COMMUNITY REFERENCE LABORATORY

"Detection of animal proteins in feedingstuffs"

CRL-AP

Walloon Agricultural Research Centre – CRA-W (Belgium)

Report of year 2010 activities

PUBLIC VERSION

1 Scientific advice and support to the European Commission (41 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)

A second report on the evaluation of the MELISA-TEK Ruminant kit for the detection of ruminant proteins in processed animal proteins was finalised in February. It was focussed on the variability of the blank control tested through a limited inter-laboratory study involving ELISA experienced labs from different institutes (CCL, CRA-W, JRC-IRMM, Rendac and RIKILT). It concluded that the decision criterion as set by CCL based on blank-subtracted figures is not applicable as such.

During the 4th CRL-AP workshop, modification of the text for the improvement of Annex VI of EC/152/2009 was held simultaneously to discussion with the NRL network. Major steps were realised on standardisation of the equipment and implementation of the microscopic method. A last version of the revised Annex VI was presented and submitted for vote by mid September. The revised protocol was validated by an interlaboratory study in Autumn this year. In June 2010 the DG - Sanco contacted the CRL-AP for a request for technical assistance regarding the testing methods related to insoluble impurities in rendered animal fat ('tallow'), an harmonisation of analytical methods for determining these insoluble impurities is required. The CRL-AP has already started with the request, but so far only the different protocols and the different laboratory

equipment was obtained. Samples of the materials to be analysed were ordered but they are still being awaited by the CRL-AP.

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. (1 p/m)

In January 2010, a lecture was given at the international meeting "Rapid Methods Europe 2010" (Noordwijkerhout, The Netherlands) about the transfer of a cut-off value in PCR. The ideas for this transfer were developed within the SAFEED-PAP FP6 European project but the validity of the approach was tested by the organisation of an inter-laboratory study by the CRL-AP.

Participation in the first CRL scientific forum organised by JRC-IRMM (Geel, 9-10 February 2010). Presentation of the CRL-AP activities in the framework of interlaboratory studies.

The 20th of May, CRL-AP participated in the Standing Committee on the Food Chain and Animal Health (SCoFCAH). Two presentations on the last developments of the classical microscopy and of the PCR methods were presented.

In September, a presentation on the status of immunological and PCR methods for the detection of PAPs was presented during the German NRL annual workshop to which the CRL-AP was invited to participate.

The results obtained by the CRL-AP during the validation of the protocol to

set a cut-off for PCR were shortly highlighted during a lecture held at Ispra during the 14th ENGL (European Network of GMO Laboratories) plenary session (9-10 November).

In November, the CRL-AP participated in the International Conference "The Future of Reference Materials-Science and Innovation" organised by JRC-IRMM (Geel, 23-25 November 2010). A poster on the need of plasmids with certified copy numbers for the calibration of PCR platforms was presented. It was also the opportunity to get in touch with JRC-IRMM responsibles for a collaboration on this topic. The access to the digital PCR device present at JRC-IRMM will be of great help.

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)

Among the possible activities in 2010 there might be a CEN working group (within CEN TC327) defining guidelines for PCR in detection of PAP in feed.

On request of EFSA and in view for the BIOHAZ panel to review its opinion about the quantitative risk assessment of the residual BSE risk in mammalian derived meat and bone meal, the CRL-AP provided an update on the performance level of different analytical methods (classical microscopy, NIRM, immuno-assays and PCR) with respect to their capacities to discriminate animal species and at which limits of detection. The CRL-AP was contacted again by EFSA in autumn 2010, just to check if some statements made by the BIOHAZ panel to be integrated in their opinion on the TSE roadmap II were correct. CRL-AP agreed with what was proposed.

The CRL-AP participated in the IAG annual meeting held in Tervuren, Belgium, in June 2010. Three lectures were presented by the CRL-AP members. In the first lecture the micrograph collection posted in the Intranet was presented to the IAG members, as they have (restricted) access to this collection. The latest updates, the software used for data management and recent advances in sea mammal´s research were presented. The second lecture summarized the SAFEED-PAP outputs with specific focus to the methods/tools to transfer to CRL-AP. The last lecture presented the results of the CRL-AP Proficiency Test 2009 as well as the recent research results for the determination of the limit of detection (LOD) for the presence of processed animal proteins in a feed matrix.

In September 27th – 29th , a representative of the CRL-AP assisted to the IAG meeting held in Hamburg, a lecture presenting the TSE Roadmap II was given.

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)

No activities during 2010.

1.5 To keep at CRL the highest standard possible of technical skill, scientific awareness and quality management under accreditation (ISO17025) 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. (16 p/m)

1.5.1 To maintain scientific awareness in general about techniques that might be helpful in relation to topics of interest of the CRL-AP

1.5.2 To maintain the accreditation scope

1.5.3 Maintenance of the competences of the CRL-AP team and formation of the new recruits

1.5.4 Extend the accreditation scope of the CRL-AP by including additional animal targets DNA in the scope for PCR analyses

1.5.5 Preparation of the dossier for the accreditation of the organisation of interlaboratory studies

In May, the CRL-AP was submitted to an audit by BELAC. No non-conformity was identified by the auditor.

CRL-AP also subscribed to a proficiency test scheme for the detection of animal proteins in animal feed by PCR organised by the VLA, Luddington, UK : 4 sets of 5 samples to be analysed per year. The sets of samples distributed in January, April, July and October 2010 were successfully analysed (no false negative). A simultaneous detection of pig and ovine material was recorded for one sample in April and in July whereas the organizers of the PT expected only presence of ovine material. The reason of this discrepancy is not clearly established. It was firstly interpreted by the organizers of the PT as a possible cross-contamination internal to the CRL-AP lab. However, another sample distributed in July 2009 and containing the same source of animal protein gave a similar result at CRL-AP. In the light of this information, it seemed more plausible for the CRL-AP to consider that the ovine material used by VLA was slightly contaminated by pig material at a level the organizer is unable to detect. In November, the organizers admitted the possibility of a low level porcine contamination in the rendering plant where the ovine sample was originally produced. More of the material was sent to the CRL-AP for additional analysis in order to confirm the hypothesis. The results are pending.

CRL-AP subscribed also to proficiency tests for the detection of animal proteins in animal feed by classical microscopy organised by the VLA. Sets of 4 samples were received in March, June, September and December 2010. The

results obtained by classical microscopy were successfully analysed for these tests with exception of one of the 16 total samples. CRL-AP notified the presence of scarce fish particles although the sample was not supposed to contain any fish. Similar notifications for fish presence by others participants of the ring test lead the organisers to inform the participants on their decision to not use the matrix again in next ring tests.

The samples were also analysed in parallel by PCR, all sets of samples were all successfully analysed: the PCR results were in accordance with the expected results (presence of fish and/or terrestrial bones) on the exception of the sample mentioned above. For this sample, the PCR signal was at the limit of the cut-off values for fish so the decision for the fish presence was negative by PCR but in classical microscopy fish presence was confirmed with few fish particles presence on each of the three analyses repetitions made.

One person of the CRL-AP team participated in the Digital Image Processing Workshop, held in London the 4/06/2010. The main focus was to participate in a hands-on, computer-based workshop in which digital image analysis was learnt by example. It allowed learning on the different possibilities that can be offered by the imaging analysis, as well as a further knowledge of the software used by the CRL-AP "Axiovision 4.8". It was also a good opportunity to discover the Royal Microscopy Society which opens a new horizon as support in microscopy imaging.

During the first half 2010 the replacement of the ICT position of Daniele Bonsignori by Alexandre Quoitot has taken place. The selected candidate started at the CRL-AP on the 9th of April 2010, he has been trained accordingly.

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

In May and June, the CRL-AP analyzed samples of meat and bone meal reputed to be of swine origin on the request of Member State #1. Due to the composition of the samples, a microscopic analysis was useless. So PCR and immunological assays were performed to check the presence of ruminant material. Ruminant material was detected by both methods in all the samples submitted to the analysis.

In June a request for analysis was formulated by NRL of Member State #2 . The CRL-AP received the sample in August, no official results were sent.

From June to August several NRLs (Member States #3, #4, #5 and #6) contacted the CRL-AP on the issue of the detection of plasmatic proteins in artificial milk. So far, there is no official method for detecting plasma proteins, the CRL-AP nevertheless tested the tetramethylbenzidine and H_2O_2 reaction on samples. The interpretation of such staining reaction does not deliver the ultimate evidence of the presence of blood products in a feed matrix, especially when the feed is a milk powder. It is certainly a research area that will need future development. Meanwhile, the CRL-AP informed the NRLs as accurate as possible with all the requested prudence on the interpretation of the results.

During 2010, several request of official counter analysis were submitted by NRLs:

- ***5 samples from Member State #1, animal meals, May 2010***
- ***2 Samples from Member State #7, poultry feed, July 2010***
- ***1 Sample from Member State #7, fishmeal, September 2010***
- ***1 Sample from Member State #8, fishmeal, December 2010***

1.7 Organisation in collaboration with TAIEX of a training/workshop for the candidate and potential countries (Turkey, Croatia, Iceland, FYR Macedonia, Albania, Bosnia and Herzegovina, Montenegro, Serbia) (2 p/m)

1.7.1 Request of financial support to TAIEX

1.7.2 Preparation and organisation of the workshop

1.7.3 Redaction of the proceeding

No activities during 2010.

2 Coordination of activities of NRL network (17 p/m)

2.1 Maintenance and update of CRL website (internet/intranet) to disseminate and share information with NRLs and others stake holders. (6 p/m)

2.1.1 Information collection and validation

2.1.2 Maintenance of the website

2.1.3 Development of additional management tools of the website

2.1.4 *Test of the information system and validation*

During the 12 months period, 5540 pages were visited through 3659 visits by 751 unique visitors coming from 57 countries and 277 network locations throughout the world.

Regarding the intranet, 13423 hits were recorded during this period. 3 new NRL members joined the network and 6 members left. 4 logins were delivered to IAG members with restricted access to the micrograph database.

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

During the second half year efforts were also spent to update totally the public website. Not only the layout and logo were renewed but also the webpage content has been updated according to the work provided for the 5 first years and the CRL-AP missions. The website will be available for the public by early 2011.

2.2 Prepare and send a six-months newsletter for NRLs. (1 p/m)

As has been already reported in the previous "CRL-AP activity report 2009", based on the lasts years experience, the CRL-AP has limited the number of newsletters to two per year instead of three.

A fisrt newsletter was prepared and diffused among the NRLs during the first half year 2010: Newsletter 9 in June 2010. This 9th newsletter content consists in the minutes of the 4th CRL-AP annual workshop which was held

in Turin, Italy by end of April 2010. We can find in this newsletter a workshop summary, including some of the decisions taken around the revision work on 152/2009 protocol which was discussed and modified simultaneously to the agreements taken in Turin. A second Newsletter was published during the second half of 2010, in this Newsletter 10 some important subjects were pointed out as the announcement of the 5th CRL-AP workshop that will be held in Vienna in April 2011, some words about the CRL-AP Interlaboratory Study 2010, the updates of the micrograph collection, advances on the LOD front, the presentation of the new EURL-AP name (European Union Reference Laboratory for Animal Proteins) and both new logos: CRA-W and EURL-AP.

(Newsletter 9 and 10 are attached to this report).

2.3 Organisation of the annual CRL-AP meeting/workshop (3 p/m)

2.3.1 Organisation of the 4rd 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 4th CRL-AP annual workshop was held in Turin, Italy, on 28th and 29th of April 2010.

This time the CRL-AP team didn´t have to deal with the logistic organisation (meeting room renting, catering, hotel, etc), it was taken in charge by the Italian NRL.

The first day, the agenda included the following items of presentation and discussions: Italian NRL network organisation and activities, the PCR detection of PAPs, CRL-AP interlaboratory study and Safeed Pap study, the transfer of PCR protocol to the NRL network, presentation of the PT-PCR detection of fish meals in feedstuffs. The CRL-AP services: activities in 2009 and imaging research; the first day continued with a presentation of the

current status of the Epidemiology of BSE, the results from the 2009 quantification of PAPs exercise, the Safeed-Pap Interlaboratory study on NIRM detection of PAPs, and a online demonstration of the new version release of ARIES 2. The day concluded with a presentation of the use of the Zeiss Microdissector laser Palm.

The second day of the workshop, the agenda was completed with: Classical microscopy and image analysis combination in sea mammals bones characterization, results of the CRL-AP PT 2009 and the results of the 2009 experiments on the determination of the limit of detection of PAPs in feed were presented. The day and the workshop ended up with the discussion on the revision work on the Annex VI of EC/152/2009 protocol.

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

Minutes of the workshop were recorded in the 9th CRL-AP newsletter (attached to this report).

2.4 Supply information, scientific advices and protocols to NRLs, testing laboratories, detection, quantification and identification of animal proteins in feed ingredients and feedingstuffs. (5 p/m)

On request of the NRLs, supply of information and scientific advice.

The interlaboratory study on the transfer protocol and the determination of the cut-off value (for PCR assays) was presented during the annual CRL-AP workshop. The report is available for the NRLs through the CRL-AP Intranet.

The CRL-AP is often contacted by NRLs in order to get protocols, or asked for advice to give on pictures which are sent to the CRL-AP microscopist via e-mail. Such scientific advice becomes really a routine activity for the CRL-AP team, it is a testimony of the good communication between the NRL network and the CRL-AP.

In order to share as much as possible all research results obtained by the CRL-AP with the NRL network, all the PPT presentations from the 4th annual workshop were posted on the CRL-AP Intranet under PDF format.

On its request, the CRA-W protocol for the detection of bovine DNA was provided to the German NRL at end of October 2010. The protocol describes extensively the primers and probe sequences as well as the thermal program so that the laboratory can start to test this target validated in the framework of the SAFEED-PAP project by an interlaboratory study. The only missing point is the determination of the cut-off of the platform as calibrants with certified copy numbers are still not produced. During the PCR training held in Gembloux from 7th till 9th December, the same protocol was also given to the participants from 6 NRLs (Belgium, Cyprus, Greece, Portugal, Romania, Slovakia).

In addition, advices, protocols and materials (PCR reagents and controls - samples and DNA extracts) were provided to the French NRL to help them to start the implementation of the PCR in the laboratory.

2.5 Participate in the annual CRL Directors co-ordination meeting.

The meeting of this year, held on 9thand 10th February in Geel (Belgium), was focussed on proficiency testing. A lecture was given on how this was organized at CRL-AP.

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

Redaction of the first 6-months report (January 2010 – June 2010) and the annual report 2010 done.

3 Interlaboratory studies and quality assurance (28 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 test. This task includes the preparation of the samples for the interlaboratory studies (12 p/m)

3.1.1 Definition of the needs

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

3.1.3 Control of the raw materials

3.1.4 Production of the samples

3.1.5 Test of the homogeneity of the samples produced

3.1.6 Report on the produced samples

3.1.7 Distribution of the samples

The ingredient and feed sample collection of the CRL-AP is continuously expanding. For this reason, the CRA-W rented a refrigerated container in order to have more place available for the correct storage of the collection samples. This container is nowadays almost entirely at the service of the CRL-AP samples due to the increasing number of samples. The correct and organized location of the material was implemented and, storage place information reported in the CRL-AP Sample Management Suite, the IT program developed internally.

An animal species sample bank for PCR analysis is also set up in order to check the specificity of PCR methods (e.g. in view of the assessment of the TNO method). New samples collected this year involve blood samples of hare, horse, donkey, and roe deer. Next to that material that was already available at CRA-W had to be renewed: blood samples of pig, sheep, goat and chicken. Furthermore material of other species among which other wildlife ruminant species has been asked too but did not arrived yet in 2010.

3.2 Organize interlaboratory study for the determination of PAPs in feed using classical microscopy. (9 p/m)

3.2.1 Redaction of the report of the CRL-AP interlaboratory study of Autumn 2009

3.2.2 Definition (with the collaboration of the DG-Sanco) of the objectives of the ring trial to perform at the end of 2010

3.2.3 Preparation of the interlaboratory study.

3.2.4 Invitation of the NRLs to participate.

3.2.5 Packaging and sending of the samples (cf. task 3.1)

3.2.6 Collection of the results.

Data from the CRL-AP Proficiency Test 2009 were analyzed and compiled in a report in January this year. A first working document version of the report was posted for download on the Intranet by mid March 2010. The document as well as the results were discussed together with the NRLs at the 4th CRL-AP annual workshop in Turin in April 2010. A final version of the report was prepared and communicated to the NRLs beginning of June 2010.

The results notably on the lowest levels of MBM adulteration (as low as 25 ppm) in a feed served for the preparation of the revised version of the Annex VI of EC/152/2009 that was submitted to the NRLs on the 22nd of March for reviewing.

By mid November a set of 10 samples has been sent to each NRL for the EURL-AP Interlaboratory Study Microscopy 2010 on the revised microscopic method. The main objective this year was the validation of the proposed revised protocol for the determination of PAPs by microscopy. The deadline for returning the results was the 10th of December. In 2010, some foreign official agencies were invited to participate, these agencies were asked to follow their own national protocol and not the European revised protocol.

Results will be analysed in January 2011.

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)

Based on the observed underperformance for the CRL-AP Proficiency Test, according to their request and after agreement by the DG Sanco, a visit to the NRL of Member State #9 was organized simultaneously with a training session on-site on 21st and 24th June 2010. This on-site option was motivated by the fact that new people joined the NRL and at related departmental sites, so training was needed. The proposed period of end of June was chosen. This official mission included helping the NRL in achieving high levels or performance. The CRL-AP was entirely satisfied by the initiatives and actions undertaken by the NRL and their professional proactivity demonstrated. CRL-AP took the opportunity to remind them that in case of hesitation or scientific issue a NRL can always ask for help to the CRL-AP for scientific support (it does not always need to be official, as a counter analysis is). This scientific assistance mission is a leading axe of the CRL-AP policy for the development of a collaborative network. CRL-AP considers the problem as closed.

Three other NRLs revealed to be underperforming during the CRL-AP PT 2009. A request for information and action plan was sent to them after the workshop. Member States #10 and #11 addressed the requests very professionnaly and no other actions need to be taken. The other NRL will participate in a training session organized in January 2011 at the CRL-AP facilities: here again new persons joining the NRL are the reasons for the training. The Member State #12' response to the request was not satisfying: we still expect, at writing time, answers and complementary details on the reasons for the underperformance.

Veterinary Laboratories Agency (VLA) that are hosting the NRL for animal proteins in UK decided to move this activity from the Luddington site to the Newcastle site. VLA asked support from the CRL-AP for organising a training of its new team with the help of the Luddington team (Mss Gillian Lilley). Based on this background, two missions were conducted: (1) a training on animal proteins detection by light microscopy organised for 3 persons from the British NRL, and (2) an advisory role played for an optimal lab layout design of the future NRL activities at the Newcastle facilities. This mission was implemented from 16th to 19th of November 2010 in Newcastle (UK). A follow up mission was asked for March-April next 2011 year. The CRL-AP team will then evaluate the knowledge level after installation of the working stations and equipment necessary to take over the work of the team from Luddington.

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. (6 p/m)

3.4.1 Definition of the needs of the NRLs

3.4.2 Provide the requested help to the NRLs

3.4.3 Preparation of a syllabus including all the information needed for an appropriate detection of processed animal proteins in feedingstuffs

3.4.4 Preparation of permanent slides set at the disposal of the NRL

A questionnaire about PCR capacities of the NRLs was sent to each NRL in June. The purpose is to evaluate the experience, the equipment and the organisation of the labs with the aim to prepare a training program to help the NRLs to implement the PCR method in their lab. On the basis of the NRL answers, a first training session gathering 6 NRLs (Belgium, Cyprus, Greece, Portugal, Romania and Slovakia) having the least experience in PCR and

needing urgently information to buy the equipment and to organize their laboratory for the implementation of the PCR method in their lab took place in Gembloux from 7th till 9th December. The PCR training program will continue in 2011 and a second training is already planned in February 2011 as invitation letters were sent in December 2010.

The study on quantification on permanent slides started end 2009 was continued during the first half year of 2010. 14 NRLs worked on this study during the first trimester of 2010. The intention of this exercise was to assess the impact of the slide preparation and the influence of the observer on the final estimation of animal constituents in feedingstuffs. Therefore, a quantification had to be performed on the permanent slide on the two samples of material sent, prepared in each NRL with the Norland Optical Adhesive 65 resin. The importance of standardization in sample preparation was supported by the distribution of the tutorial CD prepared by the CRL-AP (cf 5.4). Later on, the results and the permanent slides were sent to the CRL-AP where all NRL´s permanent slides were counted again by the CRL-AP team. Comparison of the results was performed with emphasis on the value for the repeatability and reproducibility. Results of this study were shown on the 4th CRL-AP workshop. These results demonstrated the importance of slide heterogeneity between labs (which could be solved by a higher number of field counts) and the non-negligible influence of the observer (cf 4.2).

4 Development of analytical methods and tools (34 p/m)

4.1 Contribute to the development of new methods of analysis and improvement of existing methods of analysis. (4 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

Collaboration was established with the JRC-IRMM (Geel, Belgium) on the development of fish PCR assays that might be used to quantify fish meal in feed. The rationale behind it being that in the case of fish the heat treatment is less important and the possibility exist to make a link between copy number of targets and mass of fish material. The fish target of CRL-AP (a mitochondrial target which is less suitable for the aim) and that of the JRC-IRMM (a nuclear target) were tested in a comparative scheme discussed with JRC-IRMM. Results are in the hands of JRC-IRMM. CRL-AP provided part of the experimental material, did PCR and discussed the experimental scheme. A last improvement was brought by CRL-AP to the scheme through normalization of results towards a maize target as fish meal was simply put in maize flour. It is a very preliminary study but if results are conclusive, it might be interesting to go more in depth.

All the data available on the samples used for the study on the detection of ruminant proteins in processed animal proteins with the ReVeal kit were provided to Frank Klein from Neogen Corporation.

In May, a meeting was held at CCL (Veghel, The Netherlands) to go through the last developments linked to immuno-assays. CCL explained the seven possibilities of setting a cut-off. Two approaches out of the seven appeared as the most appropriate ones but discussion based on results of the second

MELISA-TEK study of the CRL-AP could show that what might appear as the best of these two methods could nevertheless sometimes lead to false positive results. CCL will therefore consider the other one of the two best methods they selected because there are reason to think this false-positive results problem will not be encountered are at least be less frequent with the other method.

Contacts were made with JRC-IRMM Reference material Unit (Geel, Belgium) to be able to use their facilities for digital PCR or to work in collaboration with them to solve the problem of correct determination of plasmid copy numbers of the calibrants to be distributed to the NRLs. A first informal demand was addressed in May 2010 (20-21 May during the 13th plenary session of the ENGL) and was reiterated during the participation of the CRL-AP in the meeting on "Future of reference material" held at JRC-IRMM in November 2010. JRC-IRMM showed great interest for the poster presented by CRL-AP (see 1.2.) at this meeting.

4.2 Contribute to the development of complementary analytical methods necessary to assure the correct implementation of official methods and explorative or alternative methods. (6 p/m)

Studies on the LOD determination, started with the Chinese colleagues from CAU end of 2008, were continued. 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. The work is still ongoing. The analytical process followed was based on the determination of a beta-error that had to be lower than 5%. This is relevant for determining the risk of obtaining false negative results. It makes possible to evaluate the influence of the bone percentage in the PAP (f factor) and the sediment percentage of the feed matrix on the LOD. The

results will be presented at the 5th CRL-AP workshop.

Acquisition of sea mammal material continued ongoing this first half 2010, the CRL-AP received 5 different species from the Mediterranean Marin Mammals Tissue Bank, Italy. Among this material vertebrae and rib fragments, muscles and skin-bubbler from different species of dolphins and whales are included. After the procurement and the treatment of the samples, a deeply study is required. The correct discrimination against terrestrial bones based on the lacuna size, canaliculi and bone shape is mandatory. The work is being performed in collaboration with the team of the Dr Luciano Pinotti, of the University of Milano, specialized in lacuna bone characterization combining microscopic and computer image analysis. So far, the acquisition of all sea mammal material along last year and early 2010 includes samples of seal, dolphin, porpoise and whale. This material is useful not only for classical microscopy analysis but also for the further development of a target to identify cetaceans remains in feedingstuffs by PCR. The design of sea mammals PCR assays was started by the collection of sequences from the international DNA sequence banks and the analysis of interesting regions in the mitochondrial DNA. During the second half of this year, the sensitivity and the specificity of the new probe(s) was studied on the samples available in the CRL-AP animal species sample bank.

Some results of the analysis done so far by classical microscopy show the real similarity of the sea mammal bones with the terrestrial bones, a wide diversity on the lacuna shape on the same species was noticed. That makes difficult the discrimination against terrestrial material in fish meals, allowing the misidentification by classical microscopy of a natural contamination caused by sea mammal material presence in fish meals. A method of choice for this analysis would be the PCR.

NIRM method is also under investigation in order to determine its potential to detect particles from sea mammal products.

The study "Quantification on permanent slides 2009" started in December

2009 by CRL-AP continued on the first half 2010. This study was developed with the purpose to assess the impact of the slide preparation and the influence of the observer on the final estimation of animal content on feedingstuffs. The results, based on the accuracy, repeatability and reproducibility were shown on the 4th CRL-AP workshop. We can affirm nowadays that the accuracy among the NRLs and the CRL-AP is correct, but there is an important observer impact and the slide preparation also interferes and should be improved.

For the organisation of this study, the use of Norland adhesive 65 for permanent slides preparation and curing by UV source was required by all participants. Nevertheless some NRLs didn´t had this light source nor the Norland adhesive 65, this meant the need of a travelling set by post delivery of a UV lamp and Norland adhesive 65 around Member States.

After discussions held during the 4th CRL-AP workshop, the CRL-AP started in May to study the influence of the particle sizes on the sample analysis by light microscopy in its both aspects, qualitative and quantitative. Feeds were grinded at different mesh sizes: 1000, 500 and 250 µm, permanent slides were prepared and two operators observed the samples. The results showed that the optimal grinding mesh size for identifying MBM particles was at 1000 µm, at this size identification and counting was possible. Finer grindings revealed to lead to difficulties of particles identification and underestimations of quantifications. Furthermore fined grinding resulted in a loss of material during the Alizarin Red staining process.

A new horse PCR target was designed. The specificity of the test was successfully checked on DNA from the following animal species (horse, cattle, pig, sheep, goat, chicken, rat, various dolphins -Stenella coeruleoalba, Tursiops truncatus, Grampus griseus-, whales -Ziphius cavirostris- and fish species -Gadus morhua, Pollachius virens, Melanogrammus aeglefinus, Micromesistius poutassou, Sebastes spp, Mallotus villosus, Scomber scombrus, Clupea harengus, Merluccius

merluccius, Trachurus trachurus, Trisopterus minutus, Sardina pilchardus, Engraulis encrasicolus, Gadus ogac, Trisopterus esmarki, Sprattus sprattus- as well with plant species (soybean, maize, rapeseed, sugarbeet, wheat, tomato and rice).

In collaboration with the Austrian NRL (AGES-Vienna), the PCR analysis of a limited set of meat and bone particles isolated with a PALM microdissector was initiated. The collection of the particles to analyse was performed during a mission of Pascal Veys at the Austrian NRL. These first tests on 11 particles (in fact fragments of particles) gave poor results. The protocol already developed at CRA-W with particles analysed by NIRM (Fumière et al., 2010) has to be adapted to allow the isolation of fragments of particles embedded in a fixation varnish. The dissolving of this varnish with acetone before the DNA extraction seems to have no impact on the PCR efficiency but these results have to be considered as preliminary as they were obtained with particles coming from a mix. New analyses have been planned with AGES on particles of pure single-species MBM in 2011.

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 Organisation of interlaboratory studies based on alternative tests (PCR, immunology,...) developed by NRLs or by companies

4.3.2 On the basis of the former interlaboratory studies regarding PCR methods, define the strategy for the optimum implementation in the NRL

4.3.3 Preparation of CRL-AP protocol at the destination of the NRL for the implementation of the PCR methods

4.3.4 Organisation of the transfer of validated PCR methods to the NRLs network – training courses and manuals will be prepared during 2010

An interlaboratory study had to be organized with some of the PCR assays developed by TNO. To that purpose the CRL-AP needed the data of the assays from TNO. TNO first wanted to test again their methods with samples sent in blind to them by CRL-AP. Samples were sent as requested in May. Results on these samples were received in July. In parallel it was asked to TNO what they wanted to put in the confidentiality agreement. After some mail exchanges TNO provided a draft of a "Non-disclosure agreement" in June. This was submitted to the lawyers of the CRA-W because several points were difficult to accept as such and might be in disagreement with duties of the CRL-AP (there are for instance doubts about the fact that a report could be done on the results that are obtained). After several mail exchanges and some amendments to the agreement, the final version was approved by both parties and was signed in November and came into force the 1st December 2010. Therefore we expected to receive the method at the beginning of December but finally CRL-AP had to ask it. TNO replied they first wanted to implement the method in the laboratory of CRL-AP and that this would require two working days. We accepted this although it had been said in June 2010 that this was not necessary. Taking into account the available dates of each parties led us at the end of December to set this meeting on 31st of January and 1st of February 2011.

Concerning the results on the blind samples obtained from TNO in July, these were quite good. Interestingly it confirmed what was expected that the cut-off value set by TNO is not defined in a sufficient flexible manner for several platforms (and this limits its transferability). This is however a drawback that CRL-AP could improve, once we have the method.

In parallel to that it must be stressed that the University of Madrid proposed to share the PCR methods developed in their lab: they already sent to the CRL-AP the protocol for the detection of bovine material in feedstuffs. They proposed to share also their protocol for the detection of poultry material.

The results of two PCR inter-laboratory studies taking place in 2009 (the one

organized by the CRL-AP aiming at the validation of the transfer protocol of the cut-off value and the one organized by SAFEED-PAP aiming at the validation of a PCR kit) were presented to the NRLs during the CRL-AP workshop in Turin. The validation of the transfer protocol and of the determination of the cut-off value of a PCR platform can be considered as validated. The results of the validation of the CRA-W PCR method for the detection of bovine DNA in feedingstuffs indicated that a detection of bovine PAPs in feedingstuffs at a level of 0.1% is feasible with an acceptable rate of false positive (< 5%). The last remaining point for the correct implementation of the method in the NRLs is the production of calibrants with certified copy number for the determination of the cut-off platforms. The CRL-AP is in touch with the JRC-IRMM to tackle this problem using its digital PCR device.

4.4 Performing CRL available methods or adapting them on outbreak material to make them available for the NRLs network. (2 p/m)

Numerous pictures from the CRL-AP micrograph collection were used for the enrichment of a new edition of a manual dedicated to beginning microscopists and as a reference for the more experienced. The manual edited by AOCS is entitled Microscopic Analysis of Agricultural Products, 4th edition. Definitely this manual shall be a very good support material for NRLs. Recommendation to NRLs for getting the manual shall be promoted.

4.5 Extension of the samples bank with a special focus on specific animal material (e.g. sea mammals, rodents). 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 samples bank. (10 p/m)

4.5.1 Establishment of the specification for the CRL samples bank

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

4.5.3 Maintenance of informatics tools for the appropriate management of the samples

4.5.4 Collection/production of samples of animal origin

4.5.5 Test of the samples collected

4.5.6 Storing of the samples

4.5.7 Maintenance of the samples bank

The CRL-AP online micrograph collection continued to be enriched on 2010. First half year release was updated in March 2010 including pictures of placoid scales, oyster shell, fishbones and other animal structures. The second update of the collection was in October, this time a total of 166 new pictures were added. Pictures from salmon, dolphin, porpoise, seal, frog, ray and whale are available for NRL and IAG members. The Excel file for consultation has been accordingly adapted. For this purpose the CRL-AP bought the last version of the software Axiovision 4.8, and its modules z-stack, time lapse and multichannel, these modules will be used for further researches and better analyse of pictures.

The centralized and integrated management tool Sample Management Suite (SMS) was finished and is nowadays totally operational. During this period it was completed with the data concerning the pictures from the databank. Each picture is independently linked to the software, allowing the vision of the picture at the same time as the data when using the search motor of the software.

Due to the increasing number of samples and for the correct maintenance of the CRL-AP sample bank, the CRA-W rent a refrigerated container of 60 m^3 placed outdoors (cf 3.1). The new storing place was available on February 2010. The transfer of over 1000 stored materials and samples and the related database modifications took a certain workload. Even if this issue required

an investment of time for its establishment, it is already a saving time for the CRL-AP daily work.

The CRL-AP amplified its sample bank by single species MBM. A salmon meal was made at the CRL- AP facilities; two salmons were used to produce this meal. The salmon is known to have specific bone features, difficult to recognise by classical microscopy. By preparing such single species meal, the sample bank got increased, the specific bone features shall be studied and their pictures will serve as reference support. The CRL-AP treated separately the different salmon areas such as gills, skull, vertebrae, scales, lens, body bones, etc. It allowed the identification and shooting of pictures of each studied anatomical sections. These pictures were added to the micrograph collection on the Intranet, all the NRLs had, therefore, access to these pictures. The salmon meal was used as adulterant for one sample of the EURL-AP Interlaboratory Study Microscopy 2010 in order to evaluate the capacity of the NRLs to detect salmon bones as fish bones.

5 Workshops/trainings (5 p/m)

5.1 Provide specific workshop for the benefit of NRLs for the correct application of the method described in the Annex VI of the 152/2009/EC Commission regulation to detect animal proteins in feed (Classical microscopy) and any new development or regulation related to the detection, identification and quantification of animal proteins in feed. (1 p/m)

On the basis of the former interlaboratory studies regarding PCR methods it was decided of the opportunity to organise a specific workshop in 2011 for the implementation of the PCR method

On the basis of the NRLs' answers to the questionnaire about PCR capacities sent in June, a first training session gathering 6 NRLs (Belgium, Cyprus, Greece, Portugal, Romania and Slovakia) having the least experience in PCR and needing urgently information to buy the equipment and to organize their laboratory for the implementation of the PCR method in their lab took place in Gembloux from 7th till 9th December 2010.

After the IAG meeting held in Hamburg in September 2010, the CRL-AP assisted to the annual meeting of the German NRL, the CRL-AP representative made a presentation on the alternative methods for the PAPs detection.

5.2 Provide specific workshop of experts from candidate Member States for the correct application of the 152/2009/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)
See section 1.7., workshop for candidate and potential countries

No activities forecasted in 2010 but request for training are still numerous

and sometimes originate from third countries: e.g. China, Serbia, South Africa, Norway and Peru.

5.3 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 (3 p/m).

5.3.1. Presentation to the NRL of ARIES 2 (Safeed-pap)

5.3.2. Didactical support for the correct implementation of PCR 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 at the end of 2009. 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.

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 in PDF format. This file is complementary and necessary to the well understanding of the video sequences.

This CD was delivered in 2010 to the 14 NRLs that didn't receive it yet in 2009. All NRLs thus have received one copy of this CD useful for their personal training as well as for their network training. It is the most detailed support standardization of the method implementation existing at present. A project of realizing a same training CD but applied on the correct implementation of a PCR method will started in 2011.

The new version of ARIES (Animal remains identification and evaluation system) was presented at the 4th CRL-AP workshop to the NRLs by Dr Leo van Raamsdonk (The Rikilt, Netherlands). The different modules to assist

step-by step the process of identification largely improve this version which is therefore more useful than the previous one. This process of identification was presented partially online.

In 2009, the CRL-AP was contacted for participating as co-author at the revision and new larger edition of a manual entitled Microscopic Analysis of Agricultural Products. This manual edited by the AOCS Agricultural Microscopy Division end of 2010 is not only the most complete one in the market but is also a compilation of practical information written by individual microscopists toward the common goals of agricultural products identification. This manual constitutes a very valuable source of information in terms of feed ingredients detection, provided with large explanations, detailed descriptions and pictures. Two of the nine chapters of this manual were written almost by the CRL-AP which provides as well a large part of the micrographs from the CRL-AP micrograph collection. Obviously, the CRL-AP source is named below of each CRL-AP illustration. These chapters are Feed Ingredients of Marine Origin, and Detecting Animal Products in Feeds and Feed Ingredients. This last one was written with North American and EU (CRL-AP) collaboration, in this chapter both legislations regarding processed animal products are highlighted and described.