Project number: 87235701
Project title: Dutch NRL for animal proteins

Project leader: L.W.D. van Raamsdonk

Report 2008.007 May 2008

The 2008 Dutch NRL / IAG proficiency test for detection of animal proteins in feed

L.W.D. van Raamsdonk, W. Hekman, J.M. Vliege, V. Pinckaers, H. van der Voet[1], S.M. van Ruth

Business Unit: Analytical Services & Development
Cluster: Authenticity & Identity

[1]Biometris

RIKILT – Institute of Food Safety
Wageningen University and Research Centre
Bornsesteeg 45, 6708 PD Wageningen, The Netherlands
P.O. Box 230, 6700 AE Wageningen, The Netherlands
Tel: +31 317 480256
Fax: +31 317 417717
Internet: www.rikilt.wur.nl

The research described in this report was funded by the Dutch Ministry of Agriculture, Nature and Food Quality, program 438.

Distribution list:

- Dutch Ministry of Agriculture, Nature and Food Quality, Department of Food Quality and Animal Health (LNV, VD; drs.E. Pierey, dr. R. Theelen)
- Dutch Ministry of Agriculture, Nature and Food Quality, Department of Knowledge (LNV, DK; ing. T. Greutink)
- Dutch Ministry of Agriculture, Nature and Food Quality, General Inspection Service (LNV, AID; drs. M. Jansen, ing. K. van Kuijk)
- Food and Consumer Product Safety Authority (VWA; drs. W. Ooms, mr. drs. R. Herbes, dr. H.A. van der Schee)
- International Association for Feeding stuff Analysis (IAG), Section Feeding stuff Microscopy (dr. I. Paradies-Severin, dr. J.S. Jørgensen, dr. F. Wernitznig, dr. G. Frick)
- European Commission (EC; dr. K. van Dijck, dr. F. Verstraete)
- Community Reference Laboratory, Animal Proteins (CRA-W; dr. V. Baeten, dr. P. Veys, dr. G. Berben)
- Joint Research Centre, Geel (IRMM-JRC; dr. C. von Holst, dr. A. Boix-Sanfeliu)
- Veterinary Laboratory Agency (VLA; dr. S. Reaney)
- National Reference Laboratory, Italy (CReAA; dr. D. Marchia)
- European Fat Processing and Renderers Association (EFPRA; dr. S. Woodgate)
- International Fishmeal and Fish oil Organisation (IFFO; dr. A. Jackson, dr. I. Pike)
- All participants of the proficiency test

Summary

A proficiency test was organized for the detection of animal proteins in animal feed by microscopy, PCR (DNA detection) and immunoassay methods. The aim of this proficiency test was to provide information that could support RIKILT - Institute of Food Safety as Dutch National Reference Laboratory in her task to consider the organisation of comparative tests among national control laboratories. A further aim was to gather information about the application of the microscopic method. The proficiency test was carried out in the broader framework of the annual proficiency tests of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy.
Three samples were prepared: one containing no animal proteins (blank), one with 0.1% of terrestrial animal material and one with 0.05% of terrestrial animal material. Fish material was not used. All participants were requested to determine the presence or absence of land animal or fish protein material. The microscopists among the participants were asked to report the amount of sediment found (the fraction containing minerals and bones, if present) and to fill in a questionnaire on a series of the parameters of the microscopic method. Reporting the estimated amount of land animal or fish protein was optional for all participants. 45 Participants returned results using the microscopic method, three participants for PCR, and one participant for immunoassay analysis.
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. For the absence of fish meal specificity scores were reached between 0.84 and 0.98. The detection of material of terrestrial animal origin (sensitivity) was good: 0.978 for detection of 0.1%, and 0.956 for the detection of 0.05% of animal proteins. In several cases land animal material was found in the blank (specificity = 0.93).
The amount of animal protein was generally overestimated with a factor 2. This is a normal situation when an ingredient is present in a small amount.
The use of a contamination level below 0.1%, as predominantly used in proficiency tests, allows for evaluating the strength of the microscopic method. From theoretical calculations it can be concluded that below 0.01% chances for reporting false negative results will increase. Using the whole sediment instead of only a part of it, and using a starting amount of 10 grams instead of 5 grams for sedimentation are factors that could increase the quality of detection (i.e. improve the score). Some other parameters addressed in the questionnaire, such as the type of glassware used, the application of a binocular for examination of the sediment at lower magnifications, and the method of slide preparation show interesting possibilities for improvement. Training of microscopists and the use of well qualified material for preparing the samples are also important.
It is demonstrated that the DNA detection by PCR can give good results at the contamination levels used. The immunoassay method, however, failed at and below 0.1%.
The results give a good overview of current implementation of the microscopic method, and can be used for further improvement and planning of future proficiency tests. These results will be used in the framework of the European project SAFEED-PAP for method improvement. The problem of false detection of animal proteins (specificity) needs further attention.

Contents

1 Introduction

The detection of animal proteins in feed remains an important issue in the process of avoiding Mad Cow Disease. Until now, a general ban has been effective for animal proteins from terrestrial animal slaughter waste, to be used in ruminant feed. There are plans to extend the legal use of fish meal in non-ruminant feeds to ruminant feeds as well (TSE roadmap of the Commission), provided that a reliable method for quantification of fish meal is available. Further relaxations for feed include a future species-to-species ban: every “species” (in a legal sense: ruminant, pig, poultry, fish) may be fed with animal proteins of other animals, but not of their own kind. Feeding of terrestrial animal material to ruminants is excluded from this species-to-species ban. In fact, this ban is already in force, but it is overruled by the current extended feed ban. The European commission awaits good identification methods and reliable procedures for avoiding cross-contamination of the different kinds of animal proteins in practice.

Directive 2003/126/EC states that any official method should be able to detect at least a contamination level of 0.1% animal protein. This is only a practical limit that does not overrule the zero tolerance of the official bans. The limit of 0.1% forms the basis of most proficiency tests and collaborative studies to validate new methods and to establish lab performance. Nevertheless, it is desirable to test laboratory performance and method reliability at lower contamination levels, because zero tolerance is still the ultimate goal.

One of the tasks of a national reference laboratory, according to Directive 882/2004/EC, is the organisation of comparative tests among the official national laboratories. The Netherlands does not maintain a network of official laboratories for detection of animal proteins, although national legislation provides a list of five laboratories that can be involved in monitoring animal feeds in general. RIKILT, as Dutch NRL, is seeking possibilities for providing support to those national laboratories. In order to establish the possibility for organising a comparative test for a range of laboratories, a proficiency test was organised which could also fit in the framework of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy.

One of the tasks of the European project SAFEED-PAP (FOOD-CT-2006-036221; 2006-2009) is to find possibilities for the improvement of the microscopic method. The questionnaire for parameters of the implementation of the microscopic methods, as part of the current proficiency test, can serve as input for this task.

In this report the proficiency test for animal proteins is presented, organised in 2008 by RIKILT, as Dutch National Reference Laboratory, and on behalf of the IAG Section Feeding stuff Microscopy.

2 Material and methods

Materials

Three samples were produced, based on a feed that was produced in the framework of the European project STRATFEED in an approved, animal protein free, small scale feed factory (Garrido-Varo et al., 2005). The feed material contained large particles of material glued together with molasse. 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. About 10% of the total volume of the feed appeared to be contained in these large particles. Sixteen samples of this feed were tested microscopically at RIKILT again for the presence of animal proteins. No material of animal origin was found.
The proficiency test consisted of three samples with a composition as listed in Table 1.

Table 1: Composition of the samples in the NRL-IAG proficiency test 2008.

Label	Content
2008-A	Blank
2008-B	0.1 % MBM
2008-C	0.05 % MBM

The design allows for the use of DNA detection and immunochemistry detection methods, additional to microscopic detection.
The meat and bone meal (MBM) used was prepared in the framework of STRATFEED in a dedicated pilot plant owned by Prosper de Mulder (UK), as part of a set of 16 samples (coming from four different animal sources, treated at four different temperatures). The MBM used in samples B and C of this proficiency test is of bovine origin and is heat treated at 133 ^{o}C. The f-factor (share of heavy particles in the total of the MBM) of this material was 0.7. The sediment consisted of approx. 70% w/w brown coloured particles and 30% white coloured particles (Figure 1). The white particles were clearly recognisable as animal material (Figure 2). It was assumed that the brown particles suffered from heat damage. In a number of cases, the particles showed no lacunae, or they were only faintly visible (Figures 3 and 4; see documentation in ARIES for this sample and the Report of the IFFO proficiency test, van Raamsdonk and v.d. Voet, 2003). Although a part of the bone particles was not easily recognisable as bone, nevertheless, this material was chosen for the current proficiency test, since it was used in several other proficiency tests. The MBM used has a high f-factor, which was thought to compensate for possible problems with recognition.

Procedure for production

Sample 2008-B was produced according to the method of stepwise dilution. 3.5 g of MBM was used to prepare (finally) 3.5 kg of contaminated feed as follows. The initial 3.5 g of MBM was mixed in 7.7 g of feed and shaken (in a closed container) for one minute. In eight additional steps the remaining amount of feed was added. When a total amount of 500 g of contaminated feed was reached, shaking was replaced by mixing and stirring. The final jars were filled with 50 – 55 grams of material.
For the preparation of sample 2008-C, the jars were filled with 50 +/- 0.1 g of uncontaminated feed. To each jar, 25.0 mg of MBM was added to each jar, which was shaken and stirred for one minute. In this way it was assured that every individual jar contained the necessary amount of MBM, and the

possible problem of in-homogeneity in a the entire mixture, before dividing it over the jars, was avoided. Because of the adding and mixing procedure described above, the resulting concentration in the jars ranged from 0.0498% to 0.0502%. Every participant was informed about their responsibility for ensuring sample homogeneity within their own jars.
The proficiency test material was prepared in a laboratory in Wageningen for feed analysis, where animal proteins are never used.

Figure 1. Sediment of bovine sample DQ03-0031-01 (133 ^{o}C), magnification 10x

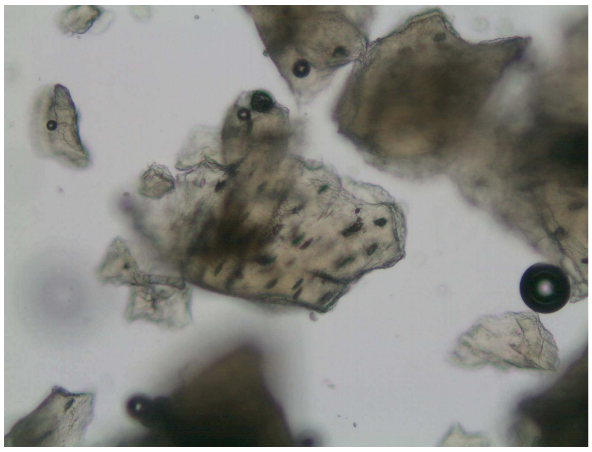
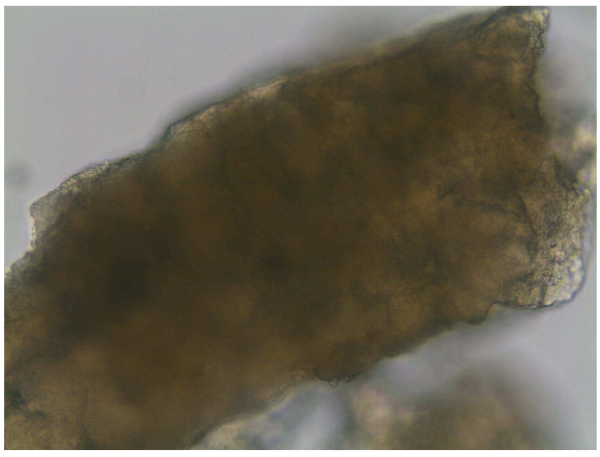


Figure 2. White particles with visible lacunae, magnification 100x



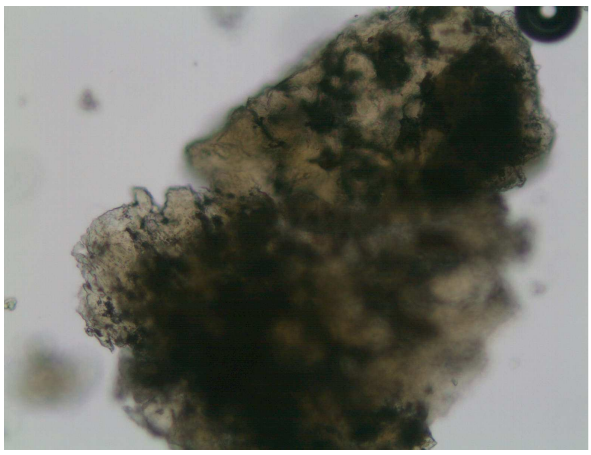


Figure 3 and 4. Two brown particles of bovine sample DQ03-0031-01 (133 ^{o}C) with hardly visible lacunae. Magnification 200x

Homogeneity study

RIKILT microscopists examined five jars of sample 2008-B and five jars of sample 2008-C. In all ten cases a correct positive result was reached, as is shown in Table 1. Based on these results it was justified to send the sets of three samples around to all participants. The microscopy research group of RIKILT did not participate in the further laboratory analysis of this proficiency test.

Table 2. Results of the homogeneity study. Sediment amounts are based on 10 grams in all cases.

Sample	Sediment amount (g)	MBM
2008-A blank (n=16)	0.109 - 0.199	16 x negative
2008-B 0.1 % MBM (n= 5)	0.104 - 0.124	5 x positive
2008-C 0.05 % MBM (n= 5)	0.103 - 0.144	5 x positive

Organization of the proficiency test

The sets of three samples with an accompanying letter (see Appendix A) were sent to all participants on the 22nd of February 2008. On Monday February 25th an E-mail message was sent around to all participants, together with an electronic report form (see Appendices B and C) and the request to confirm the receipt of the package. The report form also contained a sheet with instructions (see Appendix D).
The closing date for reporting results was fixed at April 1st. Some additional participants received the package at a later date. However, in all cases results were received not later than April 7th , and all these results were considered in the final evaluation.

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 can be presented as fractions *p* or as percentages after multiplication by 100. Accuracy (specificity or sensitivity) has been calculated for each sample type.
Theoretical calculations have been carried out for the expected chance to detect correctly the presence of animal proteins, depending on the level of contamination, the starting amount of material for sedimentation and the part of the total amount of sediment to be examined. The calculation of these chances is based on a negative binomial distribution. The results of the calculations will be used to evaluate the results of this proficiency test.

3 Results

49 Packages with three samples were sent around. Of these, 45 participants returned results for the microscopic method, three sets of results were received for PCR analysis, and one set of results was received for immunoassay analysis. In two cases only a FAX message was received, and in two other cases only an E-mail message. All results were nevertheless included. The list of participants is presented in Appendix E. The full results are presented in the tables of Appendix F, G, H and I. Blanks were considered to indicate the absence of the indicated type of animal protein.

Microscopic procedure

An inventory of nine different parameters was connected to the report of the actual results of the three samples. These results are shown in Appendix F and summarised in Table 3.

Table 3: Inventory of parameters for microscopic detection and their application.

parameter	parameter state	number of participants	amount
amount of material used for sedimentation	5 grams	16	
	10 grams	26	
	other	3	
type of glassware	chemical sedimentation funnel	22	
	beaker (flat bottom)	11	
	champagne glass	6	
	conical glass with cock	3	
	other	3	
sedimentation agent	TCE	44	
	other	1	
use of staining of sediment	yes	14	
	no	31	
use of binocular for examination at lower magnifications	yes	29	
	no	16	
number of slides used	minimum		1
	maximum		7
	other		> 10
size of cover glass used	small (e.g. 20 x 20 mm)	34	
	medium	1	
	large (e.g. 26 x 50 mm)	9	
share of the total sediment used for examination	minimum		4%
	maximum		100%
embedding agent	paraffin oil	18	
	immersion oil	8	
	glycerine	8	
	phenol glycerol	3	
	chloral hydrate	3 *	
	other (e.g. Depar 3000, water)	5	
f-factor for MBM	minimum		25%
	maximum		100%
	none estimated	27	

* in one occasion the chloral hydrate was combined with lactophenolblue.

The majority of the participants started the sedimentation procedure with an amount of 10 grams of material. Also in a majority of cases a chemical sedimentation funnel was used. Fourteen participants used staining of the sediment (alizarin) for evaluation. It was not stated if this staining procedure was used as the standard method or only additionally (for the examination of unstained material). Only the latter situation is allowed in Directive 2003/126/EC. Examination of the sediment at lower magnifications by using a binocular is requested in this Directive, but 16 participants reported to skip this part of the procedure. Usually, between one and seven slides were made for evaluation of the sample, although two participants reported the use of more than ten slides. Most of the participants used small cover glasses. It can be expected that using a larger portion of sediment for examination is correlated with a larger amount of slides and/or the use of large cover glasses in order to accommodate the sediment material. However, in one case the use of 8% of sediment material on a total of more than 10 slides was reported, while in another case with approximately the same amount of sediment 100% of the sediment material was placed on six slides with small cover glasses. So, there is an apparent diversity in the preparation of slides for the microscopic examination. Also a range of eight different embedding agents was reported, some of them hardly suited for a good examination of sediment material.

Microscopic detection

The specificity and sensitivity were at acceptable levels for most analyses (Table 4). Seven participants reported the presence of fish meal in sample 2008-C, resulting in a relatively low specificity of 0.84. From all 12 positive deviations for the detection of fish meal, only one was reported for the blank sample (2008-A), the other 11 being reported for the samples containing exclusively terrestrial animal material. Three participants found terrestrial animal material in sample A, one of them reported also a positive deviation for fish in the same sample A.
For both samples B and C only three negative deviations were found. This resulted in total sensitivity scores at or higher than 0.95. There was no correlation between the three negative deviations and the amount of sediment material used (10%, 75% and 100%, respectively) or with the number of slides examined (1, 3 and 6 respectively). However, a noticeable difference can be seen between the sensitivity for the results based on 5 grams and on 10 grams of material. More material used for sedimentation results in a higher sensitivity.

Table 4: Sensitivity and specificity for the detection of animal proteins in three samples.

		Fish			MBM		
N		A	B	C	A	B	C
Total							
45	specificity	0.98	0.91	0.84	0.93		
	sensitivity					0.978	0.956
5 gr							
16	specificity	1.00	0.81	0.81	0.94		
	sensitivity					0.94	0.94
10 gr							
26	specificity	0.96	0.96	0.85	0.96		
	sensitivity					1.00	0.96

There is no difference in the specificity and sensitivity scores between the subgroups of participants that used staining of the sediment (n=14) and those that did not (n=31).

Quantification

The sediment weights reported by the participants (Table 5) are in the range of those established in the homogeneity study (Table 2). As far as known, the sediment weight prior to the staining with Alizarin, when applied, is used in the calculations. The amount of sediment established when using 10 grams of material is not twice as high as that produced with 5 grams. The highest amount of sediment (0.28 g) was reached after using 20 grams of material (see also Appendix G).The total results indicate that apart from the amount of sample used for sedimentation, other factors may cause the variation in sediment weight, perhaps the sedimentation procedure itself.
Reporting quantitative results was facultative in this proficiency test. Nevertheless, most participants made estimations of the levels of contamination (Appendix G). The results are summarised in Table 5. The average estimates for the amount of animal proteins in the samples 2008-B and 2008-C were 0.19% and 0.12%, respectively. It appears that these are overestimations in both cases (real levels were: 0.1% and 0.05%). Almost identical quantitative results were reached when using either 5 grams or 10 grams of material for sedimentation.

Table 5: Average, standard deviation, minimum and maximum values for the total amount of sediment and estimated amounts of material of terrestrial animals after microscopic detection. Values are given for all participants and for participants using either 5 grams or 10 grams of material for sedimentation.

N		Amount of sediment (g)			Indicated amount of MBM (%)	
		A	B	C	B	C
Total						
45	average	0.118	0.121	0.117	0.194	0.120
	SD	0.053	0.054	0.044	0.002	0.001
	minimum	0.05	0.04	0.06		
	maximum	0.298	0.28	0.237		
5 gr						
16	average	0.085	0.083	0.083	0.188	0.120
	SD	0.038	0.025	0.023	0.002	0.002
	minimum	0.05	0.04	0.06		
	maximum	0.211	0.142	0.129		
10 gr						
26	average	0.136	0.139	0.135	0.212	0.124
	SD	0.050	0.048	0.039	0.002	0.001
	minimum	0.074	0.073	0.06		
	maximum	0.298	0.266	0.237		

Correlation of quantification with the several types of glassware used shows interesting results. The estimations for the content of sample B (0.1 %) range from 0.264 when using the beaker to 0.13 when using special glassware with a cock or a champagne glass (Table 6). For sample C (0.05 %) a similar range was found. The estimations after using the centrifuge tube and the Shaffnit funnel were lower. This is statistically not relevant with n=1 in both cases and therefore not shown in Table 7. Starting amounts of 5 and of 10 grams of material for sedimentation are used in all groups, except in the group using the conical glass with cock (in this group all three participants use 5 grams). A significant

analysis of the amounts of sediment obtained when using the several types of glassware is not reasonable, because of the relative low number of participants per type of glassware.

Table 6: Average of estimated amounts of animal proteins (diversified) as found for the different types of glassware used for sedimentation.

Type of glassware **N**		**Indicated amount of MBM (%)**	
		B	**C**
beaker (flat bottom) n=11	average	0.268	0.129
chemical sedimentation funnel n=22	average	0.204	0.144
conical glass with cock n=3	average	0.130	0.083
champagne glass n=6	average	0.130	0.080

Detection by other methods

The use of the bovine primer in the PCR method of two participants (Appendix H) gives correct results for MBM. Also the indication of the relative amount of animal protein is fair, although the contamination level is too high where percentages are given. One participant did not test for fish material. The fish primer as used by the other participant might show cross-sensitivity with DNA of another source.
The conclusion that vertebrate material is present in samples B and C, as reported by participant 10 is correct. However, a discrimination between terrestrial animal material and fish can not be made, although this is normal in proficiency tests. The same participant reported microscopic results. With that method no animal proteins were found in sample A.
The presence of animal material according to immunoassay analysis was carried out with the Melisa-Tek kit, containing an antibody against heat treated troponin I. The use of immunoassay analysis did not result in a positive detection of animal proteins at the current levels of contamination (Appendix I).

4 Discussion

The basic material of animal proteins used in this study show a rather fundamental problem. Part of the bone material looks like it was sterilised at a (much) higher temperature. However, the material was produced in a dedicated power plant with strictly controlled circumstances (Garrido-Varo et al., 2005). Highly comparable material was used and described in an earlier proficiency test (van Raamsdonk & van der Voet, 2003). In that test, as is found in the current test, there seemed to be no or only a limited effect of appearance of the material on the results in terms of sensitivity and specificity scores. However, it is recommended to evaluate the materials currently in the collection and the ones that are to be produced, especially when quantification or staining is at stake.

The total results indicate that in all but one case the positive deviations for the detection of fish material are found in the presence of terrestrial animal material. This result might indicate that certain fragments of land animals are misinterpreted as fish material. Total specificity scores between 0.84 an 0.98 are in the range as reported in literature (van Raamsdonk et al., 2007). The first proficiency test of the CRL (Veys et al., 2007) also indicated specificity scores of 0.88 (blank) and 0.91 (presence of terrestrial animal material) for fish. It could be concluded that an improvement should be achieved at this point.

The specificity score for the detection of land animal material in the current test (0.93) is in the range of past IAG tests (Table 7). The positive trend was not sustained in the current results. Possibly, in a few cases, plant particles may have been mistaken for animal proteins. The use of pictures is necessary for affirming or refuting misinterpretation.

The difference between the total sensitivity scores for the contamination levels 0.1% and 0.05% is not significant. The current scores are within the range of previous proficiency tests (0.92 to 1.00; van Raamsdonk et al., 2007; Table 7). The sensitivity score resulting from the first proficiency test organised by the CRL (0.985; Veys et al., 2007) is also comparable. In all these literature reports the contamination level was 0.1%. There is a difference between the sensitivity score reached after using 5 grams compared to the results based on 10 grams. The scores after using 5 grams are at the lower end of the range from previous studies.

Although proficiency tests usually use a contamination level of 0.1% or higher, the current study is not the first one in which a lower level is used. Engling et al. (2000) report the results after using a contamination level of 0.02%. In their study, among 18 participants, one laboratory reported a false negative result, resulting in a sensitivity score of 0.94, comparable to that found in this report. The results for the different contamination levels in the absence of fish meal were good in previous IAG proficiency tests, as found in the current one (Table 7).

Table 7: Results for detecting material of land animals of previous proficiency tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby) 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.

Content: fish **year** **land animal**	**blank** **blank**	**2-3%** **0.2%**	**2%** **0.1%**	**0** **0.1%**	**0** **0.05%**	**0** **0.02%**
2003 (n=29)	0.86			1.0		0.97
2004 (n=30)	0.93	1.0			0.97	
2005 (n=42)			0.95	0.95		
2006 (n=43)	0.98	1.0	1.0			
2007 (n=45)			0.933			

The detection of land animal material in the presence of fish was not tested in this study, but historical data show a diverse picture. Detection of 0.1 % land animal material in the presence of 2 % of fish material usually shows good results (Table 7), opposite to the detection of 0.1 % land animal material in the presence of 5 % of fish. In the latter combination, i.e. with a higher share of fish, focusing on results from applying the method of Directive 2003/126/EC, sensitivity scores were reached ranging from 0.66 in earlier proficiency tests (van Raamsdonk et al., 2007) to 0.88 (Veys et al., 2007). A validation study with selected laboratories indicated that a sensitivity score of 0.987 could be reached (van Raamsdonk and v.d. Voet, 2003). The results as shown in Table 7 indicate that an improvement can be achieved.

Contamination levels lower than 0.1% in the presence of fish meal were never tested.

The third sample in the 2005 study of IAG contained pure fish meal contaminated with 0.1% of land animal material. A sensitivity score of 0.67 was reached. This result is interesting in light of testing fish meal for the presence of land animal material, as it is done in the course of fish meal certification: certified fish meal parties can be supposed to pose low or minimal risk when applied in feed mixtures. Some improvements of the detection method or microscopists skills are necessary to ensure reliable distinction of land animal material from fish material.

Theoretical calculations similar to those in van der Voet et al. (1999) have been carried out to estimate the expected probabilities for presence of particles of animal origin at different contamination levels (theoretical sensitivity scores). It was assumed for these calculations that 75 or 150 mg sediment material was extracted from 5 or 10 g of sample material, respectively. Based on the results of the current inventory (Appendix F) it was assumed that either 15 mg of sediment material (making up either 20% or 10% of the total amount of sediment respectively) or 100% of the material was examined in one or more slides. In addition, an extra strategy was calculated based on the assumption that only 75 mg of sediment was extracted from 10 g of sample, in order to reach the situation that the 15 mg of material for one slide would represent also 20% of the sediment material. The results are shown in Table 8. These calculations indicate the probability that at least one particle shows up in the portion of the sediment that is being examined. At lower contamination levels the probability increases that a portion of 15 mg will not contain a particle of animal origin, although it was present in the total sample. One of the prerequisites of a further discussion of the results is the assumption of homogeneity.

The calculated results indicate that at contamination levels of 0.001% and lower, substantial differences exist between using the entire sediment or only a part of it, and between using 5 or 10 grams of starting material. From the calculations it can be concluded that the first parameter, the portion of the sediment used, is the most important of the two. In this perspective it is also important that a binocular should be used for a first examination of the entire sediment, as requested in paragraph 6.2 of the Appendix of Directive 2003/126/EC. By doing this, larger particles may be detected, that may not show up in the finer sediment, which improves the chance of detecting animal proteins in the entire sediment. Based on the total results it can be calculated that 37% of the participants did not include this screening at lower magnifications in their procedures.

Table 8: Theoretical sensitivity scores for five different situations and for five different contamination levels each, ranging from 1.0% to 0.0002%, assuming sediment examination (bones only). The calculations are based on the negative binomial distribution, assuming an average particle weight of 4 µg and a variation coefficient of 130%. The percentage is the contamination level of the total MBM, assuming an f-factor of 0.5 (50 % of bones).

sample size (g)	**5**	**10**	**10**	**5**	**10**
sediment (mg) **fraction of sediment used (mg)**	**75** **15 (20%)**	**150** **15 (10%)**	**75** **15 (20%)**	**75** **75 (100%)**	**150** **150 (100%)**
%					
1.0	0.993	0.993	0.995	0.997	0.998
0.2	0.981	0.981	0.988	0.993	0.995
0.1	0.972	0.972	0.981	0.989	0.993
0.02	0.93	0.93	0.95	0.97	0.981
0.002	0.74	0.74	0.82	0.89	0.93
0.0002	0.30	0.30	0.44	0.62	0.73

The "probability of presence" is not identical to “probability of proper or successful detection”. For calculating the probability to find a fragment of animal protein it is assumed that a (bone) fragment is always properly recognised. Since this is not always the case, the sensitivity scores as reached in practice are lower than calculated. The decreased sensitivity when using only a portion of the sediment instead of the entire sediment and when using 5 instead of 10 grams of sample material is therefore also larger in practice than theoretically calculated. A presence of fish material, which was not tested in the current proficiency test, can be expected to contribute further to lower scores.
Apart from parameters discussed above, there appears to be a large diversity in the way slides are prepared for microscopic examination. One parameter contributing to this diversity is the kind of embedding agent used. The embedding agent aids in enhancing the visibility of the particle studied. For example, chloralhydrate increases the visibility of the cell walls of plant material. It can occasionally be used to examine muscle fibres, although other reagents are more suitable for that. Embedding agents such as immersion oil or paraffin oil have a moderate viscosity. With these agents, lacunae of bone particles remain filled with air for a period of time, causing them to be highly visible. A careful selection of the proper embedding agent should be considered.
Although embedding agents such as chloralhydrate are more suited for examining the entire feed or the flotation (search for e.g. muscle fibres), it has to be stated that no attention was paid to the examination of these fractions in this proficiency test. It is generally believed that the most important information can and will be retrieved from the sediment. Nevertheless, valuable additional information

can be extracted from those other fractions and more attention for these is recommended in future studies.
The results for the quantification of the animal proteins show an overestimation. This is a usual situation for ingredients with a low share in the total composition (unpublished results of proficiency tests of IAG Section Feeding stuff Microscopy; Veys and Baeten, 2008). The CRL for animal proteins is currently developing a reliable method for the quantification of animal proteins. Further discussion of this topic should take place in the framework of this development.
Five laboratories from the Netherlands sent in their results: two using microscopy, two using PCR analysis, and one using immunoassay analysis. In combination with the overall results of this proficiency test these Dutch results will contribute to considering possibilities for the Dutch NRL for animal proteins to perform its tasks.

5 Conclusions and recommendations

a) The results of this proficiency test show that at a contamination level lower than normally used in testing (i.e. 0.05%) still a good performance can be reached.
b) All participants cooperated in an optimal way in this proficiency test. The questionnaire on the method parameters was completed in all cases, which allows a good evaluation of the implementation of the microscopic method.
c) The examination of fractions other than the sediment (e.g. flotation) should get more attention in future studies.
d) It is recommended to evaluate the materials currently in collection or those to be produced in the future for quality and recognisability of the bone fragments, especially when quantification or staining are to be used.
e) The method for microscopic detection of animal proteins can be improved for a proper detection at levels lower than 0.1%. Important improvements can be the use of the entire amount of the sediment produced, or at least a major share, and the use of a starting amount of 10 grams of material for sedimentation.
f) The use of a binocular for screening the sediment at lower magnifications is obligatory according to Directive 2003/126/EC, but not every laboratory is including such a screening in their lab procedures.
g) An improvement of the microscopic method can be reached by specifying the way the slides are being prepared for microscopic detection. The choice for the embedding agent and the minimum amount of the sediment material are some of the parameters that can be defined.
h) Although interesting results were achieved for quantification, a general overestimation was found. The current efforts of the CRL Animal proteins to develop a reliable method for quantification of fish material is vital for a future discussion on this subject.
i) The results will and can be used as a contribution to a future discussion on performing the tasks of the Dutch NRL.

6 Literature

Engling, F.-P., J.S. Jørgensen, I. Paradies-Severin & H. Hahn, 2000. Evidence of animal meal in feeds. Kraftfutter / Feed Magazine 2000(1): 14-17.

Garrido-Varo, A., D. Pérez-Marin, J.E. Guerrero, A. Gómez-Cabrera, Ch. von Holst, I. Murray, L.W.D. van Raamsdonk and J. Zegers, 2005. Construction of the Stratfeed sample bank and preparation of sample sets. In: Stratfeed, Strategies and methods to detect and quantify mammalian tissues in feedingstuffs, chapter 2. Office for Official Publication of the European communities, Luxembourg, 2005.

Raamsdonk, L.W.D. van, C. von Holst, V. Baeten, G. Berben, Ana Boix and J. de Jong, 2007. New developments in the detection of animal proteins in feeds. Feed Science and Technology 133: 63-83.

Raamsdonk, L.W.D. van and H. van der Voet, 2003. A ring trial for the detection of animal tissues in feeds in the presence of fish meal. Report 2003.012, RIKILT, Wageningen.

Veys, P. and V. Baeten, 2007. CRL-AP interlaboratory study 2006 final report. CRL-AP, Gembloux, Belgium, April 2007.

Veys, P. and V. Baeten, 2008. CRL-AP interlaboratory study 2007 Report. CRL-AP, Gembloux, Belgium, April 2008.

Voet, H. van der, J. de Jong, V.G.Z. Pinckaers, 1999. Betrouwbaarheid en herhaalbaarheid microscopische methode diermeel. Report 99.015, RIKILT, Wageningen, 13pp.

7 Acknowledgements

The authors wish to thank Mr. E. Pierey (Dutch Ministry of Agriculture, Nature and Food Quality, The Hague) and Dr. J.S. Jørgensen (Danish Plant Directorate, Lyngby) for support of this study.

Appendix A. Invitation letter

Dear colleague,

For years the annual IAG ring test for microscopic detection of animal proteins in feed was organized by the Danish Plant Directorate. With many thanks and honor to J.-S. Jørgensen and his team in Lyngby, RIKILT Institute of food safety has offered to organize the 2008 ring test.

In this package three vials with 50 grams each of a feed sample are included. The instructions for this ring test and the report form are send to you by E-mail. Please report the proper receipt of the package and of the E-mail message to leo.vanraamsdonk@wur.nl.

Your laboratory has a unique lab code: <labcode>. Please enter this code in the Excel report form.

RIKILT is the Dutch National Reference Laboratory. One of the tasks of an NRL is to support official national control laboratories. This ring test is a first attempt in this framework, which clarifies the full name of this ring test. The samples and the report forms are designed in such a way that PCR or immunochemistry detection can be applied as well. If your laboratory wants to perform other techniques in addition to microscopic analysis, please feel free to submit the results **at separate sets of report forms**.

Reports are requested both by FAX and E-mail. The closing date of this ring test is April 1st, 2008. Reports received after that date will not be considered for the final report. If you find any difficulties in the process of examining and reporting, please feel free to contact me.

With kind regards,

on behalf of the organizing team,

Leo van Raamsdonk

Appendix B. 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
NRL-IAG ring test 2008		
Please select your unique lab number		
Have you read the ring test instructions?		
What detection method do you use?	Microscopy	
Please skip this line		
Please continue here		
Please indicate your starting amount of material for sedimentation		
if other, please specify		
Indicate your glassware for sedimentation		
if other, please specify		
Describe your sedimentation agent		
if other, please specify		
Did you apply staining of the sediment (e.g. alizarin staining)?		
Did you examine at lower magnifications (using a binocular)?		
Please insert the number of slides examined at magnifications of 100x or higher		
Indicate the size of cover glass		
Please estimate the amount of sediment you have used for preparing the slide(s) (in %)		
Please describe your embedding agent for the sediment material		
if other, please specify		
When estimating amounts:		
please indicate the f-factor used for fish meal		
please indicate the f-factor used for terrestrial animal meal		

Appendix C. Report form

Please complete at least all the cells with the presence of fish material and land animal material for every sample

NRL-IAG ring test 2008

lab number

sample number	2008-A	2008-B	2008-C
weight of sediment			
presence of fish material			
if present, estimated amount			
presence of material of land animals			
if present, estimated amount			

Signature:

Date:

Appendix D. Instructions as included in the report form

NRL-IAG ring test 2008

Instructions for the NRL-IAG ring trial

1 You have received a box with an introduction letter and three 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 Directive 2003/126/EC from the European Union. Identical procedures can be found in the module Methods of the computer program ARIES. 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" 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.

All fields with a drop-down list have to be completed. Please add the exact sediment weight in 0.01 g.

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 will 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.

5 Direct any questions to leo.vanraamsdonk@wur.nl

6 Closing date is April 1st, 2008.

Appendix E. List of participants

Institute	City	Country	Background
Österreichische Agentur für Gesundheit und Ernährungsich Abt. Düngemtt. und Mikroskopie	Wien	Austria	NRL
Laboratório Regional de Veterinária, Vinha Brava	Angra do Heroismo	Azores	other
CRA-W; Dept. Qualité des Productions Agricoles	Gembloux	Belgium	CRL
Federal Voedingslaboratorium (FAVV)	Tervuren	Belgium	NRL
Bioanalysis Unit, Feed and Fertilizer Section, Ottawa Laboratory (Carling)	Ottawa, Ontario	Canada	other
Ministry of Food, Agriculture and Fisheries, Danish Plant Directorate	Lyngby	Denmark	NRL
Finish Food Safety Auth. Evira	Helsinki	Finland	NRL
Institut Européen de l'Environnement de Bordeaux	Bordeaux	France	other
Lab. DGCCRF de Rennes	Rennes	France	NRL
Oberfinanzdirektion Cottbus	Berlin	Germany	other
Landwirtschaftskammer Nordrhein-Westfalen, Tiergesundheitsdienst/Labor	Bonn	Germany	other
Q-Vis GmbH, Labor Braunschweig	Braunschweig	Germany	Commercial
Landwirtsch. Untersuch- und Forschungsanstalt Sachsen-Anhalt	Halle	Germany	other
SGS-Germany GmbH, Laboratory Services Hamburg	Hamburg	Germany	Commercial
NRG Nähr- und Rohstoffveredelung GmbH	Hamm	Germany	Commercial
Landwirtsch. Untersuch- und Forschungsanstalt Thüringen	Jena	Germany	
Food GmbH, Jena, Analytic-Consulting	Jena	Germany	Commercial
Staatliche Landwirtsch. Untersuchungs- und Forschungsanstalt	Karlsruhe	Germany	other
Hess. Dienstleistungszentrum für Landwirtschaft, Gartenbau und Naturschutz (HDLGN)	Kassel	Germany	other
Staatliches Veterinäruntersuchungsamt	Krefeld	Germany	other
Sächsische Landesanstalt für Landwirtschaft Abt. LU	Leipzig	Germany	other
Q-Vis GmbH, Zentrallabor Münster	Munster	Germany	Commercial
Bayer. Landesanstalt für Gesund- und Lebensmittelsicherheit	Oberschleissheim	Germany	other
LUFA Nord West	Oldenburg	Germany	delegated NRL
Landesamt für Verbraucherschutz und Landwirtschaft	Potsdam	Germany	other
Landwirtschaftliche Untersuchungs- und Forschungsanstalt Rostock	Rostock	Germany	other
Landwirtschaftliche Untersuchungs- und Forschungsanstalt	Speyer	Germany	other
Futtermittelinstitut Stade	Stade	Germany	other
Landesanstalt für Landwirtschaftl. Chemie, Universität Hohenheim	Stuttgart	Germany	other

Landesinstitut für Landwirtschaftliche Qualitätskontrolle	Budapest	Hungary	NRL
Official Seed testing Station, Department of Agriculture and Food, Abbotstown Laboratory Complex	Dublin	Ireland	NRL
Food and Vet. Service of Latvia, National Diagnostic Centre Diagnostic Lab of Animal Disease	Riga	Latvia	NRL
National Veterinary Lab. of the Republic of Lithuania	Vilnius	Lithunania	NRL
MasterLab B.V.	Boxmeer	Netherlands	Commercial
LabCo B.V. Laboratory Services	Rotterdam	Netherlands	Commercial
RIKILT— Institute of Food Safety	Wageningen	Netherlands	NRL
TNO Kwaliteit van Leven	Zeist	Netherlands	Commercial
Fiskeriforskning	Fyllingsdalen	Norway	other
Lab Nett AS Agriculturel Laboratory	Stjødal	Norway	other
BSI Inspectorate Peru, SAC, Jefa Laboratorio Microbiologia	Lima	Peru	other
Laboratório Nacional de Investigação Veterinária	Lisboa	Portugal	NRL
Cent. Control and Testing Inst. Agric. – Dept. feedingstuff	Bratislava	Slovakia	other
State Veterinary and Food Institute	Košice	Slovakia	NRL
National Veterinary Inst., Unit Pathologi and Animal Nutrition.	Ljubljana	Slovenia	NRL
Laboratori Agroalimentari – DAR, Generalitat de Catalunya	Cabrils (Barcelona)	Spain	other
National Veterinary Institute Dept. of Feed Chemistry	Uppsala	Sweden	NRL
Swiss Federal Research Station, Animal Production and Dairy Production	Posieux	Switzerland	NRL

Appendix F. Details of procedures applied, microscopic method

lab nr	amount*	glassware	agent	staining	binocular	# slides	size	sed. used	embedding	f-factor
2	10	chemical sedimentation funnel	TCE	no	no	4	small	10%	paraffin oil	-
3	5	beaker (flat bottom)	TCE	no	no	>10	small	8%	chloralhydrate	-
4	5	conical glass with cock	TCE	yes	yes	1	small	20%	glycerin	60%
5	10	conical champagne glass	TCE	no	yes	6	large	100%	immersion oil	
6	10	chemical sedimentation funnel	TCE	yes	no	5	small	100%	glycerin	40%
7	5	beaker (flat bottom)	TCE	no	yes	4	small	15%	immersion oil	-
8	div.	centrifugation tube	TCE	yes	yes	>10	medium	100%	paraffin oil	60%
9	10	beaker (flat bottom)	other	no	no	6	small	100%	immersion oil	-
10	10	conical champagne glass	TCE	no	yes	6	small	4%	paraffin oil	-
11	5	chemical sedimentation funnel	TCE	no	no	5	small	100%	immersion oil	40%
12	10	beaker (flat bottom)	TCE	no	no	2	small	30%	paraffin oil	-
13	5	chemical sedimentation funnel	TCE	no	yes	4	large	100%	paraffin oil	-
14	10	chemical sedimentation funnel	TCE	no	no	4	small	50%	paraffin oil	-
15	20	beaker (flat bottom)	TCE	no	no	3	small	20%	paraffin oil	65%
16	5	conical champagne glass	TCE	no	yes	4	small	100%	immersion oil	100%
17	5	conical glass with cock	TCE	no	yes	2		80%	paraffin oil	60%
18	10	conical champagne glass	TCE	no	yes	7	small	100%	paraffin oil	25%
19	10	Schaffnit funnel	TCE	no	yes	1	small	5%	phenol glycerol	-
20	10	beaker (flat bottom)	TCE	no	yes	5	small	25%	immersion oil	100%
21	10	beaker (flat bottom)	TCE	no	no	6	small	100%	phenol glycerol	100%
22	10	chemical sedimentation funnel	TCE	no	yes	2	small	30%	Depar 3000	-
23	10	chemical sedimentation funnel	TCE	yes	no	6	small	50%	photopolymer	-
24	10	conical champagne glass	TCE	no	yes	5	small	70%	glycerin	-
25	10	chemical sedimentation funnel	TCE	no	no	1	large	10%	immersion oil	-
26	10	chemical sedimentation funnel	TCE	no	no	1	small	10%	paraffin oil	-
29	10	chemical sedimentation funnel	TCE	yes	yes	3	small	70%	glycerin	40%
30	5	chemical sedimentation funnel	TCE	no	no	3	small	33%	chloralhydrate	80%
32	>5	conical champagne glass	TCE	yes	yes	>10	small	10%	glycerin	60%
33	10	chemical sedimentation funnel	TCE	yes	yes	4	large	31%	paraffin oil	70%
34	5	mensur	TCE	no	yes	6	large	100%	mineral oil	-

36	10	beaker (flat bottom)	TCE	yes	no	6	small	100%	immersion oil	-
38	10	chemical sedimentation funnel	TCE	yes	yes	4	small	9%	paraffin oil	-
40	10	chemical sedimentation funnel	TCE	yes	yes	7	small		paraffin oil	-
41	5	chemical sedimentation funnel	TCE	yes	yes	3	small	75%	glycerin	80%
42	5	beaker (flat bottom)	TCE	no	no	6	small		mineral oil	-
44	5	chemical sedimentation funnel	TCE	yes	yes	6	small	5%	glycerin	-
45	5	chemical sedimentation funnel	TCE	no	yes	3	small		water	-
47	10	chemical sedimentation funnel	TCE	no	yes	3	small	33%	paraffin oil	-
48	5	chemical sedimentation funnel	TCE	no	yes	2	large	10%	paraffin oil	-
49	5	chemical sedimentation funnel	TCE	no	yes	3	large	99%	paraffin oil	-
50	10	chemical sedimentation funnel	TCE	no	yes	4	small	29%	phenol glycerol	-
51	10	beaker (flat bottom)	TCE	yes	yes	5	small		paraffin oil	-
52	10	chemical sedimentation funnel	TCE	yes	no	3	large	25%	paraffin oil	40%

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

Appendix G. Results: presence of MBM, microscopic detection

Lab	fish			MBM			amount of sediment (g)			amount MBM	
	A	B	C	A	B	C	A	B	C	B	C
2	no	no	yes	no	yes	yes	0,148	0,19	0,175		
3	no	no	no	no	yes	yes	0,211	0,142	0,121	0,10%	0,15%
4	no	no	no	no	yes	yes	0,0787	0,0985	0,0671	0,15%	0,06%
5	no	no	no	no	yes	yes	0,16	0,135	0,164	0,45%	0,25%
6	no	no	no	no	yes	yes	0,0859	0,1217	0,0992	0,14%	0,03%
7	blank	yes	no	blank	yes	yes	0,064	0,066	0,065	0,40%	0,20%
8	no	no	no	yes	yes	yes	0,05	0,07	0,08	0,07%	0,03%
9	no	no	no	no	yes	yes	0,12	0,09	0,1	0,45%	0,15%
10	no	no	no	no	yes	yes	0,14	0,14	0,12	0,02%	0,01%
11	no	no	no	no	yes	yes	0,093	0,0967	0,0929	0,20%	0,05%
12	no	no	yes	no	yes	yes	0,13	0,13	0,11	0,50%	0,20%
13	no	no	yes	no	yes	yes	0,086	0,097	0,129	0,10%	0,05%
14	no	no	no	no	yes	yes	0,119	0,118	0,118	<0.5%	<0.2%
15	blank	blank	blank	no	yes	yes	0,2	0,28	0,21	0,15%	0,10%
16	no	no	no	no	yes	yes	0,09	0,07	0,07	0,03%	0,01%
17	no	no	no	no	yes	yes	0,088	0,106	0,116	0,04%	0,09%
18	no	no	yes	no	yes	yes	0,14	0,11	0,06	0,04%	0,05%
19	no	no	no	no	yes	yes	0,13	0,1	0,16	0,04%	0,04%
20	no	no	no	no	yes	yes	0,15	0,113	0,134	0,27%	0,13%
21	no	no	no	no	yes	yes	0,14	0,15	0,12	0,04%	0,03%
22	no	no	no	no	yes	yes	0,083	0,1	0,106	0,20%	0,10%
23	blank	yes	no	blank	yes	yes	0,0962	0,1464	0,1219		
24	no	no	no	no	yes	yes	0,12	0,15	0,12		
25	no	no	no	no	yes	no	0,08	0,13	0,11	< 0.1%	
26	no	no	no	no	yes	yes	0,105	0,076	0,128		
29	no	no	no	no	yes	yes	0,1092	0,0732	0,103	0,20%	0,10%
30	no	no	no	yes	yes	yes	0,08	0,07	0,07	0,60%	0,60%
32	no	no	no	no	yes	yes	0,09	0,08	0,07	0,11%	0,08%
33	no	no	no	no	yes	yes	0,137	0,129	0,12	0,10%	0,05%
34	no	yes	yes	no	yes	no	0,05	0,04	0,06		
36	no	no	no	no	yes	yes	0,159	0,18	0,159		
38	no	no	no	no	yes	yes	0,093	0,085	0,109	0,23%	0,33%
40	no	no	no	no	yes	yes	0,14	0,13	0,16	0,10%	0,20%
41	no	yes	yes	no	no	yes					0,02%
42	no	no	no	no	yes	yes	0,07	0,06	0,06	0,20%	0,10%
44	no	no	no	no	yes	yes					
45	no	no	no	no	yes	yes	0,06	0,09	0,09		
47	yes	no	no	yes	yes	yes	0,0742	0,132	0,1319		
48	no	no	no	no	yes	yes	0,09	0,07	0,08	0,046%	0,014%
49	no	no	no	no	yes	yes	0,07	0,07	0,07		
50	no	no	no	no	yes	yes	0,2609	0,2608	0,2373	0,23%	0,13%
51	no	no	yes	no	yes	yes	0,15	0,17	0,19		
52	no	no	no	no	yes	yes	0,298	0,266	0,214	0,30%	0,20%

Appendix H. Results: presence of MBM, DNA detection

Lab	fish			MBM			amount MBM			
	A	B	C	A	B	C	B	C	method	target
37	blank	blank	blank	no	yes	yes		low	PCR	bovine
53	yes	yes	yes	no	yes	yes	1%	0.1%	PCR	bovine, ruminant, fish

Lab	vertebrate in general						
	A	B	C			method	target
10	yes	yes	yes			PCR	vertebrate

Appendix I. Results: presence of MBM, protein detection

Lab	fish			MBM			amount MBM			
	A	B	C	A	B	C	B	C	method	target
39				no	no	no			Elisa	bovine