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Summary

Late 2006 RIKILT was appointed Dutch National Reference Laboratory (NRL) for the detection of animal proteins in animal feeds. The tasks for an NRL are a full cooperation with the CRL and support of the national government and Competent Authorities.
The Dutch NRL participated in a workshop of the CRL in April 2007. NRL actions at that workshop included active support in a discussion concerning quantification of fish meal in feed, and in support for a well-functioning network of all national NRLs. The CRL granted licenses for the expert system ARIES to all NRLs. During the workshop the system was provided to all colleague NRLs.
Two proficiency tests organized by the CRL were carried out, the results of which were submitted in 2007. Besides a proper detection and identification of material of terrestrial animals (MBM) and of fish material, the quantification of fish material was included in these proficiency tests as an important issue for support of future legislation. Large differences in the results for the amounts of fish material were reported by the NRLs, showing predominantly overestimations. It was concluded that the current method for quantification is not sufficient. The CRL has used these first impressions of the possibility of quantifications for the planning of the proficiency test 2007/2008.
Identification as part of the species-to-species ban is currently focused on the specific detection of ruminant or cattle material. For a full support of the species-to-species ban a desk study is recommended summarising the amount of variation within a species of farmed animal as well as between different species of farmed animals. In this study, the possibilities of raising specific primers and/or antibodies would need to be discussed.
An investigation to explore the possibilities of supervising the research of other national laboratories in the framework of contra expertise has been started by the Dutch NRL. The organisation of a proficiency test with samples contaminated at levels below 0.1 % will be considered.

Contents

1 Introduction

In 2006 the European Union appointed a series of Community Reference Laboratories; one of them was dedicated to the area of detection of animal proteins in feeds. Each member state has subsequently appointed a National Reference Laboratory in this area. The stakeholders, i.e. the Ministry for Agriculture, Nature and Food Quality as representative of the member state, and the competent authority (Food and Consumer Product Safety Authority (VWA)), require technical and scientific support. RIKILT, as appointed NRL in this area of expertise, is providing this support.
The CRL organised one workshop and two proficiency tests in 2007. Besides a proper detection and identification of material of terrestrial animals (MBM) and of fish material, the quantification of fish material is an important issue for supporting future legislation.
The Dutch NRL renders account of its activities in the framework of collaboration with the CRL and support of the national authorities.

2 Activities

The tasks of the NRL are laid down in Directive 882/2004/EC. RIKILT is appointed as the only official control laboratory for animal proteins in feeds in the Netherlands. Several of the tasks listed in the directive do therefore not require specific activities. Remaining tasks are:

1. collaboration with the CRL, including participation in meetings and workshops, participation in proficiency tests;
2. coordination of activities of official laboratories responsible for the analysis of samples;
3. where appropriate, the organisation of comparative tests between the official national laboratories;
4. communication of information from the CRL to the stakeholders;
5. providing technical and scientific support to the stakeholders;
6. performing other specific tasks; RIKILT has been invited to be member of the scientific advisory board of the CRL.

The performance of all the tasks meets the additional requirements of Directive 999/2001. The tasks b) and c) currently do not apply to the Dutch situation. Therefore, in this report the activities for the tasks a), d), e) and f) will be presented.

2.1 Collaboration with the CRL

2.1.1 Proficiency test 2006

The CRL started to organise its first proficiency test late 2006 for testing the microscopic detection according to Directive 2003/126/EC. The report was presented in 2007 (Veijs, 2007a). Therefore, the share of RIKILT in this proficiency test in the larger framework of the pooled results is reported in this 2007 annual report.

The proficiency test consisted of 19 samples with a composition as shown in Table 1.

Table 1: Composition of the samples in the CRL proficiency test 2006. Source: Veijs et al.(2007a).

Label	Content	Number of samples
A	blank	3
B	0.1 % MBM	3
C	0.5 % fish III	2
D	0.25 % fish III	2
E	1.5 % fish III	2
F	1 % fish III	2
G	0.1% MBM + 5% fish	3
H	1% fish I	2

Twenty-two NRLs submitted their results. RIKILT identified all samples correctly.
The pooled results of all NRLs (Table 2) showed some false positive results (positive deviations, expressed in a specificity score) in several cases, and false negative results (negative deviations, expressed in a sensitivity score) in some others.

Table 2: Pooled results of all participants for all samples related to the detection of material of terrestrial animals. Number of false positives or false negatives in brackets. Source: Veijs et al.(2007a).

		MBM		Fish	
Label	Content (n=66)	Specificity	Sensitivity	Specificity	Sensitivity
A	blank	1.000		0.879 (8)	
B	0.1 % MBM		0.985 (1)	0.909 (6)	
G	0.1 % MBM, 5% fish		0.879 (8)		1.000

It appears that in this proficiency test no particle was misinterpreted as terrestrial material, in other words, in none of the cases terrestrial material was reported while not present, which is shown by the 1.0 score for specificity in material A. However, MBM material, when present, was not detected in all cases. In addition, the presence of fish was incorrectly indicated by some participants (false positives), but it was always correctly detected when present. The same optimal score was also reached for the other samples with fish meal (samples C to F, and H; results not shown).
The CRL decided to require a minimum value for both specificity and sensitivity of 0.95 for the individual results of every laboratory. Five labs presented one or more scores below that target limit of 0.95. The CRL decided to organize a training, followed by a second small proficiency test. In order to

get a full set of data, all NRLs were requested to participate in this second proficiency test, organized in 2007.

2.1.1.1 Quantification of fish meal

A second goal of the 2006 proficiency test was an evaluation of the current procedures for quantification of fish meal. Therefore, the samples C to F, and H with various quantities of fish meal were included in this trial.
Large differences in the results of the amounts of fish material were reported by the NRLs. In general, mainly overestimations were presented. The repeatability (within-laboratory variability) ranged from 12% to 30%, which is reasonable. However, the reproducibility (between-laboratory variability) is considerably, ranging from 85% to 116% for the different samples. It is concluded that the current method for quantification is not sufficient (Veijs et al., 2007a).The CRL will consider the result of this proficiency test when planning a third proficiency test in 2007/2008.

2.1.2 Proficiency test 2007

During summer 2007 the CRL for Animal Proteins organized a small proficiency test (see 2.1.1) with four samples, aimed at the detection of animal proteins of both terrestrial animals and fish. RIKILT reported the results, which were included in the final report of the CRL-AP (Veys et al., 2007b).

2.1.2.1 Reported contents of the samples

Four samples were included in the test with a content as shown in Table 3 (Veys et al., 2007b). The CRL carried out homogeneity studies before sending them around, by investigating five or ten portions respectively for each sample with the following results as in Table 3.

Table 3: Composition of the four samples in proficiency test 2007, and the results of the homogeneity study performed by the CRL. Source: Veijs et al.(2007b).

		Fish material		Terrestrial animal material	
Label	Content	Negative	Positive	Negative	Positive
A	blank	10	0	10	0
B	challenger	9	1	7	3
C	0.1% MBM + 5% fish	0	5	0	5
D	5% fish	0	5	5	0

The challenger material appeared to have very low levels of contamination: the homogeneity study revealed that fish material was detected in only 10 % of the samples. For terrestrial material this was 30%. (in all cases only one fish bone resp. one bone fragment was found).
Twenty five NRLs submitted their results, which are summarised in Table 4.

Table 4: Pooled results of all participants for all samples related to the detection of material of terrestrial animals. Number of false positives or false negatives in brackets. The sensitivity and specificity of the challenger (sample B) was not reported in the CRL report. Source: Veijs et al.(2007b).

		MBM		Fish	
Label	Content (n=25)	Specificity	Sensitivity	Specificity	Sensitivity
A	blank	0.96 (1)		0.96 (1)	
C	0.1 % MBM, 5 % fish		0.84 (4)		1.00
D	5% fish	0.84 (4)			1.00

The results as presented in Table 4 show basically the same pattern as found in the proficiency test of 2006. In this case also one false positive was found for the detection of material from terrestrial animals as well. The differences between levels of the scores in the two proficiency tests can be due to the low number of samples in the 2007 proficiency test per sample type.
The report of the CRL (Veijs et al., 2007b) presents an evaluation of the number of particles as found in the samples. The results are shown in Table 5. There appears to be a large variety in the number of particles of terrestrial animals (MBM) detected in sample C.

Table 5: Overview of the number of particles detected in sample C(0.1% MBM + 5% fish) by the participants in the proficiency test 2007. Source: Veijs et al.(2007b).

	Number of particles detected						
	0	1	3	4 to 5	6 to 10	11 to 50	?
Number of NRLs reporting	3	2 *	3	3	6	6	2

?: no number of particles reported, nevertheless with the indication "presence" for MBM.
**: one participant considered the presence of only one particle as "absence" of MBM.*

2.1.2.2 RIKILT results

RIKILT reported one false negative for the presence of terrestrial animal material in sample C (Table 6).

Table 6: Official RIKILT results for the proficiency test 2007.The problematic result is indicated with an arrow.

Label	Content	Number of fish particles	Number of terrestrial animal particles
A	blank	0	0
B	challenger	0	11 to 50
C	0.1% MBM + 5% fish	> 50	*0* ←
D	5% fish	> 50	0

All investigations were based on 10 grams of material. These results were included in the final report of the proficiency test 2007 (Veijs et al., 2007b).

2.1.2.3 Additional investigations

RIKILT decided to repeat the investigations in October 2007 and in January 2008. In both cases a new portion of 10 grams was taken from each sample and the same procedure was followed. Only in the second run two particles were detected in sample type C, which possibly originated from terrestrial animals. In the third run the results of the first trial were reproduced, i.e. no bone fragments were found in sample C. In all cases the entire sediment material was examined macroscopically and microscopically.

The results of all three trials should be discussed in the view of the diversity in reported numbers of bone fragments, as shown in Table 5. A large variation was found among the participants of the ring trial. No conclusions can be drawn on possible inhomogeneity of the total material of sample C, because other factors such as variation in the application of the method can influence the final results as well. However, likewise no conclusion can be derived from the RIKILT results for sample C.

2.1.2.4 Examination procedure

At RIKILT, the procedure as laid out in Directive 2003/126/EC is followed. In this Directive the examination of two additional slides is required, if fish material is detected. However, the size of the cover glass or the minimal amount of material to be used for each slide is not indicated.

The RIKILT results indicated that only two slides were examined, instead of the total of three slides required, according to the Directive.

The procedure of RIKILT includes the use of large cover glasses to examine the fine sediment after sieving. These cover glasses are 24 x 50 mm in size, instead of the smaller ones commonly in use (e.g. 21 x 26 mm). With an even distribution of the fine sediment material across the major part of the large cover glass area, no material was left to examine a third slide. However, with both slides, the entire fine sediment was examined microscopically. This examination followed a full macroscopic examination of the coarse sediment according to RIKILT standard procedures. In this way, the entire sediment was examined, although not with the required number of slides.

The number of slides used by the labs is also reported in Veys et al. (2007b). The distribution in number of slides examined for sample C is shown in Table 7.

Table 7: Overview of the number of slides used for the examination of sample C(0.1% MBM + 5% fish) by the participants in the proficiency test 2007. Source: Veijs et al.(2007b).

	Number of slides used								
	1	2	3	4	5	6	7	20	?
Number of NRLs reporting:		2	8	1	1	4	3	2	4

?: no number of slides reported.

2.1.3 CRL workshop

The CRL organised a workshop in April 2007. A RIKILT delegation participated in this workshop in Gembloux, Belgium. Much attention was given to the results of the first proficiency test (Veijs et al., 2007a) and the first efforts to quantify the amount of fish material in the samples. A first outline for an improved quantification procedure was presented as a basis for the planning of the 2007/2008 proficiency test.
The CRL had decided to grant licenses of the expert system ARIES to all NRLs. RIKILT, as former partner in the project STRATFEED and coordinator of the development of ARIES, presented ARIES to all the NRL representatives. A workshop was held for the NRL representatives to gain experience for using ARIES.

2.2 Communication with stakeholders

During 2007 information has been presented and advice has been given to the official authority on the risks and benefits of including fish meal in ruminant feeds. These advises were the sequel of an official risk analysis for the competent authority (in Dutch: van Raamsdonk en Heres, 2006). This analysis was focused on aspects of allowing fish meal in ruminant feeds, and it was presented to the Dutch government in February 2007.
Political opinions vary from continuing to prohibit any fish material in ruminant feeds on the one hand to a free use on the other. Currently a compromise is worked out to allow 1 % of fish material in ruminant feeds, provided that the fish material used is checked and certified for the absence of terrestrial animal material. A problem with such a compromise solution is the possibility of occasionally present sea mammal and sea bird material (problem of identification), but also the quantification of the final share of fish material in feed (problem of quantification).
The identification problem is part of the research of the European Union project SAFEED-PAP, as far as the exclusive identification of ruminant material is concerned. In discussions with the Dutch government the issue of identifying pig material and poultry material for a full support of the species-to-species ban was addressed.
The CRL has started to develop a method for the quantification of fish material, as based on the preliminary results of the 2006 proficiency test.

2.3 Technical and scientific support to the stakeholders

Contrary to some other member states, the Netherlands does not have a network of national control laboratories for the detection of animal proteins. RIKILT is the only designated control laboratory for monitoring of feeds and feed materials in this matter. Therefore, the organisation of national proficiency tests seems to be superfluous. However, in Dutch legislation a list of in total five laboratories is given that might be consulted for official control. These laboratories are also available for cases where a contra-expertise is requested. In most of these cases the contamination levels of animal proteins are in the range of the level of detection of the microscopic method, which is much below the current 0.1%, as is stated as minimum requirement for detection methods in Directive 2003/126/EC. Reproducing the results as obtained in the official monitoring is therefore problematic. Plans are in progress to offer support to these laboratories, if they are involved in the detection of animal proteins. The Dutch NRL can offer such support e.g. by organising proficiency tests. The current efforts to establish the performance of the microscopic method or of the laboratories is related to the required level of 0.1 %. Nevertheless, zero tolerance is applied to the prohibition of animal proteins in practice, and animal proteins should be detected as low as reasonably achievable. RIKILT has considerable experience in the detection of animal proteins with other methods than microscopy, e.g. DNA detection by PCR and protein detection by immunochemistry. A paper was published with the PCR method (Aarts et al., 2006). Immunochemistry is part of the research of the project SAFEED-PAP.

2.4 Other specific tasks

The RIKILT project leader for the Dutch NRL was invited to be a member of the advisory board of the CRL for microscopy affairs. Other members are from the Danish and Belgium NRL, and from the EC Joint Research Centre. Since the start of the CRL activities, the advisory board met once. New meetings are planned for 2008.

3 Discussion

3.1 Detection

In 2003 a proficiency test was organised on behalf of International Fishmeal and Fishoil Organisation (IFFO) for testing the microscopic method as published in that year (Directive 2003/126/EC). Nine laboratories, known for their high expertise in applying microscopic examinations, were selected to participate. Because of this selection procedure, the results could be interpreted as indicative of an optimal performance (without any bias of the local expertise). The study showed a sensitivity score of 0.987 for the detection of terrestrial animals in a sample with 0.1 % MBM and 5 % of fish material (van Raamsdonk and van der Voet, 2003).
Other proficiency tests, organised later after 2003, show a progress in performance of the method and/or of the laboratories (Figure 1; see Veys et al 2007a, van Raamsdonk et al., 2007).
Progress in performance is still required. Training is an important tool. The result of the IFFO study does not necessarily mean that the current method as described in Directive 2003/126/EC can not be improved. A simple requirement of analysing two additional slides in the presence of fish material is not sufficient; the size of the cover glass and the amount of material to be examined needs attention as well. Another issue applies to the minimum amount of material to be used for sedimentation. The Directive states a minimum of 5 grams, whereas the participants in the IFFO study all used 10 grams as starting material. Other improvements may be considered.

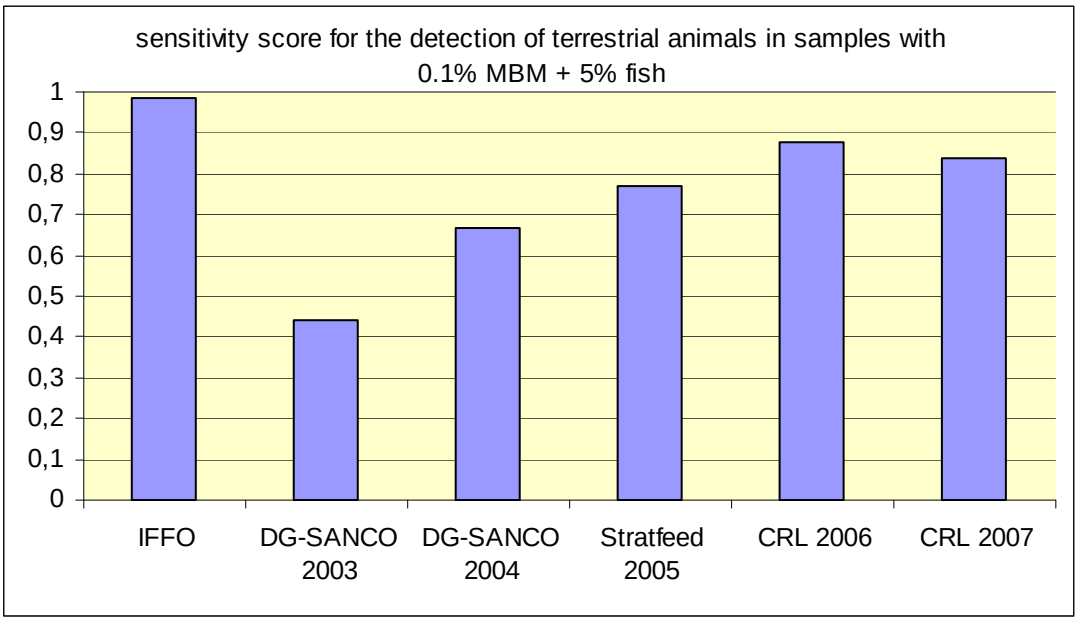

Figure 1: Sensitivity scores from a range of proficiency tests or validation studies for samples with 0.1 % of MBM and 5 % fish material. The studies discussed in this report are indicated as CRL 2006 and CRL 2007.

The aim of having a sensitivity score of at least 0.95 means a maximum of 1 error in 20 measurements (e.g. samples). When a proficiency test consists of only 19 samples of the same type, even one false negative will result in a score for sensitivity that is slightly below 0.95. If a result in a previous proficiency test showed no false negatives, this will not be used as compensation in the evaluation of

the current proficiency test results. It can be argued that a specific application of the principle of advanced means should be used here: the results of the latest proficiency test are to be completed with results of previous proficiency tests in historical order up to a minimum of 20 results. If the addition of new results gives a total of more than 20 data points, all results of the earliest proficiency test considered need still to be included, since discriminating within the results of one proficiency test will bias the final calculation. When the results of a new proficiency test become available, all the results of the oldest proficiency test can be omitted, providing that at least 20 data points will remain. The use of a few if any data points in addition to the minimum of 20 results might give a too small basis for proper evaluations. Therefore, including all results of a period of five years for calculations should be considered, assuming that a sufficient amount of data has been collected in those years. After adding the results of year 6 the results of year 1 can be skipped. In this way, a fairly balanced evaluation of a consecutive row of trial results and of NRL performance may be achieved, and possible outlier results can be put in proper perspective.

3.2 Identification

Cattle or ruminant material is the primary target for species identification in all current projects (e.g. SAFEED-PAP). However, for a successful implementation of the species-to-species ban the use of pig material or poultry material in feeds for their own animal type should be monitored. Implementation of identification procedures for protein material originating from these animals implies the development of specific DNA or protein detection, and proper primer sets or antibodies for these animals.
More specifically, the species-to-species ban applies not only to ruminants, pigs or poultry, but to other categories of animals as well. Identification of materials of sheep, goat, horse, turkey and other farmed animals is necessary for correct monitoring of their respective feeds. Other protein material i.e from rodents that may inadvertently enter the production chain should be classified, in order to identify it as contaminating material. A special case could be made for goat and sheep material. The ban for using mammalian material for ruminant feed applies to all mammalian material, according to EC Regulation 999/2001 (article 7: “1. The feeding to ruminants of protein derived from mammals is prohibited.”). The species-to-species ban is laid down as follows: “1. The following uses of animal by-products and processed products are prohibited: (a) the feeding of a species with processed animal protein derived from the bodies or parts of bodies of animals of the same species; [etc.]” (Article 22 of EC Regulation 1774/2002). The span of the prohibition depends on the definition of “species”. If cattle, sheep and goats are defined as different species, which is the correct interpretation from a biological view, monitoring should include separate identification of sheep and goat material. Even if a future implementation of the ban applies to ruminants as group in general, most of the currently used antibodies and primers are specific for cattle and not for ruminants (cf. Aarts et al., 2006). The same applies to the case of poultry, where it may be argued that primers and antibodies of the species turkey should be developed and used for separate identification.
The possible presence of sea mammal and sea bird material in fish meal batches should be addressed and dealt with as well. Primers and antibodies should also be developed for these groups of species.

3.3 Quantification

An equation for the quantification of animal material in feeds is published in EC Directive 126/2003. Main problems arise for the estimation of the f-factor and the d-factor. The f-factor indicates the share of bone material in the original MBM or fish meal. Since the original MBM or fish meal is not available after producing a feed, the f-factor can only be estimated. In general f = 0.1 – 0.2 for fish material and f = 0.5 – 0.7 for MBM is used. The d-factor indicates the share of bone fragments in the sediment. This factor is the main difficulty. Currently the CRL is developing a new measuring procedure using a counting grid, and a new proficiency test is organised for early 2008.

4 Recommendations

a) Considering the pooled results of the proficiency test of 2007, it might be valuable to evaluate the procedure for organizing, examining and reporting of proficiency tests by CRL and NRLs. Such an evaluation can only be initiated and directed by the CRL.
b) The number of slides examined varies considerably. Two labs, including RIKILT, did not comply fully to the Directive. However, the directive does not give any indication of the requirements for the size of the slides and the amount of sediment material to be used. A more precise and detailed description of the procedure as described in Directive 126/2003 is needed. Efforts for optimizing the procedure for microscopic detection are part of the project SAFEED-PAP.
c) An international project for developing identification procedures, and as part of it the development of antibodies and/or primers, for a range of species is urgently needed. As a first action a desk study for the relative differences between the DNA and amino-acid sequences, and predictions for the possibility to raise specific antibodies and primers, is highly recommended. This desk study should include information from international databases as well as from surveys of systematic or taxonomic research.
d) The principle of advanced means should be considered for evaluating the performance of labs. All proficiency test results achieved in the past five years per sample type should be included. Including a minimum of 20 data points in the calculations for specificity and sensitivity assures a proper statistical evaluation.
e) A proficiency test for support of the monitoring by other Dutch laboratories should be considered.

5 Literature

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