviation (in italics) is given. Eighteen participants did not report these results.

	estimated amount MBM		
	A 0.1%	D 0.02%	C 0% In fish meal
total	0.28% (*0.35*%)	0.09% (*0.11*%)	0.40% (*0.65*%)
n participants t statistic	35 3.043 **	35 3.765 **	9 1.846 *

*: $p < 5\%$.
**: $p < 0.5\%$.

3.4 Detection by other methods

Three participants made nine PCR runs in total, all with primer sets for different target animals (Annex IX). All wrong results are false positive reports. The presence of sheep material could not be detected by a cattle/bovine test as used by two participants, but it is remarkable that the same two participant reported the presence of pig material. No quantitative results were submitted.

One participant (nr 30) reported two sets of results for immunoassay analysis (Annex X). No correct positive result was reported. This could be reasonable considering the low contamination levels.

4 Discussion and conclusions

4.1 Method performance

The presence of contaminated samples with fish material, although at extremeley low levels (one particle), might hamper to draw reliable conclusions. The homogeneity studies in two ring tests for two subsequent years based on the same feed as matrix, the correct negative results in the blanks of the 2011 study (van Raamsdonk et al., 2011), the fully contained production of the samples, and the correct results of the blind trial of the samples at RIKILT justify the conclusion that the contamination was caused by a lab accident. Moreover, results per participant are presented "as is" and will never be reported in comparison to a performance limit.

The results as obtained in this latest version of the annual IAG ring tests for microscopic detection of animal proteins in feed is slightly less optimal as in previous years (Table 10). Considering a level of 0.95 as lower limit for the performance parameters sensitivity and specificity, the specificity in blank samples was below that limit for the first time since 2008 (land animal material) or since 2006 (fish material). The specificity for the detection of fish in the presence of 0.02% is in line with previous results. The sensitivity of the detection of 0.02% MBM could be considered as very good (Regulation (EC) 152/2009: detection limit of 0.1%), also in the view of earlier results (Table 10).

Table 10. Results for detection of material of terrestrial animals and of fish in feed samples of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008-2012) 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	4-5%	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=54) current study	0.94			0.98		0.98	0.94	0.96	0.92

The examination of fish meal fortified with 10% of salmon material was additional to the proper proficiency testing. The results for the detection of land animal material in this sample of fish meal are very remarkable: 14 out of 53 sets of results showed a false positive result. In nine occasions this presence was quantified between 0.01% and 1.93%. It is known that fish meal frequently contains DNA of avian origin (EURL, oral communication), which could implicate that some of the encountered bone fragments do originate from avian sources. The reported levels, however, are too high to accept this as the full explanation. Salmon meal consists of bone fragment structures which can mimic that of mammalian bones (Makovski et al., 2010; Veys et al., 2011). Examples are included in the EURL-AP Image Bank and in the expert system ARIES (van Raamsdonk et al., 2010). The current results indicate that fish meal parties can cause serious problems in the discrimination between land animal material and certain fish species.

The results for the PCR and immunoassay methods are not in line with recent but still unpublished results. Nevertheless, several studies indicate the occurrence of false positive results in a variety of occasions (Prado et al., 2007; Fumière et al., 2011). Further ring tests are recommended for monitoring improvements.

4.2 Method parameters

A proficiency test is meant to reveal information on the performance of individual labs. It is not possible to draw conclusions about the validity of the method(s) applied (von Holst et al., 2005). In certain occasions a questionnaire is send around with the samples, which can be used to evaluate the way in which the method is implemented. The current and previous ring tests of IAG are examples of those “extended proficiency tests”. Although method validation is principally impossible, improvements of method implementation and relationships with the results can be discussed (van Raamsdonk et al., 2012).

Table 11. Comparison between parameters distribution in the IAG 2008, 2009, 2010 and 2011 study.

Parameter	Parameter choice	2008	2009	2010	2011	2012
Amount of material used for sedimentation	5 grams	16	5	3	3	2
	10 grams	26	41	48	50	50
	other	3	3	2	3	1
Type of glassware	chemical sedimentation funnel	22	28	31	33	28
	beaker (flat bottom)	11	13	10	9	7
	champagne glass	6	5	8	7	7
	conical glass with cock	3	1	2	3	6
	other	3	2	2	3	4
Use of staining of sediment	no	31	35	34	33	31
	yes	14	14	19	22	22
Use of binocular for examination at lower magnifications	yes	29	40	45	44	42
	no	16	9	8	12	11
Number of slides used	minimum	1	1	n.d.	n.d.	n.d.
	maximum	7	14	n.d.	n.d.	n.d.
Size of cover glass used	small (e.g. 20 x 20 mm)	34	27	27	36	32
	medium	1	9	10	8	7
	large (e.g. 26 x 50 mm)	9	13	16	12	13
Share of the total sediment used for examination	minimum	4%	2%	2%	0.2%	2%
	maximum	100%	100%	100%	100%	100%
Embedding agent for sediment	paraffin oil	18	20	23	20	12
	immersion oil	8	12	14	12	12
	glycerine / glycerol	8	10	12	12	16
	Norland Adhesive	0	2	2	6	7
	chloral hydrate	3	1	0	0	0
	other (e.g. Depar 3000, water)	8	4	2	5	4

As shown in Table 11, a status quo in the shift of method parameters can be found. Still some participants use only 5 grams of material for sedimentation, the use of glassware allowing the release of the sediment at the bottom is slightly increasing, as is the number of participants that apply staining of the sediment. Eleven participants did not use a binocular, although this is requested according to the official protocol.

There is an interesting link between two parameters and the performance results. The performance of the microscopic method is generally lower after applying staining of the sediment with Alizarin, especially with respect to the false positives in the blank sample. This difference is, however, not statistically significant (Table 6, sample 2012-B: p=0.066). These results are in concordance wth those of the revious IAG ring test (van Raamsdonk et al., 2011). The detection of land animal material in the fortified fish sample (2012-C) is, however, slightly better after staining. A strong correlation (p=0.011) can be observed between the use of a binocular and the false reporting of the presence of land animal material in the fortified fish meal sample (Table 7). This correlation can be biased due to the uneven numbers of participants (11 vs 42). It can nevertheless be assumed that examination at lower magnification levels causing a first (wrong) interpretation finally can lead to wrong results. Examination by means of a binocular is still important since it allows an easy examination of the whole sediment, but it is needless to emphasize the need for a sufficient level of expertise. The differences between using or avoiding a binocular are limited for the other samples, as was found by Veys et al. (2011) as well. Confusion resulting in misinterpretation of certain types of particles was concluded in the IAG ring test of 2011 with respect to feather meal (van Raamsdonk et al., 2011). In general, salmon bone fragments as confusing elements are an example, among others, which indicate that expertise is important. Besides that, a further harmonisation of the application of the method is still recommended.

4.3 Quantification

The results of sedimentation can be compared between the years of 2011 (van Raamsdonk et al., 2011) and 2012 (current study), since the same feed has been used. Three samples has been based on this feed in both years; the three average amounts of sediment per sample within a year are not significantly different (p > 5%; results not shown). The results pooled within years are shown in Table 12.

Table 12. Amounts of sediments (in mg/g) over two years of the same feed.The calculation are applied after testing for and, if necessary, the removal of outliers.

	RIKILT			Population			
	mean	SD	n	mean	SD	n	n outliers
2011	4.900	0.385	6	6.882	3.336	79	0
2012	5.373	0.656	15	7.960	2.936	139	8

The results as obtained by RIKILT are highly comparable over the years 2011 and 2012. The amounts of sediment as obtained by the participants are clearly higher. The number of results included in 2011 is relatively low due to the sitution that all results in combination with staining of the sediment were omitted. In 2012 it was explicitly asked to report the amount of sediment prior to the application of further staining, which means that all results could be included. The population means are clearly higher than the mean sediment amounts as achieved by RIKILT. A significant difference, however, could not be proven since the population standard deviations are quite high. Nevertheless, the population mean in 2012 is notably high, even after the removal of eight outliers as based on the iterative application of the Grubbs' test. All results equal to or higher than 19 mg/g were proven to be outliers (maximum: 57.7 mg/g (participant 39)). These results can be assumed to indicate differences in the way the details of the method are implemented by the different laboratories.

The averages of the quantification results of the animal proteins of terrestrial animals show a diverse pattern. The estimation for MBM in the feed (sample A: 0.1%, sample D: 0.02%) show overestimation. The usual situation is that ingredients with a low share in the total composition are overestimated (unpublished results of ring trials of IAG Section Feeding-stuff Microscopy). The Student's t-test show significant differences between the actual and estimated amounts (below or far below p = 1.0%). This means that individual results should be expected to be no reliable indicator of the real amount of MBM in a sample.

5 General conclusions and recommendations

5.1 Conclusions

In certain occasions reporting errors were noticed. These problems mainly apply to inconsistent reporting (wrong or missing unique laboratory number: one occasion), and too late reporting (one occasion). Some problems with the custom procedures of certain countries were encountered.

The proficiency test showed generally excellent results for sensitivity for the detection of land animal material (0.98). Specificity (erroneous reporting of an ingredient) was generally less optimal (0.92 to 0.94). The challlenging sample of fish meal fortified with 10% of salmon meal showed a specificity for the detection of land animal material of 0.70. The legislation states that a method for detection of animal proteins in feeds should be able to detect a level of contamination of 0.1% at the least. The current study indicates that a level of 0.02% of MBM in a feed does not give any problems. Confusion of fish material and land animal material still needs attention.

The reported amounts of sediment show a large variation. The estimation of the amounts of MBM in either feed or fish meal show a diverse pattern. In any case a significant difference exists between the actual and estimated amount. Quantification based on microscopic observations still shows a disputable reliability.

A further harmonization of the application of the microscopic method was achieved in the past years. This is especially indicated in the predominant use of 10 grams of material for sedimentation, and the use of a stereo microscope for the examination of the entire sediment. In the current ring test no gain was achieved in a further optimisation. It was shown by the amounts of sediment achieved that the specific implementation of the method is different among the laboratories. A further harmonization is still possible for some other parameters.

5.2 Recommendations

- The specificity of the microscopic method for proper detection of confusing particles still needs attention. Training of microscopists remains important.
- Evaluation of the full application of the method (e.g. examination of sample or flotate, use of binocular) is desired.
- It is recommended to evaluate further the effect of several method parameters because of large variation of application.
- Further ring tests are recommended to confirm the results of the tests with PCR and immunoassays.

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Annex I
Invitation letter

Dear colleague, Dear IAG member,

The IAG section Feeding stuff Microscopy organizes annually a ring test for the detection of animal proteins in animal feeds. As in previous years, the presidium of the IAG section Feeding stuff Microscopy and RIKILT have agreed to organize together the 2012 ring test for animal proteins under certain conditions.

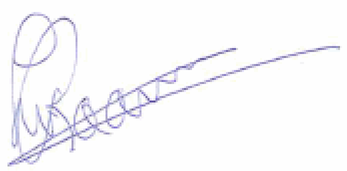
On behalf of the IAG section Feeding stuff Microscopy, RIKILT will invite you for participation in this next ring test. The share in the costs of the 2012 ring test as asked from every participant will be a fee of € 200, which is the same as in the previous years.

Three or four samples will be send around late February or early March 2012. Also a questionnaire will be sent by E-mail. A time slot of four weeks is planned for the analyses of the samples by every participants This means that late March or early April all results are expected to be returned to RIKILT. Pooling and evaluation of the results will take place during April and May, and a preliminary report will be presented during the annual IAG meeting in Tervuren (Belgium) in June. After that, a final report will be made depending on the outcome of the discussions during the meeting. All communications of the evaluation will be fully anonymous.

If you are interested to participate in the ring test 2012 for animal proteins, please return the application form and make a payment of € 200 to RIKILT. For smoothing the administrative procedure, an invoice is already included with this letter. In case of participation, please hand this invoice over to your financial department, and 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 can not be linked to participating institutes.

We are looking forward to have a nice cooperation for the next ring test and to have results which will support your laboratory quality system.

On behalf of the IAG section Microscopy and the RIKILT organizing team,



Dr. L. van Raamsdonk

Annex II
Report form for procedure details

Please complete at least all the cells with a drop down list that apply to your procedure	select your choice from a drop down list	type in your answer if necessary
IAG ring test 2012		
Please select your unique lab number	-- select --	
Have you read the ring test instructions?	yes	
Which detection method do you use?	Microscopy	
Please skip this line	-- select --	
Please continue here		
Please indicate your starting amount of material for sedimentation of FEED material	-- select --	
if other, please specify		
Please indicate your starting amount of material for sedimentation of FISH 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 --	please indicate the sediment weight BEFORE staining at the report sheet
Did you examine at lower magnifications (using a binocular)?	-- select --	
Indicate the size of cover glass	-- select --	
Please estimate the amount of sediment you have used for preparing the slide(s) (in %)		
Please describe your embedding agent for the sediment material	-- select --	

Please complete at least all the cells with a drop down list that apply to your procedure	select your choice from a drop down list	type in your answer if necessary
if other, please specify		
Did you use the expert system ARIES for identification of particles?	-- select --	
When estimating amounts:		
please indicate the f-factor used for fish meal		
please indicate the f-factor used for terrestrial animal meal		

Annex III
Report form

IAG ring test 2012

lab number: 0

sample number	2012-A	2012-B	2012-C	2012-D
weight of sediment				
presence of fish material				
if present, estimated amount				
presence of material of land animals				
if present, estimated amount				
Comment, if necessary				

Signature:

Date:

Annex IV
Instructions as included in the form

IAG ring test 2012

Instructions for the IAG ring trial

1	You have received a box with an introduction letter and four vials containing 40 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 152/2009/EC from the European Union. Identical procedures can be found in the module Methods of the computer program ARIES. It is recommended to start the sedimentation procedure with 10 grams of material. Take care to homogenise the content of each vial before taking the amount for analysis.
3	Reporting consists of the following steps:
3a	Please fill in the questionnaire on the page "Procedure". Depending on your chosen method, different questions will show up. 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. 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. Select "yes" from the drop-down list if fish or land animal material is detected, or "no" if the respective type of material is absent. You are free to give an estimation of the amount of material found. Please indicate the type of the materials found. More than one indication can apply, e.g. "bone and muscle". All fields with a drop-down list have to be completed. Please add the exact sediment weight in milligrams, without a decimal sign.
4	After completing the two forms "Procedure" and "Results", they have to be sent to the organisers in two ways:
4a	A print out of both forms have to be sent by Fax to RIKILT, Wageningen, the Netherlands. The FAX number will appear in the forms as soon as they are completed.
4b	The forms have to be sent to by E-mail as well. Save the Excel file by using "Save as ...", add your unique lab code to the end of name (just before ".xls") and send the file to leo.vanraamsdonk@wur.nl.
4c	Results will be included in the final analyses and report only if both forms are send in by FAX as well as 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 2th, 2012.

RIKILT Institute of food safety, Wageningen, the Netherlands

Annex V
List of participants

Institute	City	Country
Austrian Agency for Health and Food Safety-AGES	A-1226 Vienna	Austria
CRA-W	B-5030 Gembloux	Belgium
AFSCA/FAVV	B-4000 Liege	Belgium
FLVVT	B-3080 Tervuren	Belgium
Laboratorium ECCA nv	B-9820 Merelbeke	Belgium
Oleotest N.V.	B-2660 Antwerpen	Belgium
China Agricultural University	100083 Bejing	China
Central Institute for Supervising and Testing in Agriculture	Prague 5-Motol	Czech Republic
Danish Veterinary and Food Administration	DK-2800 Lyngby	Denmark
IDAC	44327-Nantes cedex	France
S.C.L. Laboratoire de Rennes	F-35000 Rennes	France
IPL Atlantique	F-33000 Bordeaux	France
Landesbetrieb Hessisches Landeslabor, Landwirtschaft und Umwelt	D-34128 Kassel	Germany
LTZ Augustenberg	D-76227 Karlsruhe	Germany
LUFA-Speyer	D-67346 Speyer	Germany
Landeslabor Berlin-Brandenburg	D-14473 Potsdam	Germany
SGS Germany GmbH	D-21035 Hamburg	Germany
CVUA-RRW	D-47798 Krefeld	Germany
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	D-85764 Oberschleissheim	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	D-04159 Leipzig	Germany
Universität Hohenheim, LA Chemie (710)	D-70599 Stuttgart	Germany
LUFA Nord-West	D-26121Oldenburg	Germany
LLFG Landesanstalt für Landwirtschaft	D-06120 Halle	Germany
Thüringer Landesanstalt für Landwirtschaft	D-07743 Jena	Germany
Agri Q-service GmbH	D-48155 Münster	Germany
Inst. Fur Veterinar-Pharmakologie und Toxicologie	D-16321 Bernau Bei Berlin	Germany
LUFA Rostock	D-18057 Rostock	Germany
Futtermittelinstitut Stade (LAVES)	D-21680 Stade	Germany
MGSZH ÉTBI TAKARMÁNYVIZSGÁĹĆ NEMZETI LABORATÓRIUM	H-1144 Budapest	Hungary
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Celbridge, Co. Kildare	Ireland
Equine Centre	Naas, County Kildare	Ireland
IZS PLV Torino - CReAA	I-10154 Torino	Italy
Ist. Zooprofilattico Sperimentale delle Lombardia e dell'Emilia Romagna	I-25121 Brescia	Italy
Inst. Zooprofilattico Sperimentale delle Venezie	I-35020 Legnaro	Italy
Istituto Zooprofilattico Sperimentale Abruzzo & Molise "G. Caporale"	I-64100 Teramo	Italy
Istituto Zooprofilattico della Sicilia	I-90129 Palermo	Italy
Natl. Food and Veterinary Risk Assessment Institute	LT-08409 Vilnius	Lithuania
MasterlabBV	NL-5831 JN Boxmeer	Netherlands
CCL - Nutricontrol	5462 GE Veghel	Netherlands

Institute	City	Country
Labco	3198 LC Europoort-Rotterdam	Netherlands
Nofima Ingredients	N-5141 Fyllingsdalen	Norway
International Analytical Services SAC	Lima	Peru
SGS del Perú S.A.C.	Lima	Peru
Lab. Regional de Veterinária	PT 9700-236 Angra do Heroismo	Portugal
Laboratório Nacional de Investigação Veterinária INRB, IP	PT 1549-011 Lisboa	Portugal
SC Alcoprod Services SA	RO-925300 Urziceni	Romania
Scientific Veterinary Institute "Novi Sad"	21000 Novi Sad	Serbia
State Veterinary and Food Institute	04001 Kosice	Slovakia
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	SLO-1000 Ljubljana	Slovenia
Dirección General de Produccion Agropecuaria, Laboratorio Agrario Regional	E-09071 Burgos	Spain
Trouw nutrition Espana	E-28760 Tres Cantos (Madrid)	Spain
SVA	SE-75189 Uppsala	Sweden
Agroscope (ALP), Swiss Research Station	CH-1725 Posieux	Switzerland
LGC	Middlesex TW11 0LY	UK

Annex VI
Details of procedures applied, microscopic method

Lab nr	Amount		Glassware	Agent	Staining	Binocular	Size	Sed. used	Embedding	ARIES	f-factor
	Feed	Fish								Used	MBM
1	10		chem.sed.funnel	TCE	yes	yes	small	100%	Norland	no	40%
2	10	10	chem.sed.funnel	TCE	yes	yes	large	100%	glycerol	no	25%
3	10	10	conical glass with cock	TCE	no	yes	large		glycerol	no	
4	10	10	chem.sed.funnel	TCE	yes	yes	small	80%	Norland	no	40%
5	10	10	chem.sed.funnel	TCE	yes	yes	small	50%	Norland	no	40%
6	10	10	conical champagne glass	TCE	no	yes	small	100%	immersion oil	no	60%
7	10	10	beaker (flat bottom)	TCE	no	yes	small		immersion oil	no	
8	10	5	chem.sed.funnel	TCE	no	yes	small	20%	xylol	no	
9	10	10	chem.sed.funnel	TCE	yes	yes	small	75%	glycerol	no	
10	10	10	beaker (flat bottom)	TCE	no	no	small	100%	phenol glycerol	no	50%
11	10	10	conical glass with cock	TCE	no	yes	medium	100%	immersion oil	no	
12	10	10	chem.sed.funnel	TCE	yes	no	small	20%	Norland	no	30%
13	5		chem.sed.funnel	TCE	no	yes	small		water	no	40%
14	10	10	chem.sed.funnel	TCE	no	yes	large	100%	glycerol	no	
15	10	10	beaker (flat bottom)	TCE	no	yes	small	30%	immersion oil	no	
16	10	5		TCE	no	no	small	80%	Mineral oil	no	
17	10	10	conical glass with cock	TCE	no	yes	small	2%	paraffin oil	no	50%
18	10	5	beaker (flat bottom)	TCE	yes	yes	small		immersion oil	yes	
19	10	10	beaker (flat bottom)	TCE	no	no	small	100%	immersion oil	no	50%
20	10	3	chem.sed.funnel	TCE	yes	yes	small	40%	Norland	no	
21	10	1	chem.sed.funnel	TCE	yes	yes	small		glycerol	yes	40%
22	10	10	chem.sed.funnel	TCE	no	yes	large	100%	glycerol	no	40%

Lab nr	Amount		Glassware	Agent	Staining	Binocular	Size	Sed. used	Embedding	ARIES	f-factor
	Feed	Fish								Used	MBM
23											
24	10	10	conical champagne glass	TCE	yes	yes	small	100%	glycerol	no	40%
25	10	10	conical champagne glass	TCE	no	yes	small	95%	immersion oil	no	
26	10	5	chem.sed.funnel	TCE	no	no	small	20%	glycerol	no	40%
27	10	5	chem.sed.funnel	TCE	yes	yes	medium	50%	glycerol	no	40%
28	10	10	tube	TCE	yes	yes	large	6%		no	
29	5	5	chem.sed.funnel	TCE	no	yes	large	50%	paraffin oil	no	20%
30	10	10	conical glass with cock	TCE	no	no	small	25%	immersion oil	yes	
31	10	10	conical champagne glass	TCE	no	yes	large	100%		no	50%
32	10	10	chem.sed.funnel	TCE	yes	yes	medium		glycerol	no	40%
33	10	10	conical glass with cock	TCE	no	no	small	25%	glycerol	no	
34	10		chem.sed.funnel	TCE	no	yes	medium	75%	paraffin oil	no	
35	10	10	conical champagne glass	TCE	no	yes	small	50%	paraffin oil	no	
36	10	10	chem.sed.funnel	TCE	yes	yes	medium	5%	glycerol	no	
37	10	10	chem.sed.funnel	TCE	no	yes	large	60%	paraffin oil	no	60%
38	10	5	chem.sed.funnel	TCE	no	yes	small	50%	immersion oil	no	40%
39	10	5	chem.sed.funnel	TCE	yes	yes	small		glycerol	no	
40	10	10	other	TCE	yes	yes	large	100%	paraffin oil	no	
41	10	5	beaker (flat bottom)	TCE	no	yes	small	33%	immersion oil	no	25%
42	10	10	conical glass with cock	TCE	yes	no	small	100%	Norland	no	40%
43	10	10	chem.sed.funnel	TCE	yes	yes	large	10%	immersion oil	no	40%
44	25	25	beaker (flat bottom)	TCE	no	yes	small	50%	paraffin oil	no	55%
45	10	10	chem.sed.funnel	TCE	yes	yes	small	50%	glycerol	no	
46	10	10	chem.sed.funnel	TCE	yes	no	small	10%	glycerol	no	40%
47	10	3	chem.sed.funnel	TCE	no	no	large	100%	paraffin oil	no	60%
48	10	3	conical champagne glass	TCE	no	yes	small	100.0%	paraffin oil	no	50%

Lab nr	Amount		Glassware	Agent	Staining	Binocular	Size	Sed. used	Embedding	ARIES	f-factor
	Feed	Fish								Used	MBM
49	10	10	Mensur	TCE	no	yes	large	40%	Mineral oil		
50	10	10	conical champagne glass	TCE	no	yes	medium	20%	immersion oil	no	15%
51	10	3	chem.sed.funnel	TCE	yes	no	small	25%	paraffin oil	no	40%
52	10	10	chem.sed.funnel	TCE	no	yes	medium	16%	paraffin oil	no	
53	10	10	chem.sed.funnel	TCE	no	yes			norland	no	
54	10		tube	TCE	yes	yes	large		paraffin oil		

* *The indications of the parameters are short names for the full descriptions as presented in Annex B.*

Annex VII
Results: presence of MBM, microscopic detection

Lab nr	Fish				MBM			
	A	B	C	D	A	B	C	D
1	no	no	yes	no	yes	no	no	yes
2	no	no	yes	no	yes	no	no	yes
3	no	no	yes	no	yes	no	no	yes
4	no	no	yes	no	yes	no	no	yes
5	no	no	yes	yes	yes	no	no	yes
6	no	no	yes	no	yes	no	no	yes
7	no	no	yes	yes	yes	no	yes	yes
8	no	no	yes	no	yes	no	no	yes
9	no	no	yes	no	yes	no	yes	yes
10	no	no	yes	no	yes	no	no	yes
11	no	no	yes	no	yes	no	no	yes
12	no	yes	yes	no	yes	no	no	yes
13	no	no	yes	no	yes	no	no	yes
14	no	no	yes	no	yes	no	no	no
15	no	no	yes	no	yes	no	no	yes
16	no	no	yes	no	yes	no	no	yes
17	yes	no	yes	no	yes	no	yes	yes
18	no	no	yes	no	yes	no	no	yes
19	no	no	yes	no	yes	no	no	yes
20	no	no	yes	no	yes	no	no	yes
21	no	no	yes	no	yes	no	no	yes
22	no	no	yes	yes	yes	no	yes	yes
23								
24	no	no	yes	no	yes	no	no	yes
25	no	no	yes	no	yes	no	no	yes
26	no	no	yes	no	yes	no	no	yes
27	no	no	yes	no	yes	no	no	yes
28	no	no	yes	no	yes	yes	no	yes
29	no	no	yes	no	yes	no	no	yes
30	no	no	yes	no	yes	no	no	yes
31	no	no	yes	no	yes	no	no	yes
32	no	no	yes	no	yes	no	yes	yes
33	no	no	yes	no	yes	no	no	yes
34	no	no	yes	no	yes	no	yes	yes
35	no	no	yes	no	yes	no	no	yes
36	no	no	yes	no	yes	no	yes	yes

Lab nr	Fish				MBM			
	A	B	C	D	A	B	C	D
37	no	no	yes	no	yes	no	no	yes
38	no	no	yes	no	yes	no	yes	yes
39	no	no	yes	no	yes	no	yes	yes
40	no	no	yes	no	yes	no	no	yes
41	no	no	yes	no	yes	no	yes	yes
42	no	no	yes	no	yes	no	no	yes
43	no	no	yes	no	yes	yes	yes	yes
44	no	no	yes	no	yes	no	yes	yes
45	yes	no	yes	no	yes	no	no	yes
46	no	yes	yes	yes	yes	yes	no	yes
47	no	no	yes	no	yes	no	no	yes
48	no	no	yes	no	yes	no	yes	yes
49	no	no	yes	no	yes	no	yes	yes
50	no	no	yes	no	yes	no	yes	yes
51	no	no	yes	no	yes	no	no	yes
52	no	no	yes	no	yes	no	no	yes
53	no	no	yes	no	yes	no	no	yes
54	yes	yes	yes	no	no	no	yes	yes

Annex VIII
Results: sediment and quantification

Lab nr	Amount of sediment				Amount MBM		
	A	B	C	D	A	C	D
1	100	63	485	80	0.22%		0.11%
2	49	51	1623	46	0.30%		0.10%
3	74	76	800	72			
4	89	125	1827	99	0.10%		0.05%
5	71	69	1771	61	0.36%		0.15%
6	334	38	893	40	0.10%		0.02%
7	90	70	1870	90			
8	55	53	817	65	0.30%		0.10%
9	130	111	2115	122			
10	60	80	1820	70	0.04%		0.01%
11	77	71	1768	88	0.10%		0.05%
12	80	85	1864	68	0.10%		0.04%
13					0.05%		0.02%
14							
15	69	66	1746	70	0.20%		0.02%
16	89	81	99	80	0.09%		0.04%
17	74	69	1740	69	0.15%	0.5-1.5%	0.05%
18	90	80	800	90			
19	50	50	1670	30	0.10%		0.05%
20	109	94	501	145			
21					0.23%		0.25%
22	81	66	1795	72	1.70%		0.003%
23							
24	80	91	1799	84	0.06%		0.06%
25	75	73	1866	72			
26	238	250	896	295	0.20%		0.10%
27	54	53	694	66	0.31%		0.15%
28	130	250	1720	450	0.30%		0.30%
29					0.56%		0.12%
30	95	78	1910	70			
31	121	157	1821	100	0.15%		0.05%
32	119	89	1966	93	0.07%	0.10%	0.05%
33	68	53	1628	127			
34	54	46	1121	50	1.00%	0.01%	0.06%
35	60	70	1850	90	0.10%		0.01%
36	34	29	1433	30			
37	53	46	1305	46	0.15%		0.11%
38	78	75	1661	85	0.14%	0.03%	0.06%
39	61	58	588	577			
40	131	156	2000	158			
41	50	50	674	77	0.12%	0.15%	0.05%

Lab nr	Amount of sediment				Amount MBM		
	A	B	C	D	A	C	D
42	79	78	1418	78	0.09%		0.05%
43	95	89	1489	67	0.31%	1.93%	0.05%
44	131	124	3428	136	0.15%	0.26%	0.03%
45	106	72	494	172			
46	102	86	1905	83	1.20%		0.60%
47	176	190	652	145			
48	38	48	260	50	0.05%	0.02%	0.02%
49							
50					0.50%	0.10%	0.10%
51	85	81	595	107	0.20%		0.05%
52	88	105	2191	96			
53							
54							

Annex IX
Results: presence of MBM, DNA detection

Lab	MBM A 0.1%	B 0%	C 0%	D 0.02%	Amount MBM A	D	Method	Target
9	yes	yes	yes	yes			PCR	ruminant
16	yes	no	no	yes			PCR	land animals
	no	no	no	no				cattle
	yes	no	no	yes				pig
	no	no	no	no				chicken
	no	no	no	no				turkey
30	no	yes	no	no			PCR	bovine
	yes	yes	yes	yes				pig
	no	yes	no	no				chicken

Annex X
Results: presence of MBM, protein detection

Lab	MBM				Amount MBM		Method	Target
	A	B	C	D	A	D		
	0.1%	0	0%	0.02%				
30	no	no	no	no			Melisa-Tek	ruminant
	no	no	no	no			Reveal	ruminant

RIKILT - Institute of Food Safety is part of the international knowledge organisation Wageningen UR (University & Research centre). RIKILT conducts independent research into the safety and quality 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.

RIKILT advises national and international governments on establishing standards and methods of analysis. RIKILT is available 24 hours a day and seven days a week in cases of incidents and food crises.

The research institute in Wageningen is the National Reference Laboratory (NRL) for milk, genetically modified organisms, and nearly all chemical substances, and is also the European Union Reference Laboratory (EU-RL) for substances with hormonal effects.

RIKILT is a member of various national and international expertise centres and networks. Most of our work is commissioned by the Dutch Ministry of Economic Affairs, Agriculture and Innovation and the new Dutch Food and Consumer Product Safety Authority. Other parties commissioning our work include the European Union, the European Food Safety Authority (EFSA), foreign governments, social organisations, and businesses.

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