COMMUNITY REFERENCE LABORATORY

"Detection of animal proteins in feedingstuffs"

CRL-AP

Walloon Agricultural Research Centre – CRA-W (Belgium)

Annual report 2009 activities

PUBLIC VERSION

1 Scientific advice and support to the European Commission *(36 p/m)*

1.1 Provide scientific and technical assistance to the European Commission in relation to the development of EC feed legislation. (6 p/m)

End of 2008, CRL-AP asked each NRL to realise a methodical reading of Directive 126/2003 text in order to
(1) signal existing translation errors in their respective national languages vs. the English version of the document,
(2) to report proposals for changes or improvements of the directive text content on the qualitative part of the method for the detection of animal proteins in feeding.

By end of January 2009, only few NRLs delivered the asked comments. During the 3rd CRL-AP annual workshop all NRLs were invited once more to deliver their request for change or revision for the EC/126/2003.
By the 2nd half of 2009, the CRL-AP had received all comments and after analyse of these a new protocol was written and submitted beginning 2010 to all NRLs.
During the 4th CRL-AP annual workshop in Turin, it is foreseen to drawn up a final version of this new protocol taking into account all the remarks formulated by the NRL network.

On the request of the DG SANCO, a study on the "MELISA-TEK™ Ruminant kit" (ELISA Technologies Inc., Gainesville, USA) was conducted at CRA-W to evaluate the relevance of the EFPRA (European fat processors and renderers association, M. Alm) proposal to use the test as screening method for the control of the presence of ruminant processed animal proteins in other processed animal proteins. The report contains a status of the knowledge based on literature and results of experiments conducted at CRA-W according to a protocol using the MELISA-TEK™ Ruminant kit in combination with the MELISA-TEK™ High Extraction Kit.

From the results obtained by the CRA-W and presented in a report dated 1st of July 2009, it appears that the method is not directly transferable and that the decision criteria set by CCL Nutricontrol (M. Margry) should be re-evaluated. To substantiate this conclusion, an additional study was decided after a meeting at DG SANCO together with CCL (on 9th of September 2009). The study was designed and organised by the CRA-W (design of the ELISA plates, questionnaire and results forms, order and distribution of the kits, collect and analysis of the reported results ...). The collaboration of ELISA experienced labs from different institutes (CCL-M. Margry, CRA-W-M. Berben, JRC-IRMM-M. Von Holst, Rendac-M. Derks and RIKILT-Ms Bremer) was also requested.

The study consisted in recording the variability of blank control replicates amongst 5 runs performed at different moments in an Excel file. A large set of experimental conditions identified as potentially crucial for the results also had to be recorded by the participants in a questionnaire form. On the basis of the results obtained by the participants with the blank control, it can be confirmed that the decision criteria set by CCL should be re-evaluated. Moreover the variability of the results seems to arise from a batch-dependent effect.

This conclusion was shared with the CCL during a meeting held in Gembloux the 25th of November 2009. The report on the additional study is annexed to this activity report (Evaluation of the MELISA-TEK ruminant kit for the detection of ruminant proteins in processed animal proteins - O. Fumière, V. Baeten, G. Berben).

1.2 Upon the request of the European Commission or in order to fulfil his role as Community reference laboratory, participate to international fora/committees relating to the detection of animal proteins in feedstuffs (EFSA, WHO/FAO, JRC, etc) with eventual presentations to prepare for it. As up to 2 European or international missions/year are foreseen in support to DG SANCO and/or CRL activities. (2 p/m)

1.2.1 Preparation and participation in international meeting/fora

1.2.2 Report/minutes following completion of the mission

CRL-AP further developed the close collaboration with the Chinese Agricultural University of Beijing (CAU, Professor Han Lujia) initiated on request of the Chinese authorities in 2008. This collaboration has already allowed the CRL-AP microscopy team to develop a new research model for the estimation of the limit of detection (LOD) for qualitative methods of analyses such as provided by microscopy.

In continuity of the results obtained for the estimation of the LOD of fish meals the CRL-AP team applied this first half-year the same experimental model for the estimation of the LOD of mammalian meat and bone meals. Results were presented and discussed at the 3rd CRL-AP Annual Workshop held in Gembloux in March 2009.

Furthermore, the CRL- AP went on scientific mission to China in July 2009. CRL-AP helped the CAU to install the first laboratory specialised in conventional and infrared microscopy methods in order to create a later network of expert based on the CRL-NRL networks in EU.

During the CRL-AP visit, numerous lectures were given as well as practical sessions, where about 20 scientists were trained.

The CRL-AP also attended to the 2nd SAFEED-PAP Workshop PAP Detection: Europa-Asia exchange of experiences, an international workshop organised in the framework of SAFEED-PAP project which was held in Quingdao 21-22 and 23 of April 2009. This workshop aimed to facilitate an exchange of experiences between European and Chinese research teams. A total of more than 20 oral presentations were given, including several by CRL-AP—NRL-AP members, covering many aspects of PAP detection as well as food and feed safety.

The CRL-AP attended the 3rd Feed Safety Conference: Methods and Challenges. This conference was held in Wageningen, the Netherlands on the 6th and the 7th October 2009. It was organised by the RIKILT in the framework of the SAFEED-PAP project.
End November, on 26th and 27th 2009, the CRL-AP was invited by the Italian NRL to present a lecture at the II Convegno degli Istituti Zooprofilattici Sperimentali sull'Alimentazione Animal, Torino, Italy. The slides presented at this meeting are added in annex of this report (Tools for the optimization of the detection of PaPs in feed, P. Veys - V. Baeten).

1.3 Upon the request of the European Commission or in order to fulfil his role as Community reference laboratory, participate in meetings for the standardisation of analytical methods relating to the detection of animal proteins in feedstuffs and their implementation (CEMA, ISO/CEN, OIE, IAG, etc). Up to 3 European missions/year are foreseen in support to DG SANCO and/or CRL activities. (1 p/m)

1.3.1 Participation in the CEMA, CEN and IAG meetings

1.3.2 Reports/minutes of the meetings

Participation at the AOCS Annual Conference in Orlando, Florida, USA

from the 2nd to the 7th of May 2009.

The CRL-AP head of microscopy participated in the AOCS meeting as guest speaker and presented a lecture on the ongoing researches on quantification methods.

The collaboration with AOCS scientists from Canada and USA initiated in 2007 is fruitful : Canada participated to several ring trials organised by CRL-AP and now the CRL-AP head of microscopy is redacting as co-author the new edition of the AOCS Handbook of Feed Microscopy which should be published by end of 2009. This manual will include the EU method of detection of PAPs in feed.

During the meeting qualified contacts were established with the managing team of the AOCS Feed Microscopy Section. Other international contacts were also established such as with Dr Cruywagen, from Stellenbosch University South Africa, who is willing to participate to CRL-AP proficiency tests in the future. The slides presented at this meeting are added in annex of this report (Quantification for PaPs in feedingstuffs, P. Veys-V. Baeten).

The CRL-AP participated at the IAG annual meeting held in Dublin in June. Three lectures were presented.

A first one dealt with the results of the CRL-AP Proficiency Test 2008 in which the NRL network participated. Subsequent discussions allowed to bring those results in comparison with the results obtained from the IAG ring trial 2008 in which the CRL-AP participated.

A second presentation informed on the results obtained on the LOD determination for fish meals and terrestrial meat in bone meals in collaboration with the CAU.

Finally an extended presentation of the CRL-AP Imaging Services was done. During this presentation the CRL-AP Micrograph Collection was demonstrated on-line as DG SANCO approved to give access to this collection to all IAG members.

The access to the CRL-AP intranet is nevertheless only restricted to the sole Micrograph Collection for IAG members.

Immediate interest was noted during the days following the Dublin presentation (more than 10 access requests were received from IAG members by the CRL-AP during this short period). CRL-AP expects more access requests in the future. .
The slides presented at this meeting are annexed to this report (CRL-AP Imaging Services: Intranet, P. Veys, C. Belinchon Crespo).

The CRL-AP also participated at the Annual meeting of IAG research group on feed the 29th and 30th September 2009, one representative travelled to Hamburg, Germany. This Annual meeting allowed the CRL-AP to be better informed about methods to detect and identify feed components of interest as well as the danger of vegetal contaminants for the human health.

1.4 To actively participate in technical and scientific support of the European Commission in the context of incidents or crises linked to incorrect use of animal proteins. (3 p/m)

1.4.1 Provide technical and scientific support

1.4.2 Submission of the report on the technical and scientific support provided

Analysis of samples on the request of NRLs during 2009 (unofficial requests for supporting decision):

- ***Analysis of 12 samples of fishmeal from Portugal (July 2009)***
- ***Analysis of 3 samples of fishmeal from Portugal (September-October 2009)***
- ***Analysis of 5 samples of fishmeal from Estonia (September 2009)***

1.5 To keep at CRL the highest standard possible of technical skills, scientific

awareness and quality management under accreditation (ISO17025, later on maybe even ISO9001) on analytical methods for detection, quantification and identification of animal proteins in feed ingredients and in feedingstuffs. To maintain and extend the accreditation scope of the CRL lab. (12 p/m)

1.5.1 Maintain of the accreditation scope

1.5.2 Extend of the accreditation scope : accreditation of the CRA-W PCR method for the detection of cattle DNA

1.5.3 Preparation of the file for the organisation of interlaboratory studies

This task includes the maintenance of the competences of the CRL-AP team and the training of the new recruits.

During 2009, recruitment for a new lab technician in replacement of Ms Cecilia Teller was organised. The selected candidate, Mr Benoît Scaut, started at CRL-AP on the 2nd of June 2009. Training of this person has started on the first half year and continued in July 2009. The task of this new technician is to be the responsible of the NIR microscope and also to support the present microscopy team.

The extension of the accreditation scope to PCR tests for detection of bovine material in feed was audited by BELAC on the 13th of February 2009 and accepted in April 2009. Procedures to integrate this new analysis within the scope of the Department had to be implemented. Mr Denis Roulez was also trained to be able to perform the PCR analysis under accreditation and to substitute of Ms Julie Hulin if necessary.

CRL-AP subscribed to proficiency tests for the detection of bone in animal feed by microscopy organised by VLA. Sets of 4 samples were received in March, June, September and December 2009. The results obtained by classical microscopy were successful for three of these tests. The result of September 2009 could not be considered by the proficiency test organizer because due to an interpretation error, the results were transmitted after the deadline. The samples are analysed in parallel by PCR and the sets of samples were also successfully analysed: the PCR results were in accordance

with the expected results (presence of fish and/or terrestrial animals).

CRL-AP also subscribed to a proficiency test for the detection of protein in animal feed by PCR organised by the VLA (4 sets of 5 samples to be analysed per year). The sets of samples distributed in January, April, July and October 2009 were successfully analysed.

The CRL participated at the IAG ring test on the detection of animal proteins in feed in March 2009. A set of 4 samples was successfully determined by microscopic method.

1.6 On the request of DG SANCO, to perform analyses on samples with disputed results. (12 p/m)

1.6.1 Perform the requested analyses

1.6.2 Report on the analyses performed

Official counter analysis on request of NRLs in 2009:

- Analysis of 1 sample of fishmeal from Member State#1 (September 09)

- Analysis of 1 sample of fishmeal from Member State#1 (October 09)

- Analysis of 1 sample of fish feed from Member State#2 (June 09)

- Analysis of 3 samples of fish feed from Member State#3 (July – August 09)

- Analysis of 6 samples of fishmeal from Member State#4 (July 09)

1.7 Assist TAIEX with targeted assistance towards specific training/workshop for new member states or/and candidate countries

1.7.1 Administration and participation of candidate countries (Croatia, Turkey) to the annual NRLs network workshop.

1.7.2 Request of financial support for candidate countries (Croatia, Turkey) to TAIEX

No activities during the period.

2 Coordination of activities of NRL network *(13 p/m)*

2.1 Construction, development and maintenance of CRL website (internet/intranet) to disseminate and share information with NRLs and others stake holders. (3

p/m)

2.1.1 Information collection and validation

2.1.2 Development of the website (internet and intranet)

2.1.3 Development of the management tools of the website

2.1.4 Test of the information system and validation

During the 12 months period, 6008 pages were visited through 4123 visits by 607 unique visitors coming from 59 countries and 272 network locations throughout the world.

Regarding the intranet, 15220 hits were recorded during this period. 10 new NRL members joined the network and 3 members left. 13 logins were delivered to IAG members with restricted access to the micrograph database. All those statistics are increasing (2 times higher) against the same period in 2008.

Monthly updates were carried out. Maintenance tasks were provided to maintain security and confidentiality systems and to operate backup.

2.2 Prepare and send a four-months newsletter for NRLs. (2 p/m)

2.2.1 Preparation and sending of three newsletters in 2009

One newsletter was prepared and diffused among the NRLs during the first half-year: Newsletter 7 in May.
The seventh newsletter content consisted in the minutes of the 3rd CRL-AP annual workshop that was held in Gembloux on the 11th – 12th of March 2009. It included comments from all NRL delegations at this workshop made during the discussions on the presentations but also all decisions taken about the next planned initiatives and actions regarding the CRL-AP - NRL network duties. The newsletter also presented some meeting announcement regarding among others the detection of PAPs in feed.

A second newsletter (Newsletter n° 8) was updated on the Intranet in December 2009, in which we could find interesting information about CRL-AP progress and activities: from conferences/meetings that the CRL-AP attended to, to some information about advances on the CRL-AP Intranet, sea mammals researches, advances on the LOD front or some words about the CRL-AP video preparation for PAP detection.
Newsletters 7 and 8 are annexed to this report.

Based on the two last year experience, CRL-AP has limited the number of Newsletters to two per year instead of three. This decision would be taken considering that from this year on the CRL-AP – NRL network has reached its cruise speed of functioning: so if in the first years a lot of information had to be communicated to the NRL concerning the tools that the CRL-AP had developed for them, now all NRLs are informed on their existence and hence no more than two Newsletters a year are needed. Interestingly it must be emphasized that the importance and size of the content of the annual newsletter on the CRL-AP annual workshop has increased over the years. This reflects the very active participation of all NRL members assisting to the meetings and the exchange of information and viewpoints.

2.3 Organise and host the annual NRL meeting/workshop and produce minutes of the meeting. (3 p/m)

2.3.1 Organisation of the 3rd annual CRL-AP workshop

2.3.2 Preparation of the agenda

2.3.3 Invitation of the attendees

2.3.4 Realisation of the workshop

2.3.5 Minutes of the annual workshop

The 3rd CRL-AP annual workshop was held in Gembloux on the 11th – 12th of March 2009.

As usual all NRLs were asked to participate to the workshop.

The agenda included the following items of presentation and discussions: results from the recent CRL-AP Proficiency Test 2008, state of the art on the progress of quantification method by light microscopy (based on the experimental results obtained from the collaboration asked in 2008-2009 period according to the conclusion of the 2nd CRL-AP annual workshop), presentation of an alternative approach for quantification by the Danish NRL.

Other topics presented dealt with the method for the determination of the LOD for animal particles in feed proposed by the CRL-AP and presentation of the results on the LOD. A third part of the workshop focused on alternative quantification methods such as could be provided by NIR-microscopy and PCR, respective limitations were reviewed. Presentation of the conclusion of the report on the "ReVeal for Ruminant" immunoassay kit was made. Recent legal changes were presented too and a possible network position around the LOD and the way to express results based on the observation of insignificant amount of animal particles was investigated. Minutes of the workshop were recorded in the 7th Newsletter (see annex).

2.4 Supply information, scientific advices and protocols to NRLs, testing laboratories, detection, quantification and identification of animal proteins in feed ingredients and feedingstuffs. (3 p/m)

On the request of the NRLs, supply of information and scientific advice. During 2009 period the CRL-AP has supplied several scientific advices and transfer of protocols (Staining protocols, Cystine reagent or Fheling reagent) on request of NRLs.

This reflects the active participation and the more and more trust and familiarity of NRLs members in the CRL-AP about the exchange of information and points of view.

Sharing and information exchange really becomes prominent.

As decided on the 3rd CRL-AP workshop, the lectures from the CRL-AP training sessions, were posted in PDF format on the Intranet on December. NRLs have access to "Muscles, bones and cartilage", "Hairs and feathers" and "Blood and other animal products" lectures. NRLs were reminded that these lectures are strictly intended for their training, diffusion of the information is forbidden.

2.5 Participate to annual CRL Directors co-ordination meeting.

2.5.1 Participation to the CRL directors co-ordination meeting

2.6 Prepare the six months and annual reports of activities according to the report guidelines transmitted by DG SANCO. (2 p/m)

2.6.1 Prepare and submit the 6-months report (January 2009 – June 2009) and annual report (January 2009 – December 2009)

Redaction of the first half year report and annual activity report done.

3 Interlaboratory studies and quality assurance *(21 p/m)*

3.1 Coordinate the preparation, reception, storage, maintenance and distribution to national reference laboratories (NRL) of samples containing animal proteins derived from different species and in particular from fish, poultry, pigs and ruminants to be used as reference materials or to carry out comparative testing. (6 p/m)

3.1.1 Definition of the needs

3.1.2 Set up of a planning of the samples to produce in the 2009-2010 period

3.1.3 Collection of the raw materials to use in the preparation of the samples

3.1.4 Control of the raw materials

3.1.5 Production of the samples

3.1.6 Test of the homogeneity of the samples produced

3.1.7 Report on the produced samples

3.1.8 Distribution of the samples

During the 2009 period, matrices received from the Italian NRL and BEMEFA, were tested for ensuring the absence of any animal particles or traces.

Repetitions were made for analyses by two different methods and teams: classical microscopy and PCR. More than 135 sedimentations were realized, and analyzed. In total, 47 samples were tested. Such a big work allows to the CRL-AP to have different tested feed matrices to organise interlaboratory studies.

3.2 Organize interlaboratory study for the determination of PAPs in feed using classical microscopy. (12 p/m)

3.2.1 Redaction of the report of the CRL-AP interlaboratory study of Autumn 2008

3.2.2 Definition (with the collaboration of the DG SANCO) of the objectives of the ring trial to perform at the end of 2009.

3.2.3 Preparation of the interlaboratory study.

3.2.4 Invitation of the NRLs to participate.

3.2.5 Preparation and homogeneity test of the samples (cf. task 3.1)

3.2.6 Sending of the samples (Link with task 3.1.)

3.2.7 Collection of the data.

Data from the CRL-AP Proficiency Test 2008 were analyzed in January 2009. A working document version of the report was distributed among the NRL network before the 3rd CRL-AP annual workshop. After discussions during this workshop, a final version of the report was prepared and diffused end of March to the NRL network.

In line with the successful collaboration request from last year, this year once more the NRL network collaboration was asked for fulfilling and accelerating

research issues raised during the 3rd CRL-AP annual workshop.

Thus a request for collaboration was posted in May this year. This request dealt with the production of sediments by NRLs for further analysis by NIRM in order to investigate possibilities of an alternative quantification method by NIRM.

It must be emphasized that this way of collaboration is very profitable for CRL-AP activities; gains from these requests could not be achieved by classical interlaboratory studies as they concern first tests on methods in development.

Furthermore the way those requests are formulated does not require much administrative work as it is for collaborative studies: it goes very quickly and efficiently. Furthermore NRLs appreciate this way of working as they are really considered as active partners. The final report of the CRL-AP proficiency test is annexed to this annual report.

The CRL-AP Proficiency Test 2009 took place in November-December 2009, this year 9 blind samples were sent to each NRL for testing their proficiency. After the agreement of DG SANCO and like in the previous PT 2008, some foreign countries participated this year too. That was the case of Argentina, Canada, China, Croatia, Japan, South Africa and the USA. The interest of those participations for the test can be considered once again as a further evidence of the EU leadership in this area. Data from this proficiency test will be examined and compiled in a report that will be discussed together with the NRLs at the 4th CRL-AP annual workshop in Turin in April 2010.

3.3 Audit NRLs, coordinate training on methods of analysis and assist staff from NRLs if comparative testing reveals limited experience. Up to 3 European missions/year are foreseen in support to DG SANCO and/or CRL activities (1 p/m)

3.3.1 On the basis of the results of the interlaboratory studies, organisation and planification of the audit

3.3.2 Report of the audits

Based on the observed underperformance of the CRL-AP Proficiency test 2006 and 2008, a visit of a Member State Lab (NRL), according to its request, was organized simultaneously during a training session on-site in April 2009 in order to investigate the origin of the problems observed and to propose solutions for the improvement of the proficiency of the NRL.
The intention of the visit was also an evaluation of the microscopic method implementation.
The mission allowed detecting the sources of errors of analysis which led to the underperformance, so the issue could be considered as closed.
A detailed report of this mission was delivered to DG SANCO and the Member State authorities. The report also included recommendations that must be followed by the visited NRL.

A second visit and on-site training for the same reasons was planned for another Member State Lab (NRL) during the second half-year of 2009. A training course was organised from 8th to 11th September 2009, we taught the adequate way of working according to the legal requirements and showed the participants what the CRL-AP consider as best routine techniques. It was also an opportunity for the hosting institute to get some suggestions for future equipment and laboratory organisational improvement. A report was communicated to the NRL as well as to the DG SANCO.

CRL-AP also informed officially the DG SANCO about the situation of another Member State Lab (NRL) which is repeatedly not entirely satisfying.

3.4 To help to develop, extend and keep in the NRLs the highest standard of technical skill and quality management under accreditation on analytical methods for detection, quantification and identification of animal proteins in feed ingredients and in feedingstuffs. (2 p/m)

3.4.1 Definition of the needs of the NRLs

3.4.2 Preparation of the programme, in consultation with the European Commission, including actions to be undertaken

3.4.3 Provide the requested help to the NRLs

The CRL-AP made a <u>video CD for sample preparation for PAP detection</u>. This CD aims to illustrate the implementation of the Annex VI of Regulation 152/2009 and all required information (including tips and tricks) for the preparation of permanent slides with the resin Norland Optical Adhesive 65.

The video sequences show the entire preparation processes of animal meals or feed for the microscopic detection of PAPs. It is the most detailed and illustrated information source possibly available. The most important critical points of each processes are recorded and should be useful for trying to achieve a standardized way of working within the NRL-AP network. Copy of the CD is annexed to the hard copy of the annual report send to the European Commission.

This CD has been delivered in 2009 to a first selection of 12 NRLs (first array of four) by mid December 2009 with the further intention to perform a study of quantification on permanent slides. This study started in 2009 and will continue in 2010.

The intention of this exercise is to assess the impact of the slide preparation and the influence of the observer on the final estimation of animal constituents in feedingstuffs. Therefore, 2 quantifications have to be performed on the permanent slides, prepared in each NRL with the Norland Optical Adhesive 65 resin.
Later on, the results and the permanent slides shall be sent to the CRL-AP, where all permanent slides will be counted again by the CRL-AP team.
The CRL-AP will make the same exercise as the NRLs on the NRLs' permanent slides. Comparison of the results will be performed with emphasis on the value for the repeatability and reproducibility.

4 Development of analytical methods and tools (40 p/m)

4.1 Contribute to the development of new methods of analysis and improvement of existing methods of analysis. (6 p/m)

4.1.1 Establishment/maintain of contact with the laboratory in charge of the development in order to be frequently informed about the progress of their development

4.1.2 Definition of the potential support of the CRL to these initiatives

4.1.3 Establishment of the needs in the development of methods

4.1.4 Improvement of the Neogen dipstick method of ruminant PAP detection for the screening of PAPs by immunological methods

A Zeiss microscope representative was invited from Germany to present the Zeiss Palm Microscope. The CRL-AP is interested in its performance and great scientific possibilities.

This latest technology allows the isolation of defined particles or well defined microscopic structures that let us to handle them separately and its consequently analysis.

Austrian NRL is in process to get such high-tech equipment.

CRA-W has bought a device for DGGE (denatured gradient gel electrophoresis) in 2009.

There is some interest in this equipment for the CRL-AP because it might be of possible help to set up a traceability technique to find out for instance the origin of a fish meal. The idea is based on an analogy of what was evidenced by a French group of Montpellier on fresh fish with that same technique (lecture at a TRACE meeting in Munich).

The geographical origin of the fishmeal might be traced back thanks to the DNA fingerprint of microbial consortia in the fishmeal.

A meeting with Rob Langley, Representative of the European Headquaters of Neogen Corporation, took place in Gembloux the 1st October 2009. Mr Langley wanted to be informed of the results of the study on the Neogen

dipstick assay conducted in 2008.

4.2 Contribute to the development of complementary analytical methods necessary to assure the correct implementation of official methods and explorative or alternative methods. (2 p/m)

4.2.1 Specification of the needs

4.2.2 Report of the results of the tests

Studies on the <u>LOD determination,</u> started with the Chinese colleagues from CAU end of last year, were continued. Testing the LOD of fishmeal showed that the actual LOD was far below the commonly accepted value of 0.1% as referred in EC/152/2009 text. The estimation of the LOD values obtained was about 0.005%-0.002%. A full set of investigations based on the previous protocol used for fish was realised but on feed adulterated with terrestrial MBM. The preparation of the samples at different levels of adulteration required utmost attention and represented a large part of the workload.

Analyses were made on slides prepared from Alizarin Red stained sediments and observations realised by two separated operators from the CRL-AP microscopy team. Results showed LOD values for terrestrial MBM identical to the LOD figures for fish. A compilation of the results was presented to the NRLs during the CRL-AP annual workshop and further investigations were undertaken based on the requests.
That is why the impact of the sediment percentages (or matrix effect) as well as the influence of the "f" factor of the animal meals are still under examination. This complementary work will allow to have enough information for preparing a scientific publication to which an eventually revised version of the EC/126/2003 protocol (included in the 152/2009 Commission Regulation that is in force since 26 August 2009) could refer for the questions related to LOD.

Acquisition of <u>sea mammal</u> material is still an ongoing task. Initial expectations were somehow disappointing. CRL-AP looked for other possible sources of sea mammals' materials: numerous contacts were taken with sea

research institutes and official request letters were sent. Research on the characterisation of bone fragments from sea mammals vs. other terrestrial animals required the acquisition of a 100x objective. This is in order to obtain high quality pictures for subsequent image analysis.

Concerning the quantification method development for PAPs in feed, no major work was realised during the period: previous investigations highlighted the impact of slide preparation on the repeatability and the precision. The reproducibility nevertheless still needed to be improved. Reflections on the influence of human skills were discussed during the 3rd CRL-AP annual workshop: a final test needed to be planned on a collaborative way. A potential solution to this problem could be the study "Quantification on permanent slides 2009" started on December 2009 by CRL-AP and which will continue early 2010. Results will be communicated at the 4th CRL-AP Workshop in Turin.

For the organisation of this experiment, utilization of Norland NOA 65 for permanent slide preparation and curing by UV source is required by all participants, nevertheless some NRLs didn´t have this UV lamp nor the NOA 65 at the time of writing this report, the CRL-AP will send a set of UV lamp and NOA 65 that will be transferred from one country to another by post delivery.

Thanks to NOA 65, permanent slides from each NRL already analysed can be sent later to the CRL-AP in Gembloux, where the CRL-AP team will do the analysis again on the NRLs' slides.

About the determination of protein size by HPLC, the method of reference as proposed by Dr Piva based on exclusion chromatography was tested to verify the absence of any hydrolization residues with a molecular weight which would be superior of 10 kDa. The implementation of the described method revealed to cause problems mainly the solubilisation of the mobile phase. Several attempts to solve this problem were investigated but did not succeed.

To fix this problem we had an appointment with prof G. Lognay, Head of the analytical chemistry department of the University of Gembloux. The problem could be due to the EDTA. The method does not specify the type of EDTA so we tried the acid form. Prof Lognay suggested using the disodic salt of EDTA (Na2EDTA). Indeed, the EDTA in its acid form is not very water soluble (0.500 g·L-1).

Consequently, the concentration recommended in the method (0.300 g·L-1) is very close to the limit of solubility. The influence of the other components present in the phase can also influence. Moreover, the pH of the buffer (pH = 7) does not support the dissolution of the EDTA: a slightly basic medium would have been more adapted. The hypothesis of using a disodic salt to improve solubilization of the EDTA in the buffer has to be confirmed.

The determination of the fatty acids profiles of feed fats by gas chromatography is one of the techniques that can be recommended to discriminate fats according to their "species" origin.

Some more feed samples have to be analysed. JRC-IRMM has developed a gas chromatography method to determine specifically Glycerotriheptanoate (GTH: marker for animal by-products belonging to the category 1 and 2 in processed animal by-products.

The Analytical Chemistry laboratory of CRA-W has implemented the method and participates to the Collaborative study organized by JRC in that framework.

Some preliminary trials were conducted to determine the lactose content on the basis of an enzymatic method leading to a difference in pH (Luzzana et al., 2003; ISO 2006). If this method can be easily applied for dairy products, due to interferences of other compounds, it appeared difficult to use it for mixed feedstuffs.

An HPLC method similar to the one proposed by the International Dairy Federation (Draft International Standard IDF-ISO, 2006a) will be tested and

adapted if necessary.

4.3 Coordination of evaluation studies on alternative methods. As soon as they become available, methods specifically detecting ruminant, pig or poultry proteins should be evaluated. (12 p/m)

4.3.1 Organization of an interlaboratory study for PCR methods

4.3.2 Organization of a validation study for PCR methods

4.3.3 Transfer of validated PCR methods to the CRL and to the NRLs network

The few PCR methods developed by CRA-W, TNO and VLA have already proved to be able to detect 0.1% of PAP in feed. However, it still remains to be proved that the methods can be successfully transferred to other laboratories. With the agreement of the CRL-AP working group (meeting of November 2008), the CRL-AP organised an inter-laboratory study focussing on the validation of the PCR method transferability protocol developed by CRA-W. The statistical aspects of this study were done in collaboration with the Biometry Unit of CRA-W (Dr. Robert Oger and Dr. Viviane Planchon).

A last difficulty which had not been solved within the PCR working group was to design a test that would really challenge the transfer protocol so as to prove it was really fit for purpose.

This was designed in a completely innovative way at CRA-W as up to now to the best of our knowledge nothing similar had been done by others. The protocol has been discussed together with JRC-IRMM on 17 of February 2009. Its practical applicability was tested with success at CRA-W by in-house validation.

Before using the protocol in a broad inter-laboratory trial for the validation of a test kit within the SAFEED-PAP project, the design of the protocol itself was submitted to an interlaboratory study.

A pre-validation was therefore performed with the help of CCL and RIKILT.

Although these external tests first proved to be slightly below our expectations

they were nevertheless conclusive to go further on with the validation. Nineteen laboratories (17 from the European Union, 1 from Japan and 1 from Australia) agreed to participate in the study and the protocol was tested on twenty-two thermocyclers from 4 companies (Roche Diagnostics, Applied Biosystems, BioRad and Stratagene). The material for the study was sent during the last week of May 2009. The results were collected mostly in June 2009 (and the rest in July 2009).

After the statistical analysis of the results, it can be stated that the validation is a success and the protocol is fit for purpose. A first summary of the results was presented at the Feed Safety Conference (Wageningen, 6-7 October), during the Rapid Methods Europe meeting (Noordwijkerhout, 25-27 January 2010) and at the statistical congress Agrostat (Benevento, 23-26 February 2010).
A working document with a brief overview of results was sent to the participants in November.

The final report of the study is annexed to the present activity report. Its diffusion to the NRLs is in progress and a communication on that issue will be presented during the next CRL-AP workshop
The transfer protocol will be used in the SAFEED-PAP inter-laboratory trial for testing the kit for the detection of cattle material in feed that is developed together with Diagenode. This last study conducted by the JRC-IRMM was launched in November.
The results from the participants are expected in January '10. It must be stressed that the concept of the transfer protocol is applicable to any other PCR assay. A copy of the report is annexed to this annual report (Interlaboratory study for the validation of a transfer protocol for real-time PCR methods and the determination of the cut-off of a PCR platform – O. Fumière, V. Planchon, A. Marien, R. Oger, G. Berben).

A meeting was held on 13 of May 2009 at DG SANCO gathering DG SANCO (K. Van Dijck, Z. Hajek), TNO (G. Van Duijn) and CRL-AP (V. Baeten, G.

Berben) where TNO explained to wish to make its method available to all laboratories but after a validation trial that should then be organized in collaboration with the CRL-AP.

An estimate of the costs of such an inter-laboratory study with the tasks of CRL-AP and TNO was transmitted to DG SANCO

4.4 Performing CRL available methods or adapting them on outbreak material to make them available for the NRLs network. (6 p/m)

4.4.1 Preparation of the framework for the transmission of methods to the NRL network. On the basis of the results of the validation of the PCR and immunological dipstick methods (see tasks 1.5.3.2. and 1.5.3.3.) preparation of CRL protocol to apply these methods in the NRLs labs.

A fully detailed protocol for the utilisation of Norland Optical Adhesive 65 as permanent mounting medium for microscopic slides was delivered to the NRL network in April 2009.

The use of this resin allows better preserving the lacunae of bone particles, to facilitate their observation and to keep slides permanent without any alteration by time. Undoubtedly permanent slide preparation by NRL opens new perspectives in case of discussions or disputes on samples analyses: slides can be observed back and shared by other analysts.

Even more, slides could be sent for confirmation to CRL-AP instead of sending pictures which are only representative of a single focus plan and often of poor quality. Therefore the use of this resin has also been recommended during the CRL-AP training sessions of last year and this first half year.

4.5 Construction and extension of the samples bank with a special focus on the animal meals of one single species origin (e.g. fish, poultry, pig, bovine and sheep) from different processes. Test, packaging and storage of the new samples as well as production of microscopic image representative of the particles making up the samples collected and selected to be included in the CRL-AP

samples bank. (14 p/m)

4.5.1 Establishment of the specification for the CRL-AP samples bank

4.5.2 List of the priority needs regarding the materials to include in the samples bank

4.5.3 Production and validation of informatics tools for the appropriate management of the samples

4.5.4 Collection/production of samples of animal meals of one single species origin (e.g. fish, poultry, pig, bovine, sheep)

4.5.5 Collection/production of samples of compound feeds free of MBM

4.5.6 Test of the samples collected

4.5.7 Preparation of the samples for the storing

4.5.8 Storing of the samples

4.5.9 Maintenance of the samples bank

The CRL-AP on-line micrograph collection continues being extended with state of the art pictures. During this year 2009 good work has been realised again and the micrograph collection was extended by 2 additional releases, beginning of May and mid September 2009.
The first release in May 2009 enriched the collection with new micrographs of muscles, epidermal trichomes and fishbone pictures.

The second one, in September 2009, the collection was enriched with pictures of horns, bovine hooves, teeth fragments, nails, foraminifers, otoliths and more. So by the end of the year 2009, the total number of pictures reached over 2030 wherefrom over 630 selected and published on the CRL-AP Intranet.

Access to the CRL-AP micrograph collection has been granted to the IAG feed microscopy section members in May of this year.

As established during the 3rd CRL-AP workshop in agreement between NRLs and CRL-AP, the opportunity to share excellent micrographs from NRLs with

the network was given.

The CRL-AP accepted to post the very good quality and scientific interesting micrographs coming from NRLs in the CRL-AP on-line micrograph collection with free access to the NRL and IAG members. A "Protocol for images submission to the CRL-AP collection" was posted on the Intranet beginning of December 2009, it takes into account parameters for the admission of the NRL pictures, as well as the high pertinence of the contained information, the perfect neatly, the µm scale bar correctly adapted to the final magnification, the size, the format to be used or the data to delivery about the picture.
After reviewing the pictures, the CRL-AP feels free for the final selection of pictures that will be included in the collection. The CRL-AP is the sole responsible for deciding to publish the sent micrographs or not.

A major improvement of the CRL-AP micrograph collection in 2009 concerns the addition of new modules for the identification of hairs from the most commonly occurring European rodent species, which can be a source of natural cross-contamination of feed. Studies on this topic were undertaken by CRL-AP microscopy team in 2008 and finalised begin of 2009.

The result is the delivery of 4 modules: a general information module on how to analyse hairs (with scientific and technical data), a new synthetic key text to the exact identification of those rodent hairs, a dynamic decision tree for easy and rapid identification of rodent species to which a hair belongs and finally a module with the most representative micrographs of rodent hairs.

The modules were put on the CRL-AP Intranet server by mid May 2009 in two steps: a version for MS Office 2007 and one for MS Office 2003 as compatibilities of the hyperlink navigation were not the same. These modules are also accessible to IAG members.

During the period, the centralized and integrated management tool called Sample Management Suite (SMS) is still further developed for the collection of

samples, sediments, slides and micrographs.

The developments ongoing in 2009 are related to enhancement of the search and query interface as well as extraction and export functions from the database into Excel files for daily use. The tool is now completely operational.

5 Workshops/trainings *(3 p/m)*

5.1 Provide specific workshop for the benefit of NRLs for the correct application of the 126/2003/EC directive to detect animal proteins in feed (Classical microscopy) and any new directive linked to the detection, identification and quantification of animal proteins in feed. (2 p/m)

5.1.1 Organisation of a workshop for the identification PAP particles (raw and sediment fraction) using the 126/2003/EC method

5.1.2 Invitation of the attendees

5.1.3 Preparation and submission of the minutes of the workshop

On request of a Member State Lab (NRL) a dedicated on-site training on microscopy was realised end April 2009 after the agreement of DG SANCO. This training was organised for 2 persons from the NRL.
The results of the training revealed to be utmost profitable for the participants. Certificates of participation shall be sent to each participant.

Continuation of <u>training sessions for all NRLs</u> as started last year was completely ended this first half year. In February 2009, two training sessions of three days on microscopy were organised at the CRL-AP facilities in Gembloux for NRLs. Participating NRL staff came from Belgium, Italy, the Netherlands, Cyprus, Greece and United Kingdom. The total number of persons trained was of 7.
At writing date the <u>26 NRLs thus had followed at least one training session</u> provided by the CRL-AP on microscopy.

As formulated by the NRL network, it was decided to continue providing

training sessions to the network but not on a systematic mandatory base (except in case of poor performance) but on proposals and in order to assist NRLs in case of personal turnover.

As it has been already noted in 2008, once more several NRLs asked if in the future the CRL-AP could deliver sets of training slides for maintenance of their competences and home training of their personal. The project is under reflection.

5.2 Provide specific workshop for the benefit of NRLs for the detection, identification and/or quantification of PAPs in feed according new validated method.

No activities forecasted in 2009

5.3 Provide specific workshop of experts from candidate Member States for the correct application of the 126/2003/EC directive to detect animal proteins in feed (Classical microscopy) and any new directive linked to the detection, identification and quantification of animal proteins in feed. (1 p/m)

No activities forecasted in 2009 but requests for training are still numerous and sometimes originate from third countries: e.g. China, Serbia, South Africa and Peru.

5.4 Provide training through dissemination tools like CD's or DVD's. Development of analytical support and libraries for the training and the maintenance of the skill of laboratories performing classical microscopy or other validated method.

In order to illustrate the implementation of the Annex VI of Regulation EC/152/2009, the CRL-AP decided to build up a whole video CD on the implementation's best practice.
This video called "Sample preparation for PAP detection" shows in 3 voiceless sequences from start to end the entire process of preparing feedingstuff

sample; from the sedimentation of the sample to the correct permanent slide preparation.

The reason of the voiceless is that the CRL-AP team wanted to avoid any foreign English accent on the CD. In order to describe some additional technical tips which could not be recorded by film sequence, an additional document with all the explanations and tips was prepared and included in the CD under PDF format. This file is complementary and necessary to the well understanding of the video sequences.

This CD was delivered to 12 countries in December 2009, deliveries will continue early 2010. All countries will receive one copy of this CD.

This CD is a useful training video that can be used also by NRLs for their network training. It is a really good method to standardize the way of working among our network.