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IAG ring test 2010

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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, Wageningen University and Research Centre, The Netherlands. The aim of the ring study was to provide the participants information on the performance of the method in their laboratory. This is essential information for their individual quality systems. A further objective was to gather information about a set of analytical parameters of the microscopic method.
Three samples were prepared: one containing no animal proteins (blank), one with 2% of fish material and 0.1% of terrestrial animal material, and one with 2% of fish material and 0.05% 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 answer questions on a series of parameters of the microscopic method. Reporting the estimated amount of land animal or fish protein was optional for all participants. 53 Participants returned results using the microscopic method, 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 both the absence of fish meal and the absence of land animal material were 0.98 (blank). The detection of fish material (sensitivity) was faultless. Material of terrestrial animal origin was detected with sensitivity scores of 0.98 for detection of 0.1%, and 0.91 for the contamination level of 0.05%, both in the presence of fish material. These sensitivity scores for terrestrial animal material in the presence of fish material are good to excellent.
The amount of land animal proteins was generally well estimated, but with a large variation. The amount of fish material was overestimated. These indications are different from the results of previous ring trials.
In this study the use of glassware where the sediment can be collected at the bottom (e.g. chemical sedimentation funnel) gives slightly better results than the use of glassware where the flotation and the solvent has to be decanted first (e.g. beaker). Together with the starting amount for sedimentation (10 g vs. 5 g of material) and the use of a stereo microscope, an optimization of these parameters in the microscopic method is correlated with the increase of the sensitivity and specificity of the detection of animal proteins. On the other hand, a further harmonization is still possible.
The results for the PCR (three sets of results) and the immunoassay test (one set of results) indicate that a proper detection can be achieved at relatively low levels of contamination. However, in some cases false positive results were also reported.
The results give a good overview of the performance of the laboratories performing the microscopic method, although further improvement is still possible.

Contents

1 Introduction

Member states of the European Union are requested by EU legislation to maintain an active monitoring program for the safety of feed. The monitoring of the presence of animal proteins in the framework of eradication of mad cow disease is an important part of it. A range of official control methods were in 2009 combined in one Regulation (152/2009/EC). With respect to animal proteins, the microscopic detection method is the only official control method until now. The description of the microscopic method was copied from the former Directive 2003/126/EC to Annex VI of the new Regulation without any modification.
The level of contamination of 0.1%, as stated as performance parameter for official control methods in Annex VI of Regulation 152/2009/EC, forms the basis of most proficiency tests and collaborative studies to establish lab performance and to validate new methods.
The IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy organises annually a ring test for animal proteins in feeds for all their members. In this report the ring test for animal proteins is presented, which was organised by RIKILT in 2010 on behalf of the IAG Section Feeding stuff Microscopy. The contamination level of 0.1% of animal proteins from terrestrial animals was also part of the design of this ring test. Nevertheless, a lower contamination level (0.05%) in the presence of fish meal was tested in order to document further the performance of the laboratories.
The indication “ring test” fits in the history of annual proficiency tests for animal proteins carried out under the responsibility of the IAG. The main purpose of the ring test is to monitor the performance of the participating laboratories (quality assurance). The main part of this report presents and discusses the results in terms of sensitivity and specificity scores. For a further documentation of laboratory results each participant answered questions on details of the application of the method. These results can be used to interpret the effectivity of some method parameters.

2 Methods

2.1 Materials

Three samples were produced, based on a broiler 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: wheat (46%), soy bean meal (15.0%), soy beans full fat (13.0%), corn (10.9%), rapeseed full fat (7.0%), vegetable fat (5.0%), minerals (3.1%). The ingredients were glued together with molasse to form larger particles. Therefore, the feed material was sieved at 2 mm in order to exclude these particles from the samples, avoiding the possibility of animal proteins adhering to them. The composition might be modified as a result of this procedure. Five samples of this feed have been tested microscopically at RIKILT for the presence of animal proteins. No material of animal origin was found.
The ring trial consisted of three samples with a composition as listed in Table 1.

Table 1: Composition of the samples in the NRL-IAG ring trial 2010.

Label	Content
2010-A	2% fish meal, 0.1% MBM
2010-B	Blank
2010-C	2% fish meal, 0.05% MBM

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 A and C of this ring trial is of bovine origin and had been heat treated at 133 ^{o}C. The f-factor (share of heavy particles in the total of the MBM) of this material was 70%. The fish meal is a sample from the practice, containing a mix of species (f-factor 14.5%). This fish meal sample was tested to assure the absence of animal proteins of terrestrial animals.

2.2 Procedure for production

In order to avoid any cross contamination, the samples were produced in a strict order. Jars for sample 2010-B were filled with 50-55 grams of the pure feed, closed and set aside.
Samples 2010-A and 2010-C were produced according to the method of stepwise dilution. 110 g of fish meal was used to prepare (finally) 5.5 kg of contaminated feed as follows. The initial 110 g of fish meal was mixed in 110 g of feed and stirred for one minute. In five additional steps the remaining amount of feed was added stepwise by mixing.
For the preparation of sample 2010-C, the jars were filled with 50 +/- 0.1 g of the feed/fish material. To each jar, 25.0 mg of MBM was added, 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 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.
Finally sample 2010-A was prepared. 2.75 g of MBM was used to prepare (finally) 2.75 kg of contaminated feed/fish meal (sample A) as follows. The initial 2.75 g of MBM was mixed in 2.75 g of feed and stirred for one minute. In the next step 5.5 g of feed was added and stirred to get 11 grams of contaminated feed (25%). In eight additional steps the remaining amount of feed was added stepwise by mixing. The final jars for sample 2010-A 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 three jars of sample 2010-A and of 2010-C, and five jars of sample 2010-B. In all cases a correct result was obtained, as is shown in Table 2. 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 ring trial.

Table 2: Results of the homogeneity study. Sediment amounts are based on 10 grams.

Sample		Sediment amount	fish	MBM
2010-A 2% fish meal, 0.1% MBM	(n= 3)	2.3 – 2.4%	3 x positive	3 x positive
2010-B blank	(n= 5)	2.0 – 2.2%	5 x negative	5 x negative
2010-C 2% fish meal, 0.05% MBM	(n= 3)	2.2 – 2.3%	3 x positive	3 x positive

2.4 Organization of the ring trial

The sets of three samples with an accompanying letter (see Annex I) were sent to all participants on the 24th of February 2010. On Friday February 26 an E-mail message was sent around to all participants, together with an electronic report form (see Annex II and III) and the request to confirm the receipt of the package. The report form also contained a sheet with instructions (see Annex IV). The closing date for reporting results was fixed at April 6. Some additional participants received the package at a later date. In all cases results were received not later than April 8, so that all results were considered in the final evaluation, since all results were received before any result was communicated outside RIKILT.

2.5 Participants

The 53 participants originated from 21 countries: 18 Member States of the European Union, and three other countries (Canada, Norway and Switzerland). The list of participants is presented in Annex V. Five Member States have been involved with three or more participating laboratories: Germany (15 labs), Italy (6), Belgium (4), Spain (4), France (3). With the indicated coverage, this ring test is the largest one ever reported.

2.6 Analysis of results

For binary results (yes/no, positive/negative, etc.) standard statistics are accuracy, sensitivity and specificity. The accuracy is the fraction of correct results, either positive or negative. The sensitivity is the ability of the method used, to detect the contaminant when it is present, whereas the specificity is the ability to not detect the contaminant when it is absent. The following equations have been used to calculate the statistics:

$$\text{Accuracy } AC = \frac{PA + NA}{PA + ND + PD + NA}$$

$$\text{Sensitivity } SE = \frac{PA}{PA + ND}$$

$$\text{Specificity } SP = \frac{NA}{PD + NA}$$

where PA is the number of correct positive identifications (positive agreements), NA the number of correct negative identifications (negative agreements), PD the number of false positives (positive deviations) and ND the number of false negatives (negative deviations). The statistics are presented as fractions *p*. Accuracy (specificity or sensitivity) has been calculated for each sample type.

As criterion for a good or excellent score a threshold of 0.95 for either sensitivity or specificity was applied.

3 Results

Fifty-three packages with three samples were sent to all participants. All participants returned results for the microscopic method, and three sets of results were received for PCR analysis, and one for protein detection. From all participants a FAX message was received, and in some cases some missing information was collected from the E-mail messages. The full results are presented in the tables of Annex VI, VII and VIII. 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 3a; Annex VII).

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

		Fish			MBM		
		A	B	C	A	B	C
n		2%	0	2%	0.1%	0	0.05%
53	specificity		0.98			0.96	
	sensitivity	1.00		1.00	0.98		0.91

With respect to the specificity, one false positive for fish meal and two false positives for MBM were reported. In the sample with 0.1% MBM only one false negative was observed. As far as commented by the participants the false positives were caused by only traces or low amounts of animal proteins (table 3b). Five false negatives for the sample 2010-C (with a contamination level twice as low as sample 2010-A) were found. These false negatives can obviously not be documented by the participant.

Table 3b: Participants' comments on the background of the false positives reported for the calculations in table 3a.

sample, contaminant	Participant	comment
B: fish material	41	1 fish bone
B: land animal	7	traces of hair and feather filaments
	41	1 bone and 2 feather filaments

Factors such as laboratory skills, glassware used, and lab procedures on e.g. cleaning to avoid sample pollution (in the case of false positives) might influence laboratory performance. In some cases misidentifications might be caused by some confusing plant ingredients. The results indicate a general very good performance of individual participants.

3.2 Microscopic procedure

The participants were asked to provide technical details with respect to nine different methodological parameters. The information provided is shown in Annex VI and summarised in Table 4. Through this additional information, participants are not only informed about their performance, but also get a view on the question to what extent their implementation of the method deviates from the way the method is implemented by the other participants. A combination of all information could potentially improve the overall method performance.

Table 4: Inventory of parameters for microscopic detection and their application.

parameter	parameter state	number of participants	amount
amount of material used for sedimentation	5 grams	3	
	10 grams	48	
	other	2	
type of glassware	chemical sedimentation funnel	31	
	beaker (flat bottom)	10	
	champagne glass	8	
	conical glass with cock	2	
	other	2	
sedimentation agent	TCE	52	
	TCE/Petroleumether	1	
use of staining of sediment	no	34	
	yes	19	
use of binocular for examination at lower magnifications	yes	45	
	no	8	
size of cover glass used	small (e.g. 20 x 20 mm)	27	
	medium	10	
	large (e.g. 26 x 50 mm)	16	
share of the total sediment used for examination	minimum		2%
	maximum		100%
embedding agent	paraffin oil	23	
	immersion oil	14	
	glycerine / glycerol	10	
	Norland Adhesive	2	
	other (water, glycerol:water mixture, mineral oil)	4	
f-factor for MBM	minimum		15%
	maximum		80%
	none estimated	22	

Forty-eight out of 53 participants started the sedimentation procedure with an amount of 10 grams of material. A chemical sedimentation funnel was used primarily (31 out of 53 labs). Nineteen participants used staining of the sediment (Alizarin Red) 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 official method, but eight participants out of 53 reported to skip this part of the procedure. Only in one occasion a non-suited embedding agent was used for the examination of the sediment (water). 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 exists in order to avoid the analysis of random fluctuations of results. In the process of further harmonisation of the microscopic method, almost all participants made the same choice for the application of several parameters: amount of material used for sedimentation (10 grams), sedimentation agent (TCE). Only one participant used a non-suited embedding agent.
Based on these premises only a few presentations of the results, stratified according to the different choices for the method parameters, could be 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 Embedding agent

The choice of the embedding agent is an important aspect determining the appearance of the bone particles in a sediment. This effect is primarily influenced by the viscosity (fluidity, expressed in cP, or centiPoise) of the embedding agent. In most cases the viscosity of the declared embedding agents can be deducted. Normally paraffin oil of only moderate viscosity is applied. The Norland adhesive used in Europe is a type with high viscosity (i.e. NOA 65). Glycerol (n = 12 participants) was occasionally mixed with water (n = 2) or phenol as preservative was added (n = 2). In the situation of a mixture with water the final viscosity is not known. Table 5 provides some information on the use of different embedding agents.

Table 5: Overview of the application of different embedding agents for the examination of the sediment, organised in groups of viscosity. Abbreviations: n: number of participants per group.

viscosity	n
high(> 1000 cP): Norland (NOA 65), glycerol (excluding mixtures with water)	12 (22.6%)
moderate (50-250 cP): immersion oil, paraffin oil	37 (69.8%)
low (approx. 1 cP): water	1 (1.9%)
other (unknown): mineral oil or mixtures	3 (5.7%)

The majority of participants used an embedding agent with a moderately high viscosity for the examination of the sediment material. Less than one quarter of the participants used an embedding agent with a high viscosity. The effect in terms of specificity and sensitivity can not be calculated reliably, since the exact composition of the glycerol:water mixture is not known.

3.2.2 Glassware

The types of glassware can be divided in two groups: equipment where the flotate has to be removed first before the sediment can be collected (e.g. champagne glass, beaker), and glassware that allows to remove the sediment from the bottom (e.g. chemical sedimentation funnel, conical glass with cock) without interference of the flotate. The latter type provides a better opportunity to permanently separate the sediment from the flotate. The specificity and sensitivity scores for the different types of glassware are presented in table 6. Although statistically the differences are very small, the data indicates a slightly better performance of the glass ware which allows to remove the sediment at the bottom.

Table 6: Sensitivity/specificity scores for the detection of animal proteins in three samples, separate for analyses based on the use of different types of glassware. Abbreviations: n: number of participants per group. Capitals A to C: sample indication.

Type of glassware	n		fish			MBM		
			A	B	C	A	B	C
			2%	0	2%	0.1%	0	0.05%
sediment removal at the top	18	specificity		0.94			0.89	
		sensitivity	1.00		1.00	0.94		0.89
sediment removal at the bottom	33	specificity		1.00			1.00	
		sensitivity	1.00		1.00	1.00		0.91

3.2.3 Other parameters

The use of a binocular (stereomicroscope) for examinations of the sediment at lower magnifications is required according to Regulation 152/2009/EC. Eight participants, however, reported to skip that step in the examination procedure. No relevant differences were found in the sensitivity and specificity scores between the application of the full method compared to skipping the use of a stereomicroscope. Staining of the sediment material with Alizarin Red is applied by 36% of the participants, with the goal to facilitate an initial recognition of bone particles. It is nevertheless necessary for a final decision on the nature of individual particles to consider other features of the particle such as the structure, presence of lacunae and the visibility of canaliculae. The very few false positives (see table 3) were reported for both fish and MBM in sample B without staining. There is only a slight difference between the sensitivity scores for MBM with respect to the application of stained and unstained examination.

3.3 Quantification

The starting amount of material for sedimentation will obviously 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 45 out of 53 participants for the amounts of sediment

achieved, and 29 out of 53 participants for the estimations of the amount of fish meal and terrestrial animal material in the samples. The other participants did not report these results, or 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). The amount of sediment retrieved after using only 5 grams of sample material is approximately half the amount obtained with 10 gram of starting material is (table 7a). A difference of a factor of 2 is a logic result.
The amounts of fish material in the samples were generally overestimated (table 7b). The overestimation is higher after using 5 grams of material, but the difference is not significant. The participants using 10 grams of material for sedimentation made an almost perfect average estimation of the amounts of MBM in the samples A (0.1%) and C (0.05%). The standard errors, however, are very large or even larger than the average (estimations of amount MB in sample C). Moreover, only three participants submitted results after using 5 grams of material, which makes the results of the two groups virtually incomparable.

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

	n	amount of sediment (g)		
		A	B	C
		2.1%	0%	2.05%
total	45	0.236 (*0.078*)	0.215 (*0.072*)	0.231 (*0.077*)
5 gr	3	0.121 (*0.010*)	0.111 (*0.023*)	0.116 (*0.014*)
10 gr	42	0.241 (*0.074*)	0.220 (*0.068*)	0.236 (*0.072*)

Table 7b:Estimations (in %) for the amount of fish meal and MBM in two 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. Twenty-four participants used other amounts than either 5 or 10 grams or did not report these results.

	N	estimated amount fish		estimated amount MBM	
		A	C	A	C
		2%	2%	0.1%	0.05%
total	29	3.73% (*2.05%*)	3.93% (*2.14%*)	0.091% (*0.075%*)	0.074% (*0.102%*)
5 gr	3	4.36% (*3.20%*)	5.10% (*0.95%*)	0.046% (*0.041%*)	0.195% (*0.265%*)
10 gr	26	3.48% (*1.09%*)	3.66% (*2.21%*)	0.096% (*0.078%*)	0.053% (*0.057%*)

3.4 Detection by other methods

The use of the mammalian primer set in the PCR method of two participants (Annex IX) produces correct results for one participant (no. 42) and a false positive for another participant (no. 28). No quantitative results were submitted. One participant reported results for the presence of fish and mammals separately. The report sheet of the other participant (no. 28) did not indicate whether the results for mammalian and fish proteins are retrieved with two different primer sets, e.g. for mammals and for fish, or with one primer set, e.g. for vertebrates (Annex IX).
The presence of mammalian material according to immunoassay analysis was carried out with the Feedchek kit by one participant. The use of immunoassay analysis gave also correct positive indications of mammalian proteins at the current levels of contamination (Annex X).
Both participants send in results for the microscopic detection as well, which were all correct.

4 Discussion and conclusions

4.1 Method performance

In general the results of the various laboratories in this study were very good. With respect to the detection of animal proteins of terrestrial animals, the specificity in the blank ranges between the highest values of the last eight ring trials (table 8). According to the current legislation, a method for detection of animal proteins should be able to detect at least a contamination level of 0.1%. An attempt was made in this ring trial to get information on the performance of the microscopic method at a lower level in the presence of fish meal. The sensitivity of the method in the current study (false negatives) for the detection of land animal material in the presence of fish material is very good to excellent (table 3a). 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 comparable to that of last year (table 8), which indicates a consolidation in the time frame of the last years.
A recent study of CRL-AP (Veys et al., 2010) indicates that contamination levels as low as 0.0025% of terrestrial animal material can be detected in the absence of fish meal (sensitivity score: 0.962). This level of contamination is proposed to be set as level of detection (LOD) for the microscopic method. At a contamination level of 0.005% on average slightly more than one particle per slide could be expected. These results are all based on an MBM with f = 0.48 (48% of bone fragments in the MBM; Veys et al., 2010). The number of particles to be expected on a slide depends largely on the amount of sediment applied per slide, and from the current study it appears that several parameters influencing the examination show a large variation (table 4). The current results show that even in the presence of fish material an acceptable performance can be achieved at levels lower than 0.1% (i.e. 0.05%).

Table 8: Results for detection of material of terrestrial animals of previous ring tests organised by J.S. Jørgensen (Danish Plant Directorate, Lyngby; 2003-2007) and RIKILT (2008-2010) 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	0	4-5%	2%	0	2%	0	0
year land animal	0	0	0.1%	0.1%	0.05%	0.05%	0.02%
2003 (n=29)	0.86			1.0			0.96
2004 (n=30)	0.93					0.97	
2005 (n=42)			0.95	0.95			
2006 (n=43)	0.98		1.0				
2007 (n=45)		0.89	0.933				
2008 (n=45)	0.93			0.98		0.96	
2009 (n=49)	0.96	0.98		1.0			
2010 (n=53) current study	0.96		0.98		0.91		

Only one positive deviation (false positive) for MBM was found in the blank sample (table 4: specificity 0.98). This situation is comparable to the results of 2009 (table 8).
The main problem encountered in previous studies (van Raamsdonk et al., 2008, 2009; Veys et al., 2010) was the detection of the absence of fish material in the *presence* of land animal material. These results might indicate that certain fragments of land animals were misinterpreted as fish material. This could not be verified in the current study since a sample with exclusively terrestrial animal material was not included in the design. 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.
The results for the PCR and immunoassay methods would indicate that a proper detection can be achieved at relatively low levels of contamination. However, in some cases false positive results were also reported in this study. For both methods a detection level of 0.1% in an animal feed is reported in recent studies (Woodgate et al., 2009), but for immunoassays this is in contrast with earlier publications (e.g. Myers et al., 2007). Immunoassay analysis of feed samples spiked at 0.1% with ruminant material heated at 133 ^{o}C provides correct positive results, but higher sterilization temperatures result in lower sensitivities (higher LOD; personal communication R. Margry, CCL, November 2010). Further ring tests are recommended to confirm these results.

4.2 Method parameters

A proficiency test is meant to reveal information on the performance of individual labs. It is not possible to draw conclusions about the validity of the method(s) applied (von Holst et al., 2005). In certain past occasions of proficiency tests a questionnaire was send around with the samples, which can be used to evaluate the way in which the method is implemented. The current and previous ring tests of IAG are examples of those “extended proficiency tests”. Although method validation is principally impossible, improvements of method implementation and relationships with the results can be discussed (van Raamsdonk et al., in press).
Based on the results of these questionnaires, a shift in possible choices for method parameters is found in the current ring trial compared to those of the last years (table 9; v. Raamsdonk et al., 2008, 2009). Especially a lower number of participants choose to use 5 grams instead of 10 grams for sedimentation, a higher number did use a binocular for examination at lower magnifications, a higher share of glassware equipped for removal of the sediment at the bottom, and in a lower number of cases less suited embedding agents for sediment material (e.g. chloral hydrate) was applied. These differences can generally be indicated as improvements in the implementation of the microscopic method, which is also shown in the good performance indicators (table 3).
The background of the relation between the use of glassware in which the flotate has to be removed first (e.g. beaker) and the specificity and sensitivity scores (table 6) is not easy to understand. The current result are concordant with the results achieved in a previous proficiency test under the name of “Austrian method” (Boix et al., 2004; see van Raamsdonk et al., 2007). Unintentional mixing of flotate and sediment during removal could result in the presence of unexpected particles in the sediment, and vice versa the loss of sediment material during the decantation of the flotate. In both cases an occasional lower specificity or sensitivity score, respectively, could be expected.

4.3 Quantification

The averages of the quantification results of the animal proteins of terrestrial animals show in general a very good estimation. The estimations for fish show an overestimation. This is a reversed situation to the results as obtained in last year, when the amounts for the terrestrial animal material were overestimated. The usual situation is that for ingredients with a low share in the total composition overestimations are made (unpublished results of ring trials of IAG Section Feeding stuff

Table 9: Comparison between parameters distribution in the IAG 2008, 2009 and 2010 study.

parameter	parameter choice	2008	2009	2010
amount of material used for sedimentation	5 grams	16	5	3
	10 grams	26	41	48
	other	3	3	2
type of glassware	chemical sedimentation funnel	22	28	31
	beaker (flat bottom)	11	13	10
	champagne glass	6	5	8
	conical glass with cock	3	1	2
	other	3	2	2
use of staining of sediment	no	31	35	34
	yes	14	14	19
use of binocular for examination at lower magnifications	yes	29	40	45
	no	16	9	8
number of slides used	minimum	1	1	N.d.
	maximum	7	14	N.d.
size of cover glass used	small (e.g. 20 x 20 mm)	34	27	27
	medium	1	9	10
	large (e.g. 26 x 50 mm)	9	13	16
share of the total sediment used for examination	minimum	4%	2%	2%
	maximum	100%	100%	100%
embedding agent for sediment	paraffin oil	18	20	23
	immersion oil	8	12	14
	glycerine / glycerol	8	10	12
	Norland Adhesive	0	2	2
	chloral hydrate	3	1	0
	other (e.g. Depar 3000, water)	8	4	2

Microscopy). The overestimations for fish material are in concordance with other reports, e.g. Veys and Baeten (2008). However, the standard errors are very large. This means that individual results should be expected to be no reliable indicator of the real amount of fish or MBM in a sample.

5 General conclusions and recommendations

The legislation states that a method for detection of animal proteins in feeds should be able to detect a level of contamination of 0.1% at the least. Whereas previous studies have indicated that sensitivity scores for terrestrial animal proteins are very good in the *absence* of fish meal, the current study indicates that at levels of 0.1% and 0.05% in the *presence* of fish meal most laboratories can detect MBM or fish material. The specificity levels, as far as can be discerned from the sample design, are also at very high levels. In contrast to previous studies the erroneous identification of particles of terrestrial animals as fish particles (specificity in the presence of MBM) was not tested.
As far as indications are given for the amounts of fish meal, these are in general overestimated. The estimations for the level of animal proteins are well in range with the actual spiked amount, despite the situation that a range in f-factor is applied between 15 and 80%. Further information on the process of making estimations by the participants is not available. Although at low levels, particles of other sources than animals are still identified as animal proteins.
A further harmonization of the application of the microscopic method was achieved in the past years. This is especially indicated in the predominant use of 10 grams of material for sedimentation, and the use of a stereo microscope for the examination of the entire sediment. This optimization in the application of the method is accompanied by improvements in the sensitivity and specificity of the detection of animal proteins. On the other hand, a further harmonization is still possible for some other parameters.

1) The specificity of the microscopic method for proper detection of the lack of animal proteins still needs attention. Training of microscopists remains important.
2) It is recommended to evaluate further the effect of different types of glassware, and of staining of the sediment by Alizarin Red, and of the use of different embedding agents.
3) Quantification of animal proteins by microscopic measurements needs further evaluation.
4) Further ring tests are recommended to confirm the results of the tests with PCR and immunoassays.

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Annex I Invitation letter

Dear colleague, Dear IAG member,

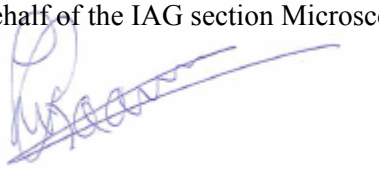
The IAG section Feeding stuff Microscopy organizes annually a ring test for the detection of animal proteins in animal feeds. As in previous years, the presidium of the IAG section Feeding stuff Microscopy and RIKILT have agreed to organize together the 2010 ring test for animal proteins under certain conditions.

On behalf of the IAG section Feeding stuff Microscopy, RIKILT will invite you for participation in this next ring test. The share in the costs of the 2010 ring test as asked from every participant will be a fee of € 200, which is the same as in 2009.

Three or four samples will be send around late February or early March 2010. Also a questionnaire will be sent by E-mail. A time slot of four weeks is planned for the analyses of the samples by every participants This means that late March or early April all results are expected to be returned to RIKILT. Pooling and evaluation of the results will take place during April and May, and a preliminary report will be presented during the annual IAG meeting in Tervuren (Belgium) in June. After that, a final report will be made depending on the outcome of the discussions during the meeting. All communications of the evaluation will be fully anonymous.

If you are interested to participate in the ring test 2010 for animal proteins, please return the application form and make a payment of € 200 to RIKILT. For smoothing the administrative procedure, an invoice is already included with this letter. In case of participation, please hand this invoice over to your financial department, and make sure that the reference number, your name and your institute's name is mentioned. This information is necessary to avoid loss of payments that can not be linked to participating institutes.

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



Dr. L. van Raamsdonk

Annex II Report form for procedure details

IAG ring test 2010

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) 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		
When estimating amounts:		
please indicate the f-factor used for fish meal		
please indicate the f-factor used for terrestrial animal meal		

Annex III Report form

IAG ring test 2010

lab number	**0**

sample number	2010-A	2010-B	2010-C
weight of sediment			
presence of fish material			
if present, estimated amount			
presence of material of land animals			
if present, estimated amount			

Comment, if necessary

Signature:

Date:

Annex IV Instructions as included in the report form

IAG ring test 2010

Instructions for the 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 Regulation 152/2009/EC from the European Union. Identical procedures can be found in the module Methods of the computer program ARIES. It is recommended to start the sedimentation procedure with 10 grams of material. Take care to homogenise the content of each vial before taking the amount for analysis.

3 Reporting consists of the following steps:

3a Please fill in the questionnaire on the page "Procedure". Depending on your chosen method, different questions will show up.

Most of the cells contain a drop-down list. These lists can be used to select an answer as follows. When clicking on a cell, the cursor changes into a hand. A second click will open the drop-down list.

Your unique lab number is mentioned in the introduction letter.

All the fields with a drop-down list have to be completed.

3b Please enter your results in the fields at page "Results". Your unique lab number automatically shows up after your have entered it at the page Procedure. Select "yes" 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 2nd, 2010.

Annex V List of participants

institute	city	country
Austrian Agency for Health and Food Safety-AGES	A-1226 Vienna	Austria
CRA-W	B-5030 Gembloux	Belgium
FLVVT	B-3080 Tervuren	Belgium
Oleotest N.V.	B-2660 Antwerpen	Belgium
AFSCA/FAVV	B-4000 Liege	Belgium
Ottawa Laboratory (Carling), Science Branch, Canadian Food Inspection Agency	Ottawa, Ontario, K1A 0C6	Canada
Central Institute for Supervising and Testing in Agriculture	Prague 5-Motol	Czech Republic
Danish Plant Directorate	DK-2800 Lyngby	Denmark
IDAC	44327-Nantes cedex	France
S.C.L. Laboratoire de Rennes	35000 Rennes	France
IPL Atlantique	F-33000 Bordeaux	France
LUFA Rostock	D-18057 Rostock	Germany
LLFG Landesanstalt für Landwirtschaft	D-06120 Halle	Germany
Landwirtschaftliches Technologiezentrum Augustenberg	D-76227 Karlsruhe	Germany
Staatliche Betriebsgesellschaft für Umwelt und Landwirtschaft, GB6-Labore Landwirtschaft / LUFA, FB62	D-04159 Leipzig	Germany
Landeslabor Berlin-Brandenburg	D-14473 Potsdam	Germany
LUFA Nord-West	D-26121Oldenbürg	Germany
SGS Germany GmbH	D-21035 Hamburg	Germany
Thüringer Landesanstalt für Landwirtschaft	D-07743 Jena	Germany
Q-vis GmbH	D-48155 Münster	Germany
Q-vis GmbH	D-38112 Braunschweig	Germany
CVUA-RRW	D-47798 Krefeld	Germany
Bayerisches Landesamt fur Gesundheit und Lebensmittelsicherheit	D-85764 Oberschleissheim	Germany
LUFA-Speyer	D-67346 Speyer	Germany
Universität Hohenheim, LA Chemie (710)	D-70599 Stuttgart	Germany
Futtermittelinstitut Stade (LAVES)	D-21680 Stade	Germany
Feedstuffs Control Laboratory, Min. of Rural Development & Food	GR-14123 Likovrissi Attikis, Athens	Greece
MGSZH ÉTBI TAKARMÁNYVIZSGÁLĆ NEMZETI LABORATÓRIUM	Budapest	Hungary
Department of Agriculture, Fisheries and Food, Backweston Agri Laboratories	Celbridge, Co. Kildare	Ireland
Equine Centre	Naas, County Kildare	Ireland
IZS PLV Torino - CReAA	I-10154 Torino	Italy
Inst. Zooprofilattico Sperimentale delle Venezie	I-35020 Legnaro	Italy
Istituto Zooprofilattico Sperimentale Abruzzo & Molise "G. Caporale"	I-64100 Teramo	Italy
Istituto Zooprofilattico Sperimentale della Sardegna	07100 Sassari	Italy
Ist. Zooprofilattico Sperimentale delle Lombardia e dell'Emilia Romagna	I-25121 Brescia	Italy
Istituto Zooprofilattico Sperimentale della Sicilia	90129 Palermo	Italy
Natl. Food and Veterinary Risk Assessment Institute	LT-08409 Vilnius	Lithuania
Labco	3198 LC Europoort-Rotterdam	the Netherlands
MasterlabBV	NL-5831 JN Boxmeer	the Netherlands
Nofima Ingredients	N-5141 Fyllingsdalen	Norway
Lab. Regional de Veterinária	PT 9700-236 Angra do Heroismo	Portugal

institute	city	country
Laboratório Nacional de Investigação Veterinária INRB, IP	PT 1549-011 Lisboa	Portugal
Institute of Veterinary medicine of Serbia	11070 Belgrade	Serbia
Scientific Veterinary Institute "Novi Sad"	21000 Novi Sad	Serbia
Central Controlling and Testing Institute of Agriculture	83316 Bratislava	Slovakia
University of Ljubljana, Veterinary Faculty, Natl. Veterinary Institute, Unit for Pathology of Animal Nutrition and Environmental Hygiene	SLO-1000 Ljubljana	Slovenia
Lab. Agroalimentari-DAR, Gen. de Catalunya	E-08348 Cabrils (Barcelona)	Spain
Dirección General de Produccion Agropecuaria, Laboratorio Agrario Regional	E-09071 Burgos	Spain
Lab. Agroalimentario de Cordoba	E-14080 Cordoba	Spain
Trouw nutrition Espana	E-28760 Tres Cantos (Madrid)	Spain
SVA	SE-75189 Uppsala	Sweden
Agroscope (ALP), Swiss Research Station	CH-1725 Posieux	Switzerland
LGC	Middlesex TW11 0LY	UK

Annex VI Details of procedures applied, microscopic method

	amount	glassware *)	agent	staining	binocular	size	sed. used	embedding	f-factor
1	10	chem.sed.funnel	TCE	yes	yes	small	100%	Norland	
2	10	centrifugation tube	TCE	yes	yes	large	100%	paraffin oil	
3	10	conical champagne glass	TCE	no	yes	small	25%	paraffin oil	40%
4	10	conical champagne glass	TCE	no	yes	large	50%	immersion oil	50%
5	10	chem.sed.funnel	TCE	no	yes	large	65%	paraffin oil	60%
6	10	conical champagne glass	TCE	no	yes	small	70%	glycerol	60%
7	10	beaker (flat bottom)	TCE	no	yes	small		immersion oil	
8	10	conical champagne glass	TCE	no	yes	small	100%	paraffin oil	15%
9	>5	conical champagne glass	TCE	yes	yes	small	100%	glycerol	40%
10	10	beaker (flat bottom)	TCE	no	no	small	100%	immersion oil	50%
11	10	chem.sed.funnel	TCE	yes	yes	medium	70%	glycerol	40%
12	10	beaker (flat bottom)	TCE	yes	yes	small		immersion oil	40%
13	10	chem.sed.funnel	TCE	yes	yes	large	40%	paraffin oil	60%
14	10	chem.sed.funnel	TCE	yes	yes	large	10%	immersion oil	40%
15	10	conical glass with cock	TCE	yes	yes	large	30%	glycerol	
16	10	chem.sed.funnel	TCE	yes	yes	large	33%	glycerol	25%
17	10	chem.sed.funnel	TCE	yes	yes	small	80%	glycerol	40%
18	10	conical champagne glass	TCE	no	yes	medium	100%	immersion oil	
19	10	chem.sed.funnel	TCE	no	yes	large	25%	paraffin oil	
20	10	chem.sed.funnel	TCE	no	yes	medium	5%	paraffin oil	
21	10	chem.sed.funnel	TCE	no	no	small	100%	paraffin oil	40%
22	10	chem.sed.funnel	TCE	no	yes	small	25%	phenol glycerol	40%
23	5	beaker (flat bottom)	TCE	no	yes	small	40%	immersion oil	
24	10	chem.sed.funnel	TCE	yes	yes	small		paraffin oil	40%
25	10	chem.sed.funnel	TCE	no	no	small	25%	paraffin oil	
26	10	chem.sed.funnel	TCE	no	yes	small	2%	paraffin oil	40%

	amount	glassware *)	agent	staining	binocular	size	sed. used	embedding	f-factor
27	10	beaker (flat bottom)	TCE	yes	yes	small		paraffin oil	
28	5	beaker (flat bottom)	TCE	no	no	small	70%	immersion oil	60%
29	10	chem.sed.funnel	TCE	no	yes	small	20%	immersion oil	40%
30	10	chem.sed.funnel	TCE	yes	yes	medium	50%	glycerol	40%
31	10	chem.sed.funnel	TCE	yes	yes	small	2%	paraffin oil	50%
32	10	beaker (flat bottom)	TCE	no	no	small	65%	phenol glycerol	15%
33	10	chem.sed.funnel	TCE	no	yes	small	40%	paraffin oil	
34	10	chem.sed.funnel	TCE	yes	yes	medium	100%	paraffin oil	40%
35	10	chem.sed.funnel	TCE	no	yes	large	100%	glycerol	
36	10	conical champagne glass	TCE	no	yes	medium	50%	immersion oil	60%
37	5	chem.sed.funnel	TCE	no	yes	small		water	40%
38	10	chem.sed.funnel	TCE	no	yes	medium	10%	paraffin oil	
39	10	beaker (flat bottom)	TCE/PE	no	yes	small	<5%	paraffin oil	
40	10	chem.sed.funnel	TCE	no	yes	large		immersion oil	
41	10	conical champagne glass	TCE	no	yes	small	100%	immersion oil	50%
42	10	chem.sed.funnel	TCE	no	yes	medium		glycerol/water	
43	10	chem.sed.funnel	TCE	yes	yes	small	80%	paraffin oil	40%
44	10	chem.sed.funnel	TCE	no	no	medium	6%	paraffin oil	
45	14	beaker (flat bottom)	TCE	no	yes	medium	50%	paraffin oil	80%
46	10	beaker (flat bottom)	TCE	no	yes	small	10%	immersion oil	25%
47	10	chem.sed.funnel	TCE	no	yes	large	20%	glycerol/water	
48	10	chem.sed.funnel	TCE	no	yes	large		immersion oil	
49	10	mensur	TCE	no	yes	large	20%	mineral oil	
50	10	chem.sed.funnel	TCE	yes	no	large	75%	paraffin oil	40%
51	10	chem.sed.funnel	TCE	no	no	large	100%	paraffin oil	
52	10	chem.sed.funnel	TCE	yes	yes	large	100%	paraffin oil	
53	10	conical glass with cock	TCE	yes	yes	small	50%	Norland	60%

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

lab nr	fish			MBM		
	A	B	C	A	B	C
38	yes	no	yes	yes	no	yes
39	yes	no	yes	yes	no	no
40	yes	no	yes	yes	no	yes
41	yes	yes	yes	yes	yes	yes
42	yes	no	yes	yes	no	yes
43	yes	no	yes	yes	no	yes
44	yes	no	yes	yes	no	yes
45	yes	no	yes	yes	no	yes
46	yes	no	yes	yes	no	no
47	yes	no	yes	yes	no	yes
48	yes	no	yes	yes	no	yes
49	yes	no	yes	yes	no	yes
50	yes	no	yes	yes	no	yes
51	yes	no	yes	yes	no	yes
52	yes	no	yes	yes	no	yes
53	yes	no	yes	yes	no	yes

Annex VIII Results: sediment and quantification

lab nr	amount of sediment (g)			amount fish (%)		amount MBM (%)	
	A	B	C	A	C	A	C
1	0.236	0.227	0.235				
2	0.2037	0.2183	0.2135				
3	0.26	0.25	0.27	3.6	4.12	0.013	0.003
4	0.248	0.25	0.3	2.5	2	0.05	0.025
5	0.23	0.22	0.22	4.83	5.85	0.24	0.04
6	0.26	0.28	0.31	2.5	2.5	0.01	0.05
7	0.228	0.197	0.223				
8	0.19	0.18	0.16	4	3.6	0.02	0.05
9	0.262	0.225	0.264	5	5	0.1	0.1
10	0.22	0.24	0.25	3	2	0.05	0.01
11	0.162	0.157	0.172	4.7	5.1	0.1	0.014
12	0.33	0.35	0.32	6.44	4.63	0.29	0.13
13	0.219	0.216	0.223	2.6	2.6	0.12	0.02
14	0.19	0.16	0.19	1.5	1.9	0.1	
15							
16	0.1554	0.128	0.16	1	3	<.1	<.1
17	0.14	0.118	0.135	4.021	2.37	0.119	0.024
18	2.21	2.06	2.16	3	2	0.03	0.01
19	0.268	0.249	0.283	5.7	10.1	<0.1	<0.1
20	0.243	0.212	0.247	3-7	3-7	0.2	0.1
21	0.26	0.209	0.242	2-4	2-4	0.1	0.1-0.2
22	0.3157	0.2313	0.2492	2.46	3.1	0.1	0.03
23	0.127	0.095	0.124	1	4	0.01	0.5
24	0.3247	0.3487	0.3926	1.91	2.04		0.02
25	0.199	0.207	0.196				
26	0.21	0.18	0.19				
27	0.176	0.176	0.14				
28	0.126	0.137	0.123	4.7	5.6	0.09	0.05
29	0.2578	0.2299	0.2385	5	4	0.05	0.01
30	0.1226	0.1227	0.1077	3.7	4.3	0.2	0.1
31	0.22	0.204	0.234	2.5-5	2.5-5	0.1-0.5	0.1-0.5
32	0.3	0.25	0.25	2-3	3-4	0.2-0.4	<0.1
33	0.3974	0.226	0.3947				
34	0.18	0.15	0.17	3.21	3.14	0.15	
35							
36	0.28	0.24	0.24	3.7	3.2	0.02	0.05
37	0.11	0.1	0.1	7.38	5.7	0.038	0.034

lab nr	amount of sediment (g)			amount fish (%)		amount MBM (%)	
	A	B	C	A	C	A	C
38	0.383	0.311	0.371				
39	0.248	0.225	0.211				
40							
41	0.23	0.2	0.25	3	3	0.2	0.25
42							
43	0.159	0.118	0.136	2.233	2.535	0.054	0.041
44	0.247	0.268	0.266				
45	0.356	0.327	0.359	7	6.57		0.15
46	0.2224	0.1742	0.2212	0.88	1.03	0.025	
47	0.33	0.26	0.26				
48							
49	0.21	0.26	0.25				
50	0.24	0.22	0.23	2.9	4.8	0.1	0.05
51	0.489	0.463	0.42	10.0	10.0	0.05	0.025
52							
53	0.11	0.1	0.11	2.05	2.183	0.012	0.121

Annex IX Results: presence of MBM, DNA detection

Lab	fish			MBM			amount MBM			
	A	B	C	A	B	C	A	C	method	target
28	yes	yes	yes	yes	yes	yes			PCR	mammal
42				yes	no	yes			PCR	mammal
42	yes	no	yes						PCR	fish

Annex X Results: presence of MBM, protein detection

Lab	fish			MBM			amount MBM			
	A	B	C	A	B	C	A	C	method	target
42				yes	no	yes			Elisa	mammal