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IAG ring test 2009

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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 in Wageningen. The aim of the ring study is 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.
Four samples were prepared: one containing no animal proteins (blank), one with 0.1% of terrestrial animal material, one with 5% of fish material, and one with both 5% of fish material and 0.1% of terrestrial animal material. All participants were requested to determine the presence or absence of land animal and/or fish protein material. The participants were also 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 parameters of the microscopic method. Reporting the estimated amount of land animal or fish protein was optional for all participants. 49 Participants returned results using the microscopic method, making this the largest ring test ever organized for animal proteins in feed.
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 absence of fish meal were 0.88 (in presence of land animal material) and 0.96 (blank), and for the absence of land animal material the specificity scores were 0.98 (in presence of fish material) and 0.96 (blank). The detection of material of terrestrial animal origin (sensitivity) was good: 1.0 for detection of 0.1% in the absence of fish material, and 0.98 in the presence of fish material. The latter sensitivity score for land animal material in the presence of fish material is near to excellent, especially in view of past results.
The amount of land animal proteins was generally overestimated with a factor 2. The amount of fish material was slightly underestimated. Especially after the application of 5 grams for sedimentation a considerable underestimation was observed (1-2% for an amount of 5%).
Using a starting amount of 10 grams instead of 5 grams for sedimentation and the compulsory use of a binocular for observations at lower magnifications are factors that could increase the quality of detection (i.e. improve the score). The use of Alizarin staining of the sediment should be discussed further. In general, for several recommended method parameters higher frequencies of use were observed compared to those of last year.
The results give a good overview of current implementation of the microscopic method, and can be used for further improvement and planning of future ring tests. These results can be used in the framework of the European project SAFEED-PAP for method improvement. The problem of false positive detection of animal proteins (specificity) needs further attention. Training of microscopists remains important.

Contents

1 Introduction

The detection of animal proteins in feed remains an important issue in the process of avoiding Mad Cow Disease. All European Union member states are demanded to carry out monitoring programs for the presence of animal proteins, among a whole range of prohibited contaminants and undesired substances. Official control methods are only accepted after evaluation by the European Commission. Until now the microscopic detection method is the only official control method.
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 establish lab performance and to validate new methods. Directive 2003/126/EC was replaced by a new Regulation 152/2009/EC. This regulation compiles a range of methods for feed analyses. The microscopic method was copied from the Directive to the new Regulation without any modification. The practical part of this ring test was carried out legally under the Directive.
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, organised in 2009 by RIKILT on behalf of the IAG Section Feeding stuff Microscopy. The level of 0.1 % of animal proteins from land animals was selected for this ring test. In view of current and possible future relaxation of the ban for fish meal, material containing both animal proteins from land animals and fish material was tested.
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 give the participating labs information of their performance (internal quality assurance), and to give them the possibility of improvement. 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 filled out an inventory with details on the individual modifications of the application of the method. These results can be used to interpret the effectivity of some method parameters.

2 Methods

2.1 Materials

Four 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 the following major ingredients: corn gluten feed (31.4 %), palmkernel meal (20.0 %), citrus pulp (16.0 %), beet pulp (10.0 %), coconut meal (5.0 %), sunflowerseed meal (5.0 %), soyabean meal (2.0 %), minerals and molasse. The ingredients 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. Sixteen samples of this feed have been tested microscopically at RIKILT in 2008 for the presence of animal proteins, in the framework of the previous ring test (van Raamsdonk et al., 2008). No material of animal origin was found.
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 2008.

Label	Content
2009-A	5% fish meal
2009-B	0.1 % MBM
2009-C	Blank
2009-D	5% fish meal and 0.1 % MBM

The meat and bone meal (MBM) used was produced by DAKA, Denmark, which runs a factory that has processed and is still processing exclusively pig material along its entire history (f-factor 60 %). The fish meal is a sample from practice, containing a mix of species (f-factor 14,5 %).

2.2 Procedure for production

Samples 2009-A, 2009-B and 2009-D were produced according to the method of stepwise dilution. 300 g of fish meal was used to prepare (finally) 6 kg of contaminated feed as follows. The initial 300 g of fish meal was mixed in 300 g of feed and stirred for one minute. In four additional steps the remaining amount of feed was added stepwise by mixing. 3 g of MBM was used to prepare (finally) 3 kg of contaminated feed (sample B) or feed/fish meal (sample D) as follows. The initial 3 g of MBM was mixed in 3 g of feed and stirred for one minute. In the next step 6 g of feed was added and stirred to get 12 grams of contaminated feed (25 %). In eight additional steps the remaining amount of feed was added stepwise by mixing. The final jars were filled with 50 – 55 grams of material.
The ring trial material was prepared in a separate laboratory of RIKILT where animal proteins are never used.

2.3 Homogeneity study

RIKILT microscopists examined five jars of sample 2009-A, of 2009-B and of 2009-D. In all 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 four samples around to all participants. 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.

Sample	Sediment amount	fish	MBM
2009-A 5% fish (n= 5)	1,5 – 2,2 %	5 x positive	5 x negative
2009-B 0.1 % MBM (n= 5)	1.1 – 1.4 %	5 x negative	5 x positive
2009-D 5% fish, 0.1% MBM (n= 5)	1.7 – 2.0 %	5 x positive	5 x positive

2.4 Organization of the ring trial

The sets of four samples with an accompanying letter (see Annex A) were sent to all participants on the 22nd of February 2009. 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 Annex D).
The closing date for reporting results was fixed at April 8th. Some additional participants received the package at a later date. However, in only two cases results were received later than April 8th , but all the results were considered in the final evaluation, since all results were received before any result was communicated outside RIKILT.

2.5 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.

3 Results

Forty-nine packages with four samples were sent around. All participants returned results for the microscopic method, and one set of results was received for PCR analysis. From all participants a FAX message was received, and in some cases some missing information was collected from the E-mail messages. The list of participants is presented in Annex E. The full results are presented in the tables of Annex F, G and H. Blanks were considered to indicate the absence of the indicated type of animal protein.

3.1 Microscopic detection

The specificity and sensitivity scores were at good to excellent levels for most analyses (Table 4a; Annex G).

Table 4a: 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	C	D	A	B	C	D
n		5%	0	0	5%	0	0.1%	0	0.1%
49	specificity		0.88	0.96		0.98		0.96	
	sensitivity	1.00			1.00		1.00		0.98

With respect to the specificity, in the case of sample B with no fish and 0.1% of MBM six false positives for the detection of fish were found. In the other samples only one or two false positive

Table 4b: Participants' comments on the background of the false positives considered for the calculations in table 4a.

sample, contaminant	Participant	comment
A: land animal	10	no comment
B: fish material	22	0.02%
	23	< 0.05 %
	11	< 0.1%
	47	1 scale, 3 bones
	6	no comment
	51	no comment
C: fish material	36	< 0.5 %
	51	no comment
C: land animal	3	< 0.01 %
	51	no comment

results were reported. As far as commented by the participants the false positives were caused by only traces or low amounts of animal proteins (table 4b). The one and only reported false negative (MBM in sample D) can obviously not be documented by the participant.
Factors such as laboratory skills, sample composition, and lab procedures on e.g. cleaning to avoid sample pollution (in the case of false positives) might influence laboratory performance. In some cases misidentifications might be caused by some confusing plant ingredients or in the case of false positives for fish material, by the presence of terrestrial animal material. The results indicate a general good performance of individual participants.

3.2 Microscopic procedure

An inventory of nine different parameters was connected to the report of the actual results of the four samples. These results are shown in Annex F and summarised in Table 3. The main purpose is to provide information for the individual participants for comparison with the general application of the method. Additionally, a set of results with both a large range of specificity and sensitivity scores, and an extended list of method parameters, is rarely available for getting further insights in method performance. The current results provides the opportunity to discuss some parameters of the microscopic method. The frequencies of application of choices for several method parameters are presented 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	5	
	10 grams	41	
	other	3	
type of glassware	chemical sedimentation funnel	28	
	beaker (flat bottom)	13	
	champagne glass	5	
	conical glass with cock	1	
	other	2	
sedimentation agent	TCE	47	
	TCE/Petroleumether	2	
use of staining of sediment	no	35	
	yes	14	
use of binocular for examination at lower magnifications	yes	40	
	no	9	
number of slides used	minimum		1
	maximum		14
size of cover glass used	small (e.g. 20 x 20 mm)	27	
	medium	9	
	large (e.g. 26 x 50 mm)	13	
share of the total sediment used for examination	minimum		2%
	maximum		100%

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

parameter	parameter state	number of participants	amount
embedding agent	paraffin oil	20	
	immersion oil	12	
	glycerine / glycerol	10	
	Norland Adhesive	2	
	chloral hydrate	1	
	other (e.g. Depar 3000, water)	4	
f-factor for MBM	minimum		40%
	maximum		80%
	none estimated	25	

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 (after the examination of unstained material). Examination of the sediment at lower magnifications by using a binocular is requested in the Directive, but nine participants reported to skip this part of the procedure. Further comments will be made in the next paragraphs discussing several parameters of the method in more detail.

Correlations between specificity and method parameters are relevant only if some sort of causal relationship may exist in order to avoid the analysis of random fluctuations of results. Although a relationship between the use of 10 grams instead of 5 grams with sensitivity can be imagined, in a discussion of specificity this parameter is not relevant. Likewise, a causal relationship seems to be absent between the specificity and the type of glassware and the embedding agent. In the latter case the use of non-suited agents should be avoided anyway.
Based on these premises only a few presentations of the results, stratified according to the different choices for the method parameters, are given in the next paragraphs. Sensitivity and specificity are presented jointly in the tables; presence and absence of fish meal and of MBM (terrestrial animal material) are indicated in the heading of all tables in order to discriminate between sensitivity and specificity.

3.2.1 Use of binocular

The use of a binocular for examinations of the sediment at lower magnifications is required according to Directive 2003/126/EC. Nine participants, however, reported to skip that step in the examination procedure. No relevant differences are found in the sensitivity and specificity scores between the application of the full method (table 5: yes) and the lack of using a binocular (no), except for sample B. Of the six previously mentioned false positives for fish in sample B, three showed up among those nine participants. In all these cases only traces (“0.02%”, “<0.05%”, “3 bones, 1 scale”) were reported. Also one out of two false positives for fish in sample C was combined with the lack of examination at lower magnification.

Table 5: Sensitivity/specificity scores for the detection of animal proteins in four samples, separate for analyses with and without application of a binocular microscope for examination at lower magnifications. Abbreviations: n: number of participants per group. Capitals A to D: sample indication.

use of binocular microscope	n	fish				MBM			
		A	B	C	D	A	B	C	D
		5%	0	0	5%	0	0.1%	0	0.1%
yes	40	1.00	0.93	0.98	1.00	0.98	1.00	0.95	0.98
no	9	1.00	0.67	0.89	1.00	1.00	1.00	1.00	1.00

3.2.2 Staining of sediment with Alizarin

Some interesting results occurred after analysing the application of Alizarin staining of the sediment. Fewer false positives were reported for fish in sample B after staining (no: 0.86 vs. yes: 0.93; table 6). In a few other cases, however, lower values for specificity and sensitivity appear. Both reports of false positives for fish and for MBM in sample C occurred after the application of staining (no: 1.00 vs. yes: 0.86 for both types of animal proteins). In the presence of fish meal one false positive (sample A) and one false negative (sample D) detection of MBM after staining was observed as well.

Table 6: Sensitivity/specificity scores for the detection of animal proteins in four samples, separate for analyses with and without application of Alizarin staining of the sediment. Abbreviations: n: number of participants per group. Capitals A to D: sample indication.

Alizarin staining	n	fish				MBM			
		A	B	C	D	A	B	C	D
		5%	0	0	5%	0	0.1%	0	0.1%
no	35	1.00	0.86	1.00	1.00	1.00	1.00	1.00	1.00
yes	14	1.00	0.93	0.86	1.00	0.93	1.00	0.86	0.93

3.3 Quantification

With respect to quantification, only the starting amount of material for sedimentation can be expected to influence the results of quantification. In most cases either 5 or 10 grams of material has been used for sedimentation. The following presentation are based on the results of 42 out of 49 participants for the amounts of sediment achieved, and 30 out of 49 participants for the estimations of the amount of fish meal and terrestrial animal material in the samples. Three participants used amounts other than 5 or 10 grams.

Considering the amount of sediment achieved after using 10 grams of material (table 7a) the results are well within the range as found in the homogeneity study (table 2). A lower amount of sediment is retrieved after using only 5 grams of sample material compared to 10 gram of starting material (table 7). A difference of a factor of 2 is a logic result.

A notable underestimation was reached for the amount of fish material in the samples. There is a significant difference between the estimated amounts of fish material of the participants using five

(n=5 participants) or 10 grams (n=25 participants) of material (table 7b; $P < 0.005$ for both samples A and D containing fish). A considerable lower amount of fish material was estimated after using 5 grams of material (sample A: $P < 0.005$; sample D: $P < 0.05$). The difference between the two groups was not achieved for the estimations of the terrestrial animal material. The results for the estimation of the amount of MBM show generally an overestimation.

Table 7a:Resulting amounts of sediment separate for the different amounts of material used for sedimentation. For every result the average (in normal) and standard deviation (in italics) is given. Seven participants used other amounts than either 5 or 10 grams or did not report these results.

	n	amount of sediment (g)			
		A	B	C	D
		5%	0.1%	0	5.1%
total	42	0.190 (*0.064*)	0.131 (*0.043*)	0.121 (*0.046*)	0.189 (*0.064*)
5 gr	5	0.109 (*0.013*)	0.069 (*0.008*)	0.064 (*0.006*)	0.108 (*0.015*)
10 gr	37	0.194 (*0.051*)	0.135 (*0.035*)	0.124 (*0.038*)	0.194 (*0.052*)

Table 7b:Estimations (in %) for the amount of fish meal and MBM in four samples, separate for the different amounts of material used for sedimentation. For every result the average (in normal) and standard deviation (in italics) is given. Nineteen participants used other amounts than either 5 or 10 grams or did not report these results.

	n	estimated amount fish		estimated amount MBM	
		A	D	B	D
		5%	5%	0.1%	0.1%
total	30	4.34% (*2.59%*)	4.10% (*2.52%*)	0.23% (*0.21%*)	0.18% (*0.15%*)
5 gr	5	1.06% (*0.32%*)	1.22% (*0.74%*)	0.20% (*0.22%*)	0.22% (*0.20%*)
10 gr	25	4.77% (*2.34%*)	4.52% (*2.47%*)	0.20% (*0.16%*)	0.16% (*0.13%*)

4 Discussion and conclusions

4.1 Method performance

The specificity score for the detection of fish material indicate that in all but two cases the positive deviations (false positives) are found in the presence of terrestrial animal material (table 4). This situation is comparable to the results of 2008 (van Raamsdonk et al., 2008), The current results, together with those of last year, might indicate that certain fragments of land animals are misinterpreted as fish material. Total specificity scores for fish detection of 0.88 and 0.96 (table 4) are in the range as reported in literature (van Raamsdonk et al., 2007). The first ring test of the CRL (Veys et al., 2007) also indicated specificity scores of 0.88 (blank) and 0.91 (in presence of terrestrial animal material) for fish.
The specificity score for the detection of land animal material in absence of fish material in the current test (0.96) is at the upper end of the range of past IAG tests (Table 8). In the presence of fish meal the specificity is even better (0.98).
The sensitivity of the method in the current study (false negatives) for the detection of land animal material as well as for fish material is very good to excellent (table 4). The detection of land animal material in the presence of fish material (sample D), which is the corner stone of the performance of the microscopic method, is near to excellent. For this combination of animal proteins a sensitivity score of 0.44 for the detection of MBM was observed in 2003, although a bench mark study in 2003 resulted in a score of 0.987 (overview in van Raamsdonk et al., 2007). The result of the current study (0.98) is a very good achievement in a time frame of six years.

Table 8: Results for detection of material of land animals of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008) 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	blank	5%	2-3%	2%	0
year: land animal	blank	0.1%	0.2%	0.1%	0.1%
2003 (n=29)	0.86				1.0
2004 (n=30)	0.93		1.0		
2005 (n=42)				0.95	0.95
2006 (n=43)	0.98		1.0	1.0	
2007 (n=45)				0.933	
2008 (n=45)	0.93				0.98
2009 (n=49) current study	0.96	0.98			1.0

The main problem encountered in the current study is the detection of the absence of fish material in the presence of land animal material (specificity; sample B). Examination at lower magnification of the entire sediment should give a first impression of the presence of fish material, which could help to improve the specificity score. Furthermore, it can be imagined that staining of the sediment may mask

certain details of particles, i.e. necessary markers on bone particles. The staining capability of certain minerals, especially those derived from animal materials such tricalciumphosphate, is yet not fully understood. A further discussion is presented in van Raamsdonk et al. (2009).

4.2 Method parameters

For several parameters a shift in possible choices for method parameters is found in the current ring trial compared to that of last year (table 9; v. Raamsdonk et al., 2008). Especially a lower number of

Table 9: Comparison between parameters distribution in the IAG/NRL 2008 and 2009 study.

parameter	parameter choice	2008	2009
amount of material used for sedimentation	5 grams	16	5
	10 grams	26	41
	other	3	3
use of staining of sediment	no	31	35
	yes	14	14
use of binocular for examination at lower magnifications	yes	29	40
	no	16	9
number of slides used	minimum	1	1
	maximum	7	14
size of cover glass used	small (e.g. 20 x 20 mm)	34	27
	medium	1	9
	large (e.g. 26 x 50 mm)	9	13
share of the total sediment used for examination	minimum	4%	2%
	maximum	100%	100%
embedding agent for sediment	paraffin oil	18	20
	immersion oil	8	12
	glycerine / glycerol	8	10
	Norland Adhesive	0	2
	chloral hydrate	3	1
	other (e.g. Depar 3000, water)	8	4

participants choose to use 5 grams instead 10 grams for sedimentation, a lower number did not use a binocular for examination at lower magnifications, on average larger cover glasses were used to accommodate more material, and in a lower number of cases less suited embedding agents for

sediment material (e.g. chloral hydrate) were applied. These differences can generally be indicated as improvements in the implementation of the microscopic method.

4.3 Quantification

The results for the quantification of the animal proteins show in general an overestimation, especially for material of land animals. This is a usual situation for ingredients with a low share in the total composition (unpublished results of ring trials of IAG Section Feeding stuff Microscopy). The use of 5 grams for sedimentation instead of 10 grams resulted in a significant underestimation of the amount of fish meal (table 7). In contrast, Veys and Baeten (2008) found overestimations for fish material. In that study a specifically developed method for quantification was applied (Veys and Baeten, 2008). In the current ring trial one third of all participants did not report quantitative results, and a general formalised method was not used. Method parameter variations did not appear to affect estimations considerably in the present study.

5 General conclusions and recommendations

For the detection of material of terrestrial animal origin good results have been achieved in this ring trial. Sensitivity scores are 1.0 for detection of 0.1% in the absence of fish material, and 0.98 in the presence of fish material. The latter sensitivity score for land animal material in the presence of fish material is near to excellent, especially in view of past results. Also other sensitivity scores (presence of fish meal) and specificity scores were good. A specificity score for the absence of fish meal of 0.88 (in presence of land animal material) might indicate the need of improvement.
The amount of land animal proteins was generally overestimated with a factor 2. The amount of fish material was slightly underestimated. Especially after the application of 5 grams for sedimentation a considerable underestimation was observed (1-2% for an amount of 5%).
Using a starting amount of 10 grams instead of 5 grams for sedimentation and the compulsory use of a binocular for observations at lower magnifications are factors that could increase the quality of detection (i.e. improve the score). In general, for several recommended method parameters higher frequencies of use were observed compared to those of last year.

Recommendations

1) The specificity of the microscopic method for proper detection of the lack of fish material needs continuous attention for improvement.
2) It is recommended to use 10 grams of sample material for sedimentation, and to examine as much material (sediment and sieve fractions of the sample) as possible.
3) The use of a binocular for examinations at lower magnifications, although required according to the official method, needs more attention for a proper implementation of the method.
4) Staining of the sediment by alizarin needs further investigations before a general application is recommended.
5) The problem of false positive detection of animal proteins (specificity) needs further attention. Training of microscopists remains important.

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7 Acknowledgements

The board of IAG section Feeding Stuff Microscopy (dr. I. Paradies-Severin (LUFA, Hameln), dr. J.S. Jørgensen (DPD, Lyngby), dr. F. Wernitznig (AGES, Vienna), and dr. G. Frick (ALP, Posieux)) supported this study as advisory board for communication with the scientists and laboratories working in this research field, and in the final report activities. Their contributions are greatly acknowledged.

Annex I Invitation letter

Dear colleague <name>,

After a first organization of the IAG ring trial for animal proteins by RIKILT – Institute of food safety in 2008, RIKILT presents the set of samples for the 2009 version of this ring test.

In this package four 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.

The first aim of his ring test is to provide information that support you and your laboratory for your internal quality systems. The set of four samples is designed for that purpose. Secondly, a ring test can be used to collect information on the performance of the method(s). Therefore the report form consists of two sheets: a table where your can put in your results, including a quantification if you desire, and a form with details on your implementation of the method. Both forms are requested for a full report.

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. Please inform me if you want to apply PCR or immunochemistry methods, in order to get a proper result and way of reporting.

Reports are requested both by FAX and E-mail. The closing date of this ring test is April 7th, 2009. Forms 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

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
NRL-IAG ring test 2009		
Please select your unique lab number		
Have you read the ring test instructions?		
What detection method do you use?		
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) as standard procedure?		
Did you examine at lower magnifications (using a binocular)?		
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		
Did you use the computer program ARIES for identifying particles ?		
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

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

NRL-IAG ring test 2009

lab number 0

sample number	2009-A	2009-B	2009-C	2009-D
weight of sediment				
presence of fish material				
if present, estimated amount				
presence of material of land animals				
if present, estimated amount				
Please indicate the number of examined slides per sample				

Signature:

Date:

Annex IV Instructions as included in the report form

NRL-IAG ring test 2009

Instructions for the NRL-IAG ring trial

1	You have received a box with an introduction letter and four vials containing 50 grams of possibly contaminated animal feed. Please report the receipt of your package as soon as possible by E-mail to the address mentioned below.
2	The samples have to be analysed according to Directive 2003/126/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" 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 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 8th, 2009.

Annex V List of participants

Institute	City	Country
AGES	Vienna	Austria
Oleotest	Antwerpen	Belgium
CRA-W	Gembloux	Belgium
AFSCA/FAVV	Liege	Belgium
FLVVT	Tervuren	Belgium
Canadian Food Inspection Agency	Ottawa	Canada
Danish Plant directorate	Lingby	Denmark
IPL Atlantique	Bordeaux	France
S.C.L. Laboratoire de Rennes	Rennes	France
Inst. für Veterinär-Pharmakologie und Toxikologie	Bernau (Berlin)	Germany
Q-vis GmbH	Braunschweig	Germany
SGS Germany GmbH	Hamburg	Germany
LTZ Augustenberg	Karlsruhe	Germany
CVUA-RRW	Krefeld	Germany
Lab. Landwirtschaft / LUFA	Leipzig	Germany
Q-vis GmbH	Münster	Germany
Bayerisches Landesamt fur Gesundheit und	Oberschleisheim	Germany
LUFA Nord-West	Oldenbürg	Germany
Landeslabor Berlin-Brandenburg	Potsdam	Germany
LUFA Rostock	Rostock	Germany
Futtermittelinstitut Stade (LAVES)	Stade	Germany
Landesanstalt fürLandwirtschaftliche chemie, Universität Hohenheim	Stuttgart	Germany
Food-feedstuff Control Laboratory, Min. of Rural	Likovrissi, Athens	Greece
Feedstuff Microscopy Laboratory	Kildare	Ireland
Equine Centre	Naas, County Kildare	Ireland
Ist. Zooprofilattico Sperimentale delle Lombardia e dell'Emilia Romagna	Brescia	Italy
Ist. Zooprofilattico Sperimentale delle Venezie	Legnaro	Italy
Istituto Zooprofilattico Sperimentale della Sicilia	Palermo	Italy
Istituto Zooprofilattico Sperimentale della Sardegna	Sassari	Italy
Istituto Zooprofilattico Sperimentale Abruzzo & Molise "G. Caporale"	Teramo	Italy
IZS PLV Torino - CReAA	Torino	Italy
Natl. Food and Veterinary Risk Assessment Institute	Vilnius	Lithuania
Masterlab	Boxmeer	Netherlands
Labco	Europoort-Rotterdam	Netherlands
Nofima Ingredients	Fyllingsdalen	Norway
Lab. Regional de Veterinária	Angra do Heroismo	Portugal
Laboratório Nacional de Investigação Veterinária	Lisboa	Portugal
Institute of Veterinary medicine of Serbia	Belgrade	Serbia
Scientific Veterinary Institute	Novi Sad	Serbia
Central Control and Testing Institute of Agriculture	Bratislava	Slovakia
Natl. Veterinary Institute, Unit for Pathology	Ljubljana	Slovenia
Regional Agrarian Laboratory o Castilla & Leon	Burgos	Spain
Lab. Agroalimentario de DAR, Gen. de Catalunya	Cabrils (Barcelona)	Spain

Institute	City	Country
Lab. Agroalimentario de Cordoba	Cordoba	Spain
Lab. De Produccion y Sanidad Animal de Cordoba	Cordoba	Spain
Laboratorio Agroalimentario Y de Sanidad Animal	El Palmar Murcia	Spain
Tragsega (Cantabria)	Santander-Cantabria	Spain
SVA	Uppsala	Sweden
ALP Posieux Swiss Research Station	Posieux	Switzerland
LGC	Teddington	UK

Annex VI Details of procedures applied, microscopic method

lab nr										# slides			
	amount*	glassware	agent	staining	binocular	size	sed. used	embedding	f-factor	A	B	C	D
1	10	chem.sed.funnel	TCE	yes	yes	small	13%	NOA 65	-	4	3	4	3
2	10	chem.sed.funnel	TCE	no	yes	large		paraffin oil	60%	3	3	3	3
3	>5	conical glass with cock	TCE	yes	yes	small	100%	glycerol	40%				
4	5	beaker (flat bottom)	TCE	no	no	small	80%	immersion oil		6	6	6	6
5	10	chem.sed.funnel	TCE	no	yes	large	90%	glycerol		3	3	3	3
6	10	beaker (flat bottom)	TCE	no	yes	small	15%	immersion oil	-	7	6	5	8
7	10	mensur	TCE	no	yes	large	20%	mineral oil		2	2	2	2
8	10	beaker (flat bottom)	TCE/PE	no	yes	small	25%	paraffin oil	40%	4	4	4	4
9	10	chem.sed.funnel	TCE	no	yes	small	40%	Depar 3000	-	4	4	4	4
10	10	beaker (flat bottom)	TCE	yes	yes	small		paraffin oil					
11	10	beaker (flat bottom)	TCE	no	no	small	3%	paraffin oil	-	2	2	2	2
12	10	chem.sed.funnel	TCE	no	yes	large		paraffin oil	60%	4	8	8	4
13	10	chem.sed.funnel	TCE	no	yes	large	20%	glycerol/water		3	2	3	2
14	10	chem.sed.funnel	TCE	no	yes	medium	100%	paraffin oil	60%				
15	10	chem.sed.funnel	TCE	no	yes	small	40%	immersion oil	40%	10	10	10	10
16	10	conical champagne glass	TCE	no	yes	medium	45%	immersion oil	40%	3	3	3	3
17	10	chem.sed.funnel	TCE	no	yes	small	16%	phenol-glycerol	40%	4	4	4	4
18	10	chem.sed.funnel	TCE	yes	yes	small	30%	paraffin oil	40%		3	3	3
19	10	chem.sed.funnel	TCE	yes	yes	large	10%	immersion oil	40%	3	3	3	3
20	10	chem.sed.funnel	TCE	no	no	small	25%	paraffin oil		4	4	4	4
21	15	beaker (flat bottom)	TCE	no	yes	medium	50%	paraffin oil	80%	5	5	5	5
22	5	beaker (flat bottom)	TCE	no	yes	medium	100%	Chloralhydrate		6	6	6	6
23	5	beaker (flat bottom)	TCE	no	no	small	100%	immersion oil		6	6	6	6

lab nr										# slides			
	amount*	glassware	agent	staining	binocular	size	sed. used	embedding	f-factor	A	B	C	D
24	10	conical champagne glass	TCE	no	yes	small	20%	paraffin oil		3	3	3	3
25	10	chem.sed.funnel	TCE	yes	yes	medium	50%	glycerol	40%	3	3	3	3
26	10	chem.sed.funnel	TCE	no	no	small	8%	glycerin	40%				
27	10	conical champagne glass	TCE	no	yes	small	100%	paraffin oil	30%	4	3	4	4
29	10	chem.sed.funnel	TCE	no	yes	small	50%	glycerin	40%	5	5	5	5
30	10	chem.sed.funnel	TCE	yes	yes	small	70%	immersion oil	40%	4	4	4	4
31	10	conical champagne glass	TCE	no	yes	small	72%	glycerol	40%	3	3	3	3
33	10	beaker (flat bottom)	TCE/PE	no	yes	small	27%	paraffin oil	40%	4	4	4	4
34	5	beaker (flat bottom)	TCE	no	yes	small	5%	immersion oil		6	6	6	6
35	10	chem.sed.funnel	TCE	yes	yes	large	31%	paraffin oil	70%	4	4	4	4
36	15	beaker (flat bottom)	TCE	yes	no	medium	50%	glycerin/water		5	5	5	5
37	10	chem.sed.funnel	TCE	yes	yes	medium	80%	glycerol	40%	3	3	3	3
40	10	chem.sed.funnel	TCE	no	yes	large		immersion oil		3	3	3	3
41	10	beaker (flat bottom)	TCE	no	yes	small		immersion oil		12	11	14	10
42	10	chem.sed.funnel	TCE	no	no	large	25%	paraffin oil	40%	4	4	4	4
43	10	chem.sed.funnel	TCE	no	yes	medium	2%	glycerol/water		6	6	6	6
44	10	chem.sed.funnel	TCE	no	yes	medium	8%	paraffin oil		1	1	4	1
45	10	conical champagne glass	TCE	no	yes	small	10%	immersion oil	50%	11	9	9	12
46	10	chem.sed.funnel	TCE	yes	yes	small	80%	paraffin oil	40%	4	4	4	4
47	10	glass tubes	TCE	no	no	large		paraffin oil		8	8	7	1
48	10	chem.sed.funnel	TCE	yes	yes	small	40%	paraffin oil		4	4	4	4
49	5	chem.sed.funnel	TCE	no	yes	small		water		6	3	3	6
50	10	chem.sed.funnel	TCE	yes	no	large	20%	paraffin oil	40%	3	3	2	3
51	10	chem.sed.funnel	TCE	yes	yes	small	15%	NOA		3	3	4	3
52	10	chem.sed.funnel	TCE	no	yes	large	100%	paraffin oil		4	4	4	4
53	10	beaker (flat bottom)	TCE	no	yes	large	90%	immersion oil		6	6	6	6

* 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	yes	no	no	yes	no	yes	no	yes
2	yes	no	no	yes	no	yes	no	yes
3	yes	no	no	yes	no	yes	yes	yes
4	yes	no	no	yes	no	yes	no	yes
5	yes	no	no	yes	no	yes	no	yes
6	yes	yes	blank	yes	no	yes	blank	yes
7	yes	no	no	yes	no	yes	no	yes
8	yes	no	no	yes	no	yes	no	yes
9	yes	no	no	yes	no	yes	no	yes
10	yes	no	no	yes	yes	yes	no	no
11	yes	yes	no	yes	no	yes	no	yes
12	yes	no	blank	yes	no	yes	blank	yes
13	yes	no	no	yes	no	yes	no	yes
14	yes	no	no	yes	no	yes	no	yes
15	yes	no	no	yes	no	yes	no	yes
16	yes	no	blank	yes	no	yes	blank	yes
17	yes	no	no	yes	no	yes	no	yes
18	yes	no	blank	yes	no	yes	blank	yes
19	yes	no	no	yes	no	yes	no	yes
20	yes	no	no	yes	no	yes	no	yes
21	yes	no	no	yes	no	yes	no	yes
22	yes	yes	no	yes	no	yes	no	yes
23	yes	yes	no	yes	no	yes	no	yes
24	yes	no	no	yes	no	yes	no	yes
25	yes	no	no	yes	no	yes	no	yes
26	yes	no	no	yes	no	yes	no	yes
27	yes	no	no	yes	no	yes	no	yes
29	yes	no	no	yes	no	yes	no	yes
30	yes	no	blank	yes	no	yes	blank	yes
31	yes	no	no	yes	no	yes	no	yes
33	yes	no	no	yes	no	yes	no	yes
34	yes	no	blank	yes	no	yes	blank	yes
35	yes	no	no	yes	no	yes	no	yes
36	yes	no	yes	yes	no	yes	no	yes
37	yes	no	no	yes	no	yes	no	yes
40	yes	no	no	yes	no	yes	no	yes
41	yes	no	no	yes	no	yes	no	yes
42	yes	no	no	yes	no	yes	no	yes
43	yes	no	no	yes	no	yes	no	yes
44	yes	no	no	yes	no	yes	no	yes
45	yes	no	no	yes	no	yes	no	yes
46	yes	no	no	yes	no	yes	no	yes
47	yes	yes	no	yes	no	yes	no	yes
48	yes	no	no	yes	no	yes	no	yes
49	yes	no	no	yes	no	yes	no	yes

lab nr	fish				MBM			
	A	B	C	D	A	B	C	D
50	yes	no	blank	yes	no	yes	blank	yes
51	yes	yes	yes	yes	no	yes	yes	yes
52	yes	no	no	yes	no	yes	no	yes
53	yes	no	no	yes	no	yes	no	yes

Annex VIII Results: sediment and quantification

lab nr	amount of sediment (gram)				estimated amount of fish		estimated amount of MBM	
	A	B	C	D	A	D	B	D
1	0.22	0.13	0.18	0.2				
2	0.21	0.12	0.15	0.14	6.3%	5.0%	0.22%	0.21%
3	0.122	0.097	0.066	0.121	4.6%	4.6%	0.45%	0.15%
4	0.1	0.07	0.07	0.1	0.8%	0.4%	0.05%	0.04%
5	-	-	-	-				
6	0.12	0.08	0.05	0.1				
7	0.209	0.135	0.19	0.244				
8	0.2	0.14	0.13	0.24	3.6%	3.8%	0.13%	0.14%
9	0.171	0.118	0.093	0.151	3.0%	3.0%	0.50%	0.10%
10	0.17	0.13	0.09	0.17				
11	0.176	0.129	0.13	0.205	4.6%	5.2%	0.1-0.5%	0.1-0.5%
12	0.2	0.134	0.124	0.189	7-8%	7-8%	0.20%	0.10%
13	0.194	0.084	0.132	0.17				
14	0.365	0.224	0.201	0.255	10.0%	10.0%	0.10%	0.05%
15	0.274	0.193	0.178	0.256	9.0%	8.8%	0.10%	0.03%
16	0.18	0.15	0.16	0.22	1.1%	1.0%	0.12%	0.55%
17	0.23	0.149	0.149	0.213	4.3%	4.6%	0.21%	0.21%
18	0.14	0.09	0.09	0.15	4.8%	4.3%	0.28%	0.27%
19	0.187	0.125	0.12	0.186	4.9%	4.7%	0.20%	0.10%
20	0.174	0.153	0.063	0.232				
21	0.308	0.218	0.225	0.306	9.2%	6.7%	0.08%	0.13%
22	0.123	0.073	0.07	0.13	1.5%	2.0%	0.50%	0.30%
23	0.1	0.08	0.06	0.09	1.0-1.5%	1.5-2.0%	0.2-0.5%	0.1-0.4%
24	0.21	0.15	0.11	0.24	5.0%	5.0%	0.10%	0.10%
25	0.151	0.099	0.074	0.121	4.6%	3.9%	0.14%	0.14%
26	0.202	0.13	0.112	0.224	4.0%	2.0%	0.20%	0.10%
27	0.13	0.08	0.11	0.17	6.1%	5.0%	0.12%	0.07%
29	0.248	0.172	0.168	0.269	4.8%	4.4%	0.25%	0.44%
30	0.098	0.088	0.046	0.113	5.2%	6.5%	0.80%	0.25%
31	0.2	0.15	0.14	0.23	1.5%	1.6%	0.14%	0.10%
33	0.17	0.12	0.1	0.16	2.1%	1.8%	0.17%	0.11%
34	0.124	0.063	0.059	0.108	1.0%	1.5%	0.05%	0.50%
35	0.185	0.125	0.113	0.189	7.5%	8.1%	0.11%	0.05%
36	0.374	0.237	0.232	0.356	1-10%	1-10%	0.5-5%	0.1-1%
37	0.098	0.076	0.073	0.075	4.0%	2.2%	0.20%	0.20%
40	-	-	-	-				
41	0.2	0.13	0.13	0.18				
42	0.23	0.18	0.16	0.21	0.0%	0.1%	0.01%	0.01%
43	-	-	-	-				

lab nr	amount of sediment (gram)				estimated amount of fish		estimated amount of MBM	
44	0.247	0.191	0.139	0.279				
45	0.2	0.14	0.13	0.19	3.0%	3.0%	0.10%	0.10%
46	0.137	0.099	0.079	0.132	5.9%	4.7%	0.17%	0.07%
47	0.2	0.12	0.13	0.19				
48	0.209	0.153	0.14	0.269				
49	0.1	0.06	0.06	0.11	0.7%	0.5%	0.03%	0.03%
50	0.283	0.184	0.135	0.282	6.5%	7.0%	0.14%	0.22%
51	0.21	0.15	0.13	0.2				
52	-	-	-	-				
53	0.16	0.16	0.15	0.13				