Project number: 872.357.01
BAS-code: WOT-02-438-IV-018
Project title: National Reference Laboratory for animal proteins

Project leader: L.W.D. van Raamsdonk

Report 2009.012 September 2009

Animal proteins

Annual Report 2008 of the Dutch National Reference Laboratory

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

Distribution list:

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- Dutch Ministry of Agriculture, Nature and Food Quality, General Inspection Service (LNV-AID; J.A. van Wendel de Joode, R. Gerlofsma.
- Food and Consumer Product Safety Authority (VWA); W. Ooms, R. Herbes, H.A.van der Schee, dr. R.M.C. Theelen.
- European Commission (EC); K. van Dijck, F. Verstraete.
- Community Reference Laboratory, Animal Proteins (CRA-W); V. Baeten, P. Veys, G. Berben.
- Joint Research Centre, Geel (IRMM-JRC); C. von Holst, A. Boix-Sanfeliu.

Summary

RIKILT Institute of food safety is appointed as Dutch National Reference Laboratory. The tasks for an NRL are a full cooperation with the CRL and support of the national government and Competent Authorities.
One ring trial was organized by the CRL in 2007, the results of which were presented in 2008. This ring trial focused entirely on the quantification of fish material in feeds. It appears that in this ring trial a serious overestimation occurred of the amount of fish in the contaminated feeds. RIKILT reported overestimations as well for all samples, but well within the normal limits of all results. The z-score and the Relative Laboratory Performance were good. The RIKILT investigation time of a sample for the quantification of animal proteins will double the normal handling time for detection only. Staining of the sediment did not result in a conclusive improvement of the method.
Additionally the Dutch NRL organised a ring trial jointly with the IAG section Microscopy and the team of the European Union funded project SAFEED-PAP. Three samples were produced, viz. a blank feed (sample A), a feed contaminated with 0.1% of terrestrial animal material (sample B), and a feed contaminated with 0.05% of terrestrial animal material (sample C). The specificity and sensitivity were at acceptable levels for most analyses. From all 12 positive deviations for the detection of fish meal, only one was reported for the blank sample, the other 11 being reported for the samples containing exclusively terrestrial animal material. For both samples B and C only three negative deviations were found. This resulted in total sensitivity scores of 0.95 or higher. A noticeable difference can be seen between the sensitivity for the results based on 5 grams and on 10 grams of material used for sedimentation. A higher amount of material used for sedimentation results in a higher sensitivity (better result).
The Dutch NRL participated in a workshop of the CRL in April 2008. The main part of the agenda was dedicated to the results of the Ring trial 2007 and to quantification in particular. All factors influencing a proper quantification and the overestimation have been discussed. The CRL invested strongly in the development of a protocol for quantification, and even considering the overestimation, this protocol is a major step forwards for tackling this considerable problem. NRL actions at that workshop included active support in a discussion concerning quantification of fish meal in feed. A further proposal was the distribution of permanent slides for quantification. The advantage is that exactly the same material is investigated by several laboratories, when the slides are forwarded in the chain of NRLs. The permanent slides were received at the Dutch NRL at the end of October and were forwarded to the next laboratory early November. Results will be reported and discussed in 2009.
Several additions and modifications of legislation have been published in 2008 or are announced for 2009. The Dutch NRL will initiate further contacts with the Dutch Ministry for Agriculture, Nature Management and Food Safety and the Dutch competent authority to support the implementation of these modifications in the Dutch control and monitoring program.

Contents

1 Introduction

In 2006 the European Union appointed a series of Community Reference Laboratories, one of them dedicated to the field of Detection of animal proteins in feeds. Each member state has raised a National Reference Laboratory in this field. The stakeholders, i.e. the Ministry for Agriculture, Nature and Food Quality as representative of the member state, and the competent authority, need technical and scientific support for their tasks. RIKILT, as appointed NRL in this field, is providing this support.

The CRL organised one workshop and one ring trial in 2008. The results of the latest ring trial of 2007 were published in 2008 and, hence, are presented and discussed in this report as well. Quantification of fish material remains an important issue for supporting future legislation. Besides that, other technical aspects received attention for improving the performance of the microscopic method. An important activity was the ring trial, organised jointly between the NRL, the IAG section Microscopy and the team of the European project SAFEED-PAP.

The Dutch NRL renders account of its activities in the framework of collaboration with the CRL and support of the national authorities in this report.

2 Description of work

The tasks of the NRL are laid down in Directive 882/2004. RIKILT serves as the one and only official control laboratory for animal proteins in feeds in the Netherlands. Several of the listed tasks do not require activities due to the single laboratory situation. Remaining tasks are:

- collaboration with the CRL, including participation in meetings and workshops, participation in ring trials;
- communication of information from the CRL to the stakeholders;
- providing technical and scientific support to the stakeholders;
- performing other specific tasks; RIKILT acts as member of the scientific advisory board of the CRL;
- Support of the national network of official control laboratories. The Netherlands does not maintain a network of official laboratories for detection of animal proteins, although national legislation provides a list of five laboratories that can be involved in monitoring animal feeds in general.. RIKILT as NRL seeks possibilities to support these laboratories in the area of detection of animal proteins.

The performance of all the tasks fits in the additional requirements of Directive 999/2001.

3 Results

3.1 CRL ring trial 2007

3.1.1 General results ring trial 2007

At the end of 2007 the CRL for Animal Proteins organized a proficiency test with ten samples aimed at the quantification of fish material in feeds, and at the effect of staining of the sediment. A specific counting grid for a microscope was requested and purchased to carry out the task of quantification. Simultaneously the effect of alizarin staining of the sediment was tested. For both aspects the time investment was an issue. RIKILT reported the results as requested in the form. The deadline was January 21st, 2008.
It was requested to follow strictly the method as described in Directive 2003/126, with the addition to use 10 grams of material for sedimentation and to apply the Alizarin staining of the sediment. Furthermore a detailed protocol for counting and quantification was developed for strict application. This protocol requires random selection of twenty fields at four slides per sample and counting of the number of dots in the grid that are covered by the particles. This protocol results in an indication of the surface area per particle (Veys et al., 2008).
It was felt at RIKILT that an additional note with extra details of the RIKILT observations could be helpful for further evaluation. This report was sent to the CRL and will be discussed here.

The ring test consisted of 10 samples with a composition as shown in table 1.

Table 1 Composition of the samples in the CRL ring test 2007. Source: Veys et al.(2008).

Label and content	number of samples
A 0.15 % fish	2
B 0.4 % fish	2
C 0.7 % fish	2
D 1 % fish	2
E 1 % fish, alternative matrix	2

Twenty-two NRLs submitted the results. The time investment to examine and quantify the ten samples varied from 1.5 to 16 days among the participants. As mean value an examination time of 5.6 days was extracted from the reports, giving a time investment of approx. 0.5 day per sample (2 samples/day).
The pooled results on the estimated amounts of fish material are presented in table 2.

Table 2 Pooled results of all participants for all samples. Source: Veys et al.(2008).

Label and content	estimated amount	
	average	median
A 0.15 % fish (n=44)	0.62%	0.54%
B 0.4 % fish (n=44)	1.14%	0.95%
C 0.7 % fish (n=44)	1.46%	1.19%
D 1 % fish (n=44)	1.98%	1.52%
E 1 % fish, alternative matrix (n=44)	1.58%	1.43%

It appears that in this ring trial a serious overestimation occurred of the amount of fish in the contaminated feeds. Furthermore, considering the results for sample D and E an effect of the matrix (the composition of the feed) should be considered in the evaluation of these results.
The staining of the sediment material results in a red colouring of presumed bone fragments, leaving minerals and other particles largely uncoloured, and in a reduction of the total sediment weight due to bleaching and rinsing of the sediment material. The results of this ring trial showed that the original sediment material was reduced to an amount of approx. 60 - 65 % of the original weight. However, a large variation was found among the participants results. All but four participants reported a reduction between 40 and 80 % after staining. One participant achieved a reduction of more than 95% (remains between 0.5 and 3%), and three other laboratories had a reduction of less than 10% for most samples. Besides the variation in the effect of the staining, the estimation of the share of fish bones compared to all particles in the sediment (e.g. minerals; factor *d*) appears to generate a lot of variation as well. This factor depends on the ability of the microscopist to identify correctly all particles in a counting field, and on the random selection of the fields for counting. It can be expected that in the process of selection of the counting fields, the fields with recognisable fish particles are preferred, which is a violation of real random sampling and results in an over-estimation of fish particles.
The development of the protocol for counting particles using a grid and estimating the share of fish particles on the total of all particles was a major investment of the CRL. The use of this protocol by all NRLs and the discussion of the results was major step forward in the development of a quantitative method.

3.1.2 RIKILT results

RIKILT reported overestimations for all samples well within the normal limits of all results. The z-score and the Relative Laboratory Performance were good. The reduction of the amount of sediment achieved after applying the staining procedure by RIKILT was higher than the average of all participants in the ring trial. Alizarin staining is not part of the normal procedure at RIKILT.

3.1.3 Reported investment of time

RIKILT implemented the procedure of Directive 2003/126/EC with several additions allowed in the framework of the Directive, as agreed by the Dutch official authority. 10 grams are always used for the sedimentation, and two microscopists evaluate the same material as an internal control and approval of the results. This is a choice for our RIKILT quality assurance system as an addition to external standards, and might result in a higher evaluation time per sample.

The amount of time spent at RIKILT for the different steps for processing and evaluating the ten samples of the ring trial are as presented in Table 3.

Table 3 The time investment for the usual examination of samples, the extra time for Alizarin staining and the extra time for quantification extracted from the RIKILT investigations of the CRL ring trial 2007.

Activity	time (hr)	extra time (hr)	
		staining	quantification
production of staining solution	1,5	1,5	
sedimentation and weighing	2		
staining	6	6	
second weighing	0,5	0,5	
examination and quantification first microscopist	5	0,5	2,5
examination and quantification second microscopist	5	0,5	2,5
reporting	1		
total	**21**	**9**	**5**

The total time to be spent for processing a single sample can be estimated approximately:
Processing, staining, evaluation, quantification and reporting: 2.1 hrs (4 samples/day)
Processing, evaluation, quantification and reporting: 1.2 hrs (7 samples/day)
Processing, evaluation and reporting (current RIKILT procedure): 0.7 hrs (10-12 samples/day)

Considering these rough calculations based on RIKILT results, quantification might almost double the costs for the examination of one feed sample, provided that the feed material contains fish as ingredient, and additional staining together with quantification might triple the cost for one feed sample.
It might be assumed that increasing experience with the different procedures might result in shorter periods of time for the entire process per sample. RIKILT made an attempt to record the times for the individual samples, but it appeared that deviations in time are primarily caused by the number of fish particles that were found in the slides.
In the view of an average for all participants in this ring trial of approx. 2 samples/day, the RIKILT performed quite well. Although the current results of this ring trial does not give any reason, it might be necessary to keep monitoring results in relation to time investment in order to avoid unnecessary errors.

3.1.4 RIKILT results of staining

After examination of the samples of the ring trial, certain particles appeared to be stained with alizarin, of which the animal origin could not be confirmed by other characteristics (Image 1 to 3). Other particles which might have a mineral nature, are present with incomplete staining (Image 5 and 6). Due to the partial lack of alizarin staining it was possible to establish the lack of lacunae, the lack of natural coloration and of other typical features of animal material.
The sediment of a frequently used bovine sample was examined as well in order to collect additional information. This sediment consists of approx. 70% brown particles and 30% white coloured particles

(bone fragments in the sediment; Image 1). The white particles are clearly recognisable as animal material. It is assumed that the brown particles suffer from heat damage. These particles show in most cases no or faint lacunae (Report NRL/IAG ring trial 2008: van Raamsdonk et al., 2008). The alizarin staining intensity is different between the two types of material, as visible at low magnification (Image 1 and 2). At higher magnifications the different effects of alizarin staining is more prominent. The white particles show a clear and bright staining. The visibility of the lacunae and other details is suppressed by the high intensity of the coloration (Image 3). The possibly excessively heated brown particles show a considerable lower intensity of staining (Image 4).

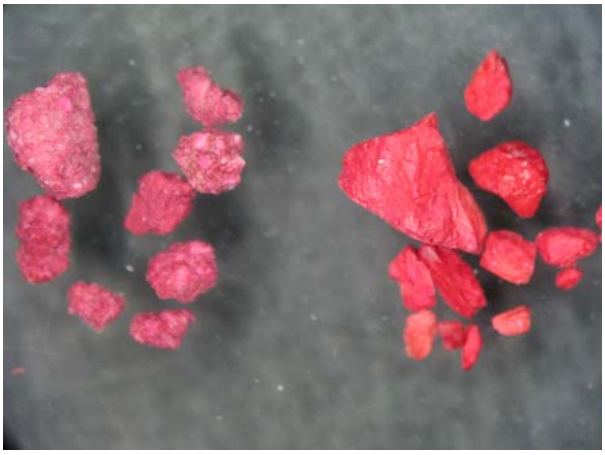
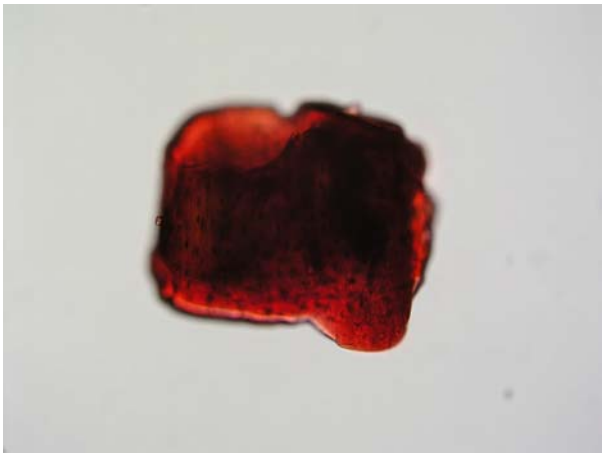


Image 1 and 2. Brown and white bone particles of a bovine sample at low magnification without staining (left; 10 x) and with alizarin staining (right; 20 x).



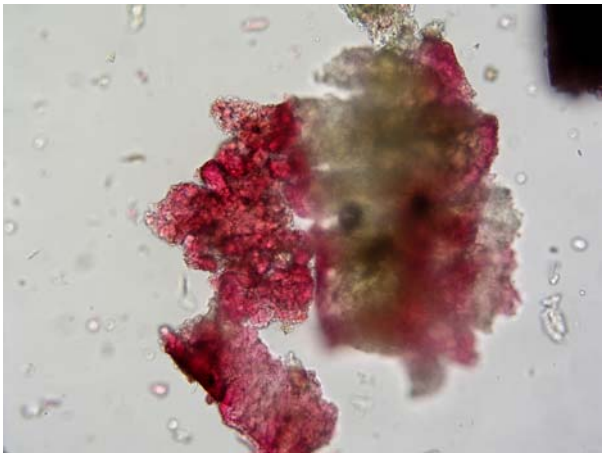


Image 3 and 4. Bone particles of a bovine sample with well established colouring (left; Lacunae are faintly visible and are mostly covered by a dark staining), and with faint staining (right). Magnification 200x, alizarin staining.

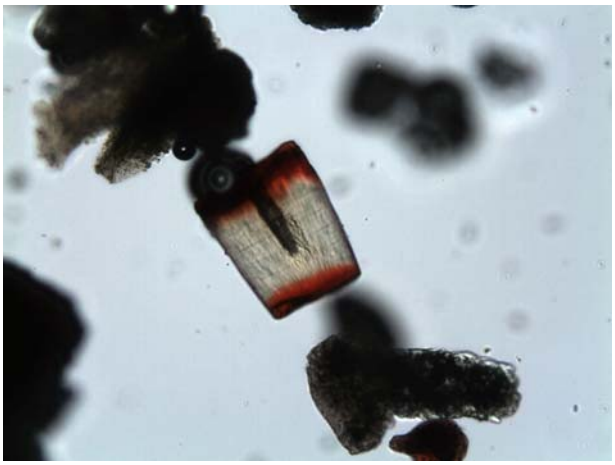
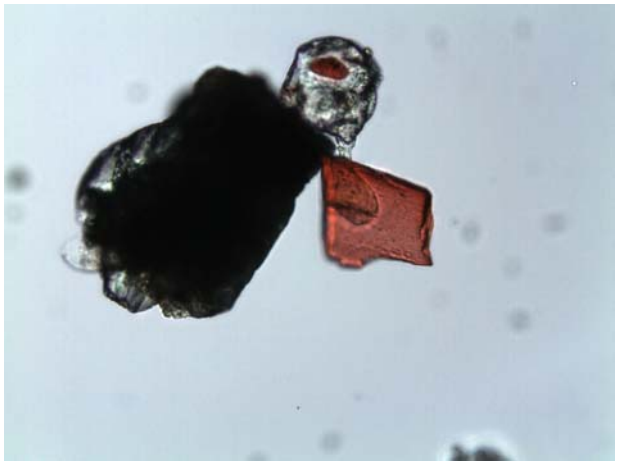



Image 5 and 6. Particles with incomplete, partial staining. The particles might have a mineral origin. The fully coloured particle (right) is a fish bone fragment. Magnification 100x, alizarin staining.

3.2 Permanent slides

A spin-off of the ring trial for quantification was the distribution of permanent slides among NRLs. The production of slides that can store for a longer period is an improvement of the daily paractice of microscopic investigations of feeds, where slides are made on a non-permanent basis (storage time one or a few days). The purpose of these permanent slides is to have results of several laboratories for exactly the same slide. The permanent slides were received at the Dutch NRL end of October and forwarded to the next laboratory early November. Results will be reported and discussed in 2009.

3.3 CRL workshop 2008

The annual workshop for all NRLs was organised from 15 to 17 April 2008 in Namur, Belgium. Several subjects were discussed, such as Level of Detection, Hydrolysed proteins, Near-Infrared Spectroscopy and DNA analysis (PCR).
The main part of the agenda was dedicated to the results of the Ring trial 2007 and to quantification in particular. All factor influencing a proper quantification and the overestimation were discussed. One major aspect is the transformation form two-dimensional examination to three-dimensional conclusions. Using a counting grid and counting the number of dots in the grid covered by the several different particles means that an indication of the surface area is collected, for the fish particles and for the other particles. The relative share is taken as representative for the amount of fish in the sediment. Normally this share is indicated by a weight percentage, which is comparable to the (three-dimensional) content of those particles, assuming that both fish and other ingredients have an identical specific density. However, a lot of fish particles are flattened, resulting in another ratio between (2-D) surface and (3-D) content than applies to globular mineral particles and more or less globular particles of terrestrial animals. This different ratio might result in an overestimation of the relatively flat fish particles.

The Dutch delegation has proposed to continue these investigations to quantification by examination a series of slides with feeds contaminated with terrestrial animals. Further investigations in this matter will be carried out by the CRL.

3.4 NRL/IAG ring trial 2008

One of the tasks of a national reference laboratory, according to Directive 882/2004/EC, is the organisation of comparative tests among the official national laboratories. The Netherlands does not maintain a network of official laboratories for detection of animal proteins, although national legislation provides a list of five laboratories that can be involved in monitoring animal feeds in general. RIKILT, as Dutch NRL, is seeking possibilities for providing support to those national laboratories. In order to establish the possibility for organising a comparative test for a range of laboratories, a proficiency test was organised which could also fit in the framework of the IAG - International Association for Feeding stuff Analysis, Section Feeding stuff Microscopy.
One of the tasks of the European project SAFEED-PAP (FOOD-CT-2006-036221; 2006-2009) is to find possibilities for the improvement of the microscopic method. The questionnaire for parameters of the implementation of the microscopic methods, as part of the current proficiency test, can serve as input for this task.
The NRL/IAG ring trial is reported separately (van Raamsdonk et al., 2008). In the present annual report the results will only be presented briefly.

3.4.1 Procedure

Three samples were produced with a composition as listed in Table 4.

Table 4 Composition of the samples in the NRL-IAG ring trial 2008.

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

The design allowed for the use of DNA detection and immunochemistry detection methods, additional to microscopic detection.

RIKILT microscopists examined five jars of sample 2008-B and five jars of sample 2008-C for homogeneity. In all ten cases a correct positive result was reached, as is shown in Table 5. 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 5 Results of the homogeneity study. Sediment amounts are based on 10 grams in all cases.

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

The sets of three samples with an accompanying letter were sent to all participants on the 22nd of February 2008. On Monday February 25th an E-mail message was sent around to all participants, together with an electronic report form and the request to confirm the receipt of the package. The report form also contained a sheet with instructions.
The closing date for reporting results was fixed at April 1st. Some additional participants received the package at a later date. However, in all cases results were received no later than April 7th , and all these results were considered in the final evaluation.

3.4.2 Results

The specificity and sensitivity were at acceptable levels for most analyses (Table 6). Seven participants reported the presence of fish meal in sample 2008-C, resulting in a relatively low specificity of 0.84. From all 12 positive deviations for the detection of fish meal, only one was reported for the blank sample (2008-A), the other 11 being reported for the samples containing exclusively terrestrial animal material. Three participants found terrestrial animal material in sample A, one of them reported also a positive deviation for fish in the same sample A.
For both samples B and C only three negative deviations were found. This resulted in total sensitivity scores of 0.95 or higher. A noticeable difference can be seen between the sensitivity for the results based on 5 grams and on 10 grams of material. More material used for sedimentation results in a higher sensitivity.

Table 6 Sensitivity and specificity for the detection of animal proteins in three samples. N: a total of 45 participants reported their results, 16 of them used 5 grams of sample material for sedimentation, and 26 used 10 grams for sedimentation. Three participants used other amounts or did not report on this aspect.

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

Three participants did not report the amount of material used for sedimentation, or used variable amounts. This clarifies the total of 45 participants for the over-all results (26 + 16 + 3).
There is no difference in the specificity and sensitivity scores between the subgroups of participants that used staining of the sediment (n=14) and those that did not (n=31).

RIKILT tested the samples blind with real time-PCR using bovine primers to detect and semi-quantify specifically presumed bovine DNA. The result for sample A was negative, for sample B and C positive with the indication that sample B contained more DNA than sample C. Also a second participant tested the presence of bovine material correctly. RIKILT did not test for fish material. The fish primer as used by the other participant might show cross-sensitivity with DNA of another source.

3.4.3 Method implementation and performance

The majority of the participants started the sedimentation procedure with an amount of 10 grams of material. Fourteen participants used staining of the sediment (alizarin) for evaluation. It was not stated if this staining procedure was used as the standard method or only additionally (for the examination of unstained material). Examination of the sediment at lower magnifications by using a binocular is requested in this Directive, but 16 participants reported to skip this part of the procedure. Usually, between one and seven slides were made for evaluation of the sample, although two participants reported the use of more than ten slides. Most of the participants used small cover glasses. It can be expected that using a larger portion of sediment for examination is correlated with a larger amount of slides and/or the use of large cover glasses in order to accommodate the sediment material. However, in one case the use of 8% of sediment material on a total of more than 10 slides was reported, while in another case with approximately the same amount of sediment, 100% of the sediment material was placed on six slides with small cover glasses. So, there is an apparent diversity in the preparation of slides for the microscopic examination.
Other factors in the microscopic method are variable, such as the use of glassware for sedimentation, the embedding agent and the use of a binocular for examinations at a lower magnification level. The procedure according to the Directive requires to examine the sieve fractions of the sample as well as the sediment by a binocular (stereo microscope). Although not mentioned explicitly in the Directive, the normal amount of sediment allows to examine it entirely. The possible error of overlooking possible animal proteins and a final false negative result can be minimised when examining a share of the sediment as large as possible. The same apply to the examination of the sieve fractions. There is no clear statistical relationship of the use of glassware and the type of embedding agent and the presented results of the participants due the low frequency of using some peculiar types of laboratory equipment, although it can be expected that some of them might result in questionable results (e.g. laboratory tubes for sedimentation, chloralhydrate (meant for histological cell structures) used for embedding sediment material).
More details can be found in van Raamsdonk et al. (2008).

3.5 Support of the national authority

In 2008 changes in legislation have been published or are still in discussion. These are:

- The existing tolerance of insignificant contamination levels with animal proteins in tuber and root crops (Regulation 1292/2005/EC), provided a risk analysis proving low or zero risk, is proposed to be extended under certain circumstances to all ingredients of plant origin (planned as Regulation 163/2009/EC). This proposal could have implication for the official control of the zero tolerance.
- The application of fish protein for animal feeds has been extended. Fish can now be used as milk replacer in calf feeds (Regulation 956/2008/EC).

- The quantification of fish in animal feeds, as developed and tested in the latest ring trial of the CRL, is meant to support a future lift of the ban of fish material in ruminant feeds. A tolerance of e.g. 1% in ruminant feeds is discussed.

RIKILT as Dutch NRL will discuss the possible implications and effects of the new additions to the legislation with the national government and the national authority. If necessary additional risk assessments can be carried out in addition to the risk assessment of 2007.

4 Discussion

4.1 Method performance

Theoretical calculations have been carried out for estimation of the effect of using the entire sediment or only a part of the sediment material, and of the starting amount of material for sedimentation. More detailed information on these theoretical calculations have been presented in the NRL/IAG ring trial report (van Raamsdonk et al., 2008). Both aspects, the use of more material for examination (up to 100% of the sediment material) and using more material for sedimentation (10g vs. 5 g), have an increasing effect on the performance of the microscopic detection (Figure 1). Results of the past five years (only results obtained applying Directive 2003/126/EC are considered) generally indicate lower sensitivity scores than theoretically achievable. In the situation of an absence of fish meal, the sensitivity scores are mainly above 0.95 for all contamination levels between 0.1% and 0.02%. In the presence of 5% of fish meal, however, the first documented study in which a method comparable to that of D 2003/126/EC has been used (2003), resulted in a sensitivity score of only 0.44. More recent results increased to a level of 0.84 in the most recent study (2007). A bench mark study, carried out in 2003 with a selected set of nine laboratories resulted in a sensitivity score for 0.1% of animal protein in the presence of 5% fish meal of 0.985 (see v. Raamsdonk et al., 2007; 2008 for documentation).

The results from proficiency tests as well as from theoretical calculations indicate that an improvement of the sensitivity can be achieved. The most likely parameters to be improved are the starting amount of material for sedimentation and the amount of sediment material for examination. The results for the other parameters such as the glassware used and the viscosity of the embedding agent are less conclusive. The use of a binocular for examination at lower magnification is already stated as compulsory in D 2003/126/EC. Specificity, as the other aspect of the proficiency of the microscopic method, can be improved by training to distinguish fragments of land animals from those of fish or from other sources.

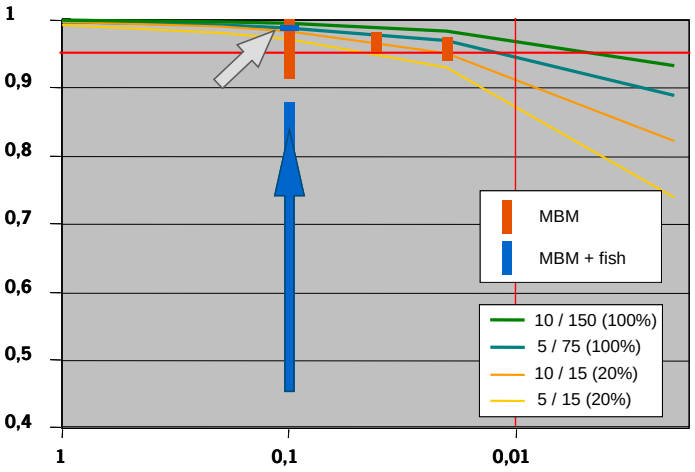


Figure 1 Sensitivity scores (y-axis) for different contamination levels of animal proteins of land animals (logarithmic x-axis). Lines: theoretical calculations for 5 or 10 grams of starting material for sedimentation, and the use of 20% or 100% of the sediment material for microscopic examination. Bars: Sensitivity for the detection of animal proteins without (orange) or with the presence (blue) of fish meal at 5%. Blue arrow: increase from 2003 to 2007. White arrow: bench mark study.

4.2 Sediment staining

Several types of particles can apparently occur in the sediment of feeds in terms of response to alizarin staining.

- Particles of animal origin show basically a colour reaction after staining with alizarin. There is a correlation between the natural colour and the staining response: the darker the natural colour (from white to brown) the more faint the staining reaction. This feature might be explained by the intensity of the heat treatment (sterilization). Tricalciumphosphate (TCP) is known to be produced from bone material after excessive treatment (heat treatment a.o.). TCP is not recognisable nor show a response as animal material as far as known parameters are concerned.
- Mineral particles might show a certain response to alizarin staining. This assumption is not approved by the present results, since no sediments have been used with an approved lack of animal material in this brief report.

A causal relationship might be expected between the nature of a certain particle and its response to alizarin staining. However, certainly not all conditions that determine the intensity of the coloration are known. The response of all kinds of minerals, vitamins, non-vertebrate shell fragments, foraminifers, etc. is insufficiently known.
Alizarin staining hides typical features of animal particles, such as general structure, lacunae and canaliculae. If a sharp limitation can be established between animal particles showing a staining response, and non-animal particles showing a recognisable lower response, alizarin staining might be considered. Alternative to a full staining application, it might be considered to conclude that staining is more effective for recognition at lower magnifications instead of application at higher magnifications, as is illustrated in Image 1 and 2.

4.3 Quantification

The time investment in the quantification and the alizarin staining is considerable. It has to be discussed thoroughly if the extra investment of time (and hence of costs per sample) can be justified against the pros and cons of staining. However, quantification might be unavoidable considering the current political goals to allow low levels of fish meal, e.g. 1%, in animal feeds for ruminants. For optimizing the costs of quantification, a certification system might be considered in which parties of pure fishmeal get individual certificates for further processing in feeds. Control of feeds with fish meal can be limited in this way. Another approach is to limit quantification as a step additional to mere detection exclusively to ruminant feeds and/or to specific monitoring actions.

5 Conclusions and recommendations

The microscopic method as presented in Directive 2003/126/EC should be improved, considering the following aspects:

- Compulsory examination of the entire sediment using a binocular at lower magnifications,
- Compulsory use of 10 grams of material for sedimentation,
- Examination of the entire sediment, or at least examination of as much sediment material as possible, at higher magnifications.

The CRL invested heavily in the development of a protocol for quantification, and even considering the overestimation, this protocol is a major step forwards for tackling this considerable problem. The costs and benefits of a method for quantification should be further explored.

The time investment of alizarin staining is only justified after an extensive testing of the positive versus negative effects of staining. The effect of staining after heat treatment of animal material, and the staining response of a large series of different materials (TCP, minerals, vitamins, non-vertebrate shell fragments, foraminifers, starch, etc.) needs further research.

RIKILT will initiate further communication with the Ministry for Agriculture, Nature Management and Food Safety and the Official Authority on new development of legislation.

6 Literature

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