Animal proteins

Annual Report 2012 of the Dutch National Reference Laboratory

L.W.D. van Raamsdonk, I. Scholten, J.M. Vliege and V. Pinckaers

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P.O. Box 230, 6700 AA Wageningen, The Netherlands, T +31 (0)317 48 02 56,
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Contents

Summary

RIKILT serves as the only official control laboratory for animal proteins in feeds in the Netherlands in the framework of Directive 882/2004/EC.

There has been a long-time desire from society as well as from legislators to lift the extended feed ban, as a whole or partly. In 2012 processes for the relaxation of measures were mainly focusing on Regulation (EC) 152/2009 for improvement of the microscopic method and the implementation of the identification of animal proteins of ruminant origin by means of PCR, and Regulation (EU) 1069/2009 concerning the acceptance of pig and poultry proteins for aquafeed. Besides the general annual interlaboratory study for the microscopic method, EURL organised two interlaboratory studies for validation and implementation of the PCR method to detect ruminant proteins in animal feeds. The method for identification of ruminant proteins in animal feeds is successfully validated and implemented. Further attention is necessary for some specificity issues (false positive signals) and for the microscopic identification of feather meal. RIKILT participated successfully all studies. In general, very good results were achieved and the collected data appeared to be useful for method improvement. The process for developing methods for the identification of pig, poultry and fish proteins is carried out in cooperation with the EURL, in a dedicated project for method development and these methods are expected to be finalised in 2013.

RIKILT participated in the annual meeting of the NRL network on animal proteins and in the meetings on the development of the new protocol on PCR procedures. RIKILT employees served as member of both the Expert panel on microscopy as well the Expert Panel for PCR methods. Furthermore, the internal procedures of RIKILT for sample preparation as well as microscopic analysis have been evaluated and improved when necessary. One of the reasons is the situation that fish meal can be both a contaminant as well a matrix (pure samples). A major achievement is the separation, physically and as work flow, of samples that are not contaminated or only at a low level from those samples that are contaminated at a high level. Also samples of pure animal proteins are handled separately.

1 Introduction

In 2006 the European Union appointed a series of European Union Reference Laboratories, one of them dedicated to the field of Detection of animal proteins in feeds. Each member state has appointed a National Reference Laboratory (NRL) in this field. The stakeholders, i.e. the Ministry for Economic Affairs (EZ) 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 by means of technical and strategic advice, method development and participation in international networks of experts.

In order to check the quality and performance of microscopic detection of animal proteins, the EURL organises an annual proficiency test. Also, the RIKILT validation of the ruminant PCR test was completed. Further National activities include the support of the competent authorities, participation in the national monitoring program and specific studies in case technical (interpretation) problems occur. The results of the annual proficiency test for microscopy are being published in the year following the year in which the test was organised. As an effect, every proficiency test was accounted for in two subsequent report of the Dutch NRL. Usually the draft report is available prior to finishing the NRL annual report. Therefore, besides the presentation of the 2011 result, the current report will present the results of the 2012 proficiency test for microscopy as well, which became available in the first quarter of 2013.

The Dutch NRL gives account of its activities in the framework of collaboration with the EURL 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/EC. RIKILT serves as the official control laboratory for animal proteins in feeds in the Netherlands. Several of the tasks listed in the Directive do not require activities due to the single laboratory situation. Remaining tasks are:

- Collaboration with the EU-RL, including participation in meetings and workshops, participation in ring trials;
- Communication of information from the EU-RL 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 EURL;
- 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 identified the desire 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/EC.

3 Results and discussion

3.1 EURL proficiency test microscopy 2011

RIKILT - Wageningen UR has participated as Dutch NRL animal proteins in the interlaboratory study for animal proteins 2011, organised by the EURL, Gembloux, Belgium. This ILS consisted of seven blind samples of feed contaminated with animal proteins of land animals and/or of fish. The results are published and discussed at the annual meeting of EURL and NRLs in 2012. Therefore these results are presented in the NRL annual report for 2012. The composition of the samples and the overall results are listed in Table 1 (Veys *et al.*, 2012).

Table 1

Results of the proficiency test of 2011. The accuracy indicates specificity in the case of absence of the target, and sensitivity in the case of the presence of the target. Optimal values are 1.0. Nr: number of reported results.

Sample number	Composition	Nr	AC terrestrial	AC fish
1	Blank I	26	1.00 (0)	0.89 (3)
2	Blank II (pellets)	26	0.92 (2)	0.92 (2)
3	Blank I + 0.05% animal proteins	26	1.00 (1)	0.96 (1)
4	Fat (+ 0.1% of dicalcium phosphate)	26	1.00 (1)	1.00 (0)
5	Fat + 0.1% animal proteins	26	0.96 (1)	0.96 (1)
6	Fish feed, containing fish (pellets)	26	0.96 (1)	1.00 (0)
7	Fish feed, containing fish + 0.5% hydrolysed feather meal (pellets)	26	0.31 (18)	1.00 (0)

The overall results of RIKILT as reported in November 2011 are shown in Table 2.

Table 2

Results of RIKILT in the EURL proficiency test 2011.

Sample number	Unique ID	Composition	RIKILT Terrestrial animals	RIKILT Fish
1	656	blank I	correct	correct
2	223	blank II (pellets)	correct	*7 fish bone fragments*
3	287	blank I + 0.05% animal proteins	correct	*1 fish bone fragment*
4	330	fat (+ 0.1% of dicalcium phosphate)	correct	correct
5	492	fat + 0.1% animal proteins	correct	correct
6	234	fish feed (pellets)	correct	correct
7	137	fish feed + 0.5% hydrolysed feather (pellets)	*not found*	correct

The EURL notified RIKILT of underperformance in this study because of reporting two false positives and one false negative, and requested a report with an explanation of possible causes and an action plant to avoid errors.

The procedure as required according to Annex VI of Regulation (EC) 152/2009 was applied in November 2011. A part of every pelleted feed sample (nrs. 1, 2, 3, 6 and 7) was ground after grinding a washing batch of pure maize grains in a Retsch mill. The normal cleaning procedure was applied. Sedimentation was achieved in thoroughly cleaned sedimentation funnels. The remaining part was kept apart for later examination. The two fat samples (4 and 5) were processed according to the

additional protocol. In addition to the reported results, RIKILT produced in March 2012 a new set of materials and slides of samples 2, 3, and 7. Special attention was given to sample 2 (223).

3.1.1 Sample 2 (223)

Overall accuracy fish: 0.923 (2 false positives).

EURL homogeneity study: no fish material found.

The part of sample 2 (223) that was ground in November 2011 was re-examined by making a new sediment and new slides. In total 12 fish bone and gill fragments were found in this second sediment. In order to find a possible cause of the false positive findings, several alternative procedures were carried out with newly ground material, in all cases based on the official amount of 10 grams. All these alternative analyses turned out to be negative.

Evaluation: all the positive findings are related to the grinding of November 2011. However, a range of precautionary measures were already taken to prevent any contamination. The whole procedure was verified in March 2012 and no possible causes for the contamination were found. In the same period during November 2011 no fish meal sample was processed with a comparable composition as shown by the fish particles found in sample 2 (predominantly herring).

3.1.2 Sample 3 (287)

Overall accuracy fish: 0.962 (1 false positive).

EURL homogeneity study: sample 3 is based on Blank I. The EURL homogeneity study revealed 1 fish scale and 1 fish bone in Blank I.

The first set of slides (November 2011) contained only one fish particle, a bone fragment. This is comparable to the results of the EURL homogeneity study, in which one fish bone and one scale was found in Blank 1; this blank sample was the basis of sample 3 (Veys *et al.*, 2012). In the second sedimentation (March 2012) no further fish material was found. This is to be expected in cases of very low levels of contamination. Since a major investment of time was used to examine sample 2, no further examinations were carried for sample 3.

Evaluation: basically RIKILT was able to confirm the EURL finding of one bone particle and one scale fragment in Blank I, which was the basis for sample 3.

3.1.3 Sample 7 (137)

Overall accuracy land animals: 0.308 (18 false negatives).

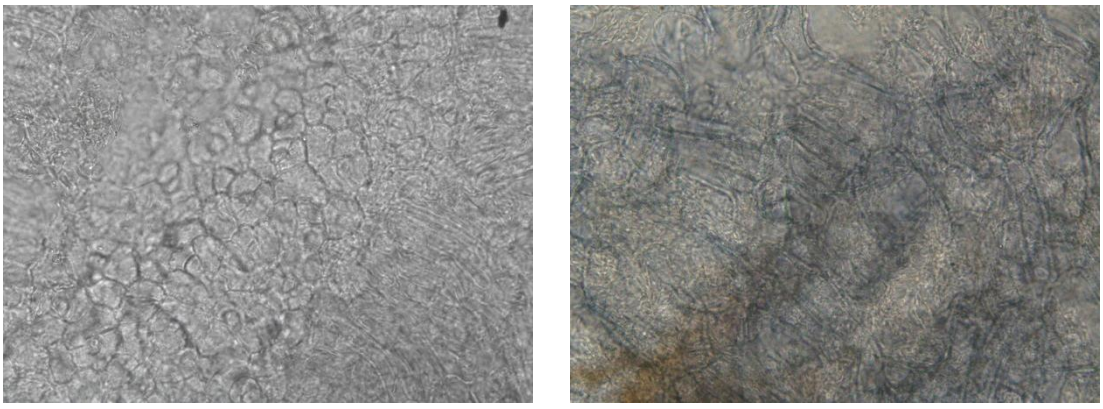

Figure 1 *Two images of plant epidermis with stomata (left), and epidermis cells after cysteine staining (right).*

EURL homogeneity study: mammalian material present: fish and hydrolysed feather meal.

Examination in November 2011 did not reveal any presence of (hydrolysed) feather particles, nor of the rare bone fragments that are usually included in feather meal. Also, re-examination in March 2012 gave the same result. A few particles turned grey in the cysteine staining method with lead acetate. These grey particles were further examined at higher magnifications, and appeared to be parts of plant epidermis fragments (Figure 1).

Evaluation: approx. 70% of the participants were not able to detect the feather meal in the presence of fish material in this sample in the ILS of 2011 (Veys *et al*., 2012). A feed sample containing feather meal in the ABsence of fish meal was included in the proficiency test of 2008 (Veys *et al*., 2009). Except for one participant, correct positive results were achieved (AC = 0.981, 1 false negative), although ten participants reported exclusively the presence of bone fragments and no feather material (filaments or hydrolysed particles; Veys *et al*., 2009). 15 correct reports for feather material out of 26 participants results in a percentage of 58%, almost double the score for the 2011 study (30%) (Veys *et al*., 2012). At a level of 0.5%, the feather meal was apparently hard to recognise. This could be caused by a very low presence of bone fragments (which are normally a good marker), and/or by extensive hydrolysis of the material.

3.2 EURL proficiency test microscopy 2012

RIKILT participated in the annual interlaboratory study for microscopy in 2012, which was organised in November. The results were published in draft in the first quarter of 2013, and are presented in Table 3.

Table 3

Results of the proficiency test of 2012. The accuracy indicates specificity in the case of absence of the target, and sensitivity in the case of the presence of the target. Optimal values are 1.0. Nr: number of reported results.

Sample number	Composition	Nr	AC terrestrial	AC fish
1	Blank I	27	0.70 (8)	0.93 (2)
2	Blank II	54	0.96 (2)	0.96 (2)
3	Blank III	54	0.87 (7)	0.91 (5)
4	Blank III + 0.05% poultry PAP	27	0.93 (2)	0.85 (4)
5	poultry PAP	27	1.00 (0)	0.78 (6)

In general problems with specificity (false indications of presence) have to be noted. This is shown by an accuracy between 0.7 and 0.96 for material of terrestrial animals in the blanks (samples 1-3), as well by an accuracy between 0.78 and 0.85 for fish in the presence of poultry material (samples 4 and 5). RIKILT produced correct results in all cases.

3.3 Other proficiency tests for microscopy

RIKILT participates annually in several proficiency tests pertaining to the detection of animal proteins or, more in general, to composition. In the latter situation the samples are considered blanks for the presence of animal proteins.

3.3.1 KDLL blind tests on animal proteins

KDLL is a Dutch organisation organising a proficiency test for animal proteins twice a year. Each of these tests consists of four samples of feed. The results of RIKILT in this bi-annually proficiency test are presented in Table 4.

Table 4

Contents of the KDLL proficiency tests with RIKILT results.

Sample	Composition	RIKILT result	
		Fish	Terrestrial
MIK12-1A	0.7% feather meal	*present*	present
MIK12-1B	1.7% fish meal, 1.7% poultry meal	present	present
MIK12-1C	Microscopic examination; no animal proteins	absent	absent
MIK12-1D	Label check; no animal proteins	absent	absent
MIK12-2A	1.0% meat meal	absent	present
MIK12-2B	2.08% fish meal, 0.42% poultry meal, 0.82% meat meal	present	present
MIK12-2C	Microscopic examination; no animal proteins	absent	absent
MIK12-2D	Label check; no animal proteins	absent	absent

The RIKILT results were correct in most cases except one. The false positive for fish in sample MIK12-1A (Table 4) can be assumed to be related to the presence of poultry material, which is comparable to the results presented in Table 2 (sample 137).

3.3.2 IAG blind tests on composition

IAG is a European organisation for supporting microscopic research. One of its activities is to organise several ring tests (proficiency tests) for composition. The results for two tests on composition are presented in Table 5.

Table 5

Contents of the IAG proficiency tests with RIKILT results.

Sample	Description	Composition	RIKILT result	
			Fish	Terrestrial
SFR S1-2012	Pig feed	No animal proteins	absent	absent
LUFA 1-2012	Dairy feed	No animal proteins	absent	absent

The usual composition of feeds in these proficiency tests does not include animal proteins. The RIKILT results were in agreement with the composition of the two samples.

3.3.3 IAG blind tests on animal proteins

The IAG is organising annually a ring test for the detection of animal proteins. RIKILT as organiser for this ring test is usually not participating in this test. However, in 2012 three of the four samples of the 2012 test were blindly put in the regular monitoring program. These samples consisted of a blank, a fish meal fortified with 10% of salmon meal, and a feed with 0.02% MBM (meat and bone meal). The salmon meal was used for their relative similarity to material of terrestrial animals. In all three cases RIKILT produced correct results (van Raamsdonk *et al.*, 2012).

3.4 General background to RIKILT microscopy procedures

RIKILT always applies the full instructions as laid down in Annex VI of Regulation (EC) 152/2009. This means that both the sediment and the original sample are examined. The examination at lower magnification is carried out extensively. In this way all types of prohibited types of material can be found in the full sample.

The general facilities at RIKILT for sample preparations, such as grinding and drying, are separated for highly contaminated samples and other regular samples. This means that e.g. fish meal is never ground in the same rooms and mills as other samples are.

At the end of 2011 RIKILT started a general evaluation of the implementation of the procedures for sedimentation and examination of all samples for microscopic analysis. As one of the results, four different sets of sedimentation funnels are applied from mid 2012: a) for general samples, b) for fish materials, c) for samples belonging to a ring test, d) for pure samples originating from terrestrial animals. The separate set of funnels for fish is based on the situation that fish can be either a legal ingredient or a contaminant.

It goes without saying that the preparation of contaminated samples for other ring tests, such as that of IAG, is always carried out in facilities that are at a large physical distance from the microscopic lab. Furthermore, RIKILT applies weekly examination of internal blank standards, according to our own quality assurance system. These blank samples always turn out to be negative.

3.5 DNA detection and identification

The EURL organised two tests for the detection of ruminant DNA. The first test, started in December 2011 and finalised in February 2012, was aimed at the validation of the TNO Triskelion ruminant test (Fumiere,2012a). Twelve laboratories analysed a set of 10 samples of DNA extracts (no feed samples included), consisting of four different blanks (without ruminant DNA) and six contaminated samples with ruminant DNA at three levels. The description of the samples and the overall results of the twelve laboratories is given in Table 6.

Table 6

Results of the ruminant PCR validation test of 2012. The performance of the twelve laboratories is indicated by the percentage of correct results at two detection levels. Every sample was analysed in 20 replicates, divided over two runs, and pooled per type. Nr: total number of replicates per lab.

Sample number	Composition	Nr	Laboratory performance at cut-off = 15 copies	Laboratory performance at cut-off = 10 copies
	DNA extracts			
2	Blank 1: compound feed	20	Two out of 12 labs < 95%	Four out of 12 labs < 95%
4	Blank 2: fish meal	20		
8	Blank 3: rapeseed oilcake	20		
9	Blank 4:maize + 5% w/w pig DNA	20		
1, 10	0.1% w/w bovine DNA in Blank 1	40	All labs > 95% correct	All labs > 95% correct
5, 7	0.025% w/w bovine DNA in Blank 1	40	All labs > 95% correct	All labs > 95% correct
3, 6	0.0125% w/w bovine DNA in Blank 1	40	All labs > 95% correct	All labs > 95% correct

The test appeared valid for the detection of ruminant DNA at low levels (sensitivity). The number of false positives for the blanks (specificity), however, depended on the laboratory and on the detection level (cut off). The report (Fumiere, s.n.) did not provide a stratification of the result per blank sample, which means that the possible source of the false positives cannot be reconstructed. The report is publicly available as draft, but a final (approved) version is not yet published. RIKILT produced correct results for all samples and in all four runs.

The ILS for PCR 2012 was announced in February 2012 and sample analysis took place in April 2012. The final deadline was May 11th. This ILS was aimed at the implementation of the ruminant test.

Different feed matrices were used for the preparation of the sample set:
- Blank 1: feed for sow (used in samples #1, #2, #3, #4 and #8);
- Blank 2: mix made of 60% of barley, 16% of maize, 16% of flax and 8% of alfalfa (used in samples #5, #6, #9 and #10);
- Blank 3: ground maize kernels (used in sample #7).

Table 7

Results of the ruminant PCR implementation test of 2012. The accuracy indicates specificity in the case of absence of the target, and sensitivity in the case of the presence of the target. Optimal values are 1.0. Nr: number of reported results.

Sample number	Composition	Nr	SE ruminant	SP ruminant
	Feed samples			
1	0.1% sheep PAP in blank 1	21	1.0	
2, 3	0.1% cattle PAP in blank 1	42	1.0	
4	1% pig PAP in blank 1	21		0.76 (5)
	DNA extracts			
5	0.2% cattle PAP in blank 2	21	1.0	
6, 10	0.1% cattle PAP in blank 2	42	1.0	
7	5% pig PAP in blank 3	21		1.0
8	0.1% sheep PAP in blank 1	21	1.0	
9	Blank 2	21		1.0

Blank 1 and blank 3 were not included in the design. The PAPs were heat treated at 133 °C (sheep, pig) or on 141 °C (cattle). A total of 21 NRLs participated in this study. Overall the result was excellent for most samples. RIKILT had correct results in all cases. Five laboratories reported false positive results for sample 4. This is presumably caused by cross-contamination during the DNA extraction steps. This is in concordance with the situation that no false positives have been reported in all samples which are submitted as already extracted DNA samples (samples 5-10) (Fumière *et al*., 2012b).

3.6 Cooperation with the EURL animal proteins

The RIKILT delegation, consisting of two persons, participated in the annual meeting of the EURL/NRL network in April in Berlin. Several issues were discussed, including the annual proficiency test for microscopy of the year 2011, the proposed improvements of the microscopic method (amendment of Regulation (EC) 152/2009), the consequences for monitoring after lifting the ban for non-ruminant animal proteins in fish feed, and the analytical problems for PCR in the presence of milk products or other legally allowed ruminant materials. In the latter case DNA was extracted from the heavy fraction, assuming that this fraction does not contain any remains of 'allowed' ruminant DNA. It was announced at this meeting that even heavy procedures for clean-up does not assure the absence of this ruminant DNA. RIKILT participated actively in all these discussions. After the meeting in Berlin RIKILT discussed an alternative strategy for solving the analytical problem of legally present ruminant DNA using immunoassays. This strategy was included in the annual work plan for method development for the detection of animal proteins in 2013.

As a spin-off of the annual meeting, RIKILT made an inventory of the possible treatments of bone particles for removing DNA of allowed ingredients and saving the native bone DNA material.

The EURL has organised two meetings (April en October) to discuss the implementation of the PCR methods for identification of animal proteins. RIKILT employees participated in both meetings, and were involved in the process of development of Standard Operational Procedures (SOPs), which were intended to be part of the official procedures as guiding documents of the official legislation. RIKILT is represented in the two Expert Panels advising the EURL in matters of microscopy and PCR.

An EURL delegation (director and microscopy coordinator) visited RIKILT in June 2012. Several topics were discussed, such as cooperation in the areas of method development, strategy for monitoring, and an inspection of the RIKILT facilities for sample preparation and microscopic research was carried out. The measures taken in the RIKILT laboratories for improvement of the work flow were considered as valid and sufficient by the EURL delegation. The further development of methods for the identification of pig, poultry and fish material was effectuated in the research plans of the WOT project Method development animal proteins.

3.7 Support of the national authority

RIKILT had frequent contact with the competent authority and the Netherlands Food and Consumer Product Safety Authority (NVWA) concerning general advices and support for the discussion in the procedures for establishment of new EU legislation. These processes were mainly focusing on Regulation (EC) 152/2009 for improvement of the microscopic method and the implementation of the identification of animal proteins of ruminant origin by means of PCR, and Regulation (EG) 1069/2009 concerning the acceptance of pig and poultry proteins for aquafeed.

3.8 Future developments

An impressive update has been made for the legislation, both with respect to the ban on the use of animal proteins in animal feed as well as in the implementation of control methods. The measures will become effective in 2013. Several aspects concerning the implementation needs further attention. The performance of the microscopic detection is generally good. Only minor aspects such as the detection of feather meal need attention. The implementation of the ruminant test in the NRL labs was carried out successfully, although the specificity of the test in the presence of pig DNA deserves further attention.

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RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

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RIKILT Wageningen UR is part of the international knowledge organisation Wageningen University & Research centre. RIKILT conducts independent research into the safety and quality of food. The institute is specialised in detecting and identifying substances in food and animal feed and determining the functionality and effect of those substances.

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To explore the potential of nature to improve the quality of life

RIKILT Wageningen UR
P.O. Box 230
6700 AE Wageningen
The Netherlands
T +31 (0)317 48 02 56
www.wageningenUR.nl/en/rikilt

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