COMMUNITY REFERENCE LABORATORY

"Detection of animal proteins in feedingstuffs"

CRL-AP

Walloon Agricultural Research Centre – CRA-W (Belgium)

Annual report 2008 activities

PUBLIC VERSION

1 Scientific advice and support to the European Commission (35.5 p/m)

1.1 Provide scientific and technical assistance to the European Commission in relation to the development of EC feed legislation. (3 p/m)

On the request of the DG-SANCO, a study on the immunoassay "ReVeal® for Ruminant" (Neogen Corporation, Lansing, USA) was conducted at CRL-AP. Beside the status of the knowledge based on literature, public documents and personal communications with the main labs involved in the field, experiments were realised to evaluate the relevance of the EFPRA proposal to use the "ReVeal® for Ruminant in Feed" kit as the screening method for the control of the re-entry of non-ruminant processed animal proteins in feeds for aquatic species (Aqua-feeds). A draft document was submitted to the Commission end of June 2008. The study is confirming most of the findings of EFPRA but in addition it clearly showed that in cases of less heat-treated ruminant material, there may be a strong masking effect of the non-ruminant PAP on the presence of ruminant meat and bone meal even at rates as high as 20%. Without better knowledge of the reason of this phenomenon, the Neogen dipstick methods appear presently as non-fit for the purpose of screening. The final version of the report including an addendum dedicated to preliminary tests with the "Melisa-Tek" Ruminant kit (ELISA Technologies Inc., Gainesville, USA) was delivered to the Commission at the end of October 2008. This ELISA test is also under evaluation in the Dutch

lab CCL Nutricontrol. The preliminary results performed by the CRL aimed to check whether the Melisa-Tek test could be a good alternative to the Neogen assay for the detection of ruminant PAPs in PAPs. The first results confirmed that the test could improve the classification of the samples when using the decision criteria proposed by the CCL. It is a less user-friendly test and takes more time than the Neogen dipsticks but it seems a much more reliable alternative. Further investigations will be probably needed in 2009.

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, this means one for the six months period of 2008. (1 p/m)

1.2.1 Preparation and participation to international meeting/fora

1.2.2 Report/minutes following completion of the mission

On request of the Chinese authorities, the CRL-AP developed a close collaboration with the Chinese Agricultural University (CAU). This collaboration was initiated with Prof. Liu Han. The CAU took in charge a two months stay of 4 PhD level scientists at the CRL-AP facilities from mid October by mid December. This collaboration 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. Clearly defining of the LOD is an absolute prerequisite for any further determination of limits of quantifications upon which those should be based. This model would therefore be a first step towards the possibility of introduction of tolerance levels. The building of the model allowed rapidly obtaining scientific evidences on the LOD of fish meals (based on real assessment of the fish bone proportion of market available fish meals). Based on this first study, the CRL-AP microscopy team will now reproduce the experimental scheme on the mammalian meat and bone meals.

Results are expected for mid March 2009.

In parallel to classical microscopic studies realized in the field of the collaboration with Chinese Agricultural University, the ability of Near Infrared Microscopy (NIRM) to quantify the rate of adulteration of feed samples has been investigated. Works were done on feeding stuff contaminated by different percentages of various types of fish meal (0.5 %, 1%, 1.5 %, 2% and 5%). Only the raw fraction (1200 particles for each sample) was studied and both the fine (< 250 µm) and the gross fractions (> 250 µm) were analyzed in reflexion mode. Particles were pointed, spectra collected then interpreted to identify the positive particles. Results are encouraging but do not allow yet proposing the NIRM for a validated approach for the quantification of PAPs.

1.3 Upon the request of the European Commission or in order to fulfil his role as Community reference laboratory, participate to 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 to the CEMA, CEN and IAG meetings

1.3.2 Reports/minutes of the meetings

The CRL-AP participated to the IAG annual meeting held in Budapest in June. An oral communication on the state of the art of the quantification method development has been presented. The presentation included a comparative analysis from the results of the two CRL-AP studies from 2006 and 2007 to which the NRL network participated. A second oral communication dealt with unusual bone characteristics discovered recently. During this IAG annual meeting numerous new contacts were established such as with representatives from FEFAC (European Feed Manufacturers Federation) and foreign delegations (a.o. Serbia, Croatia).

In September, the CRL-AP participated to the second IAG working meeting in Hamburg. During this meeting new perspectives in the field of 3D microscopy for enhancement of bone particle identification were achieved. A request from the IAG board to open the CRL-AP on-line micrograph collection to its member was formulated. In January 2009 the DG-Sanco approved this request. A Direct but secured access to the sole micrograph collection on the CRL-AP intranet will be given in the first quarter of 2009.

The CRL-AP also participated to the 3rd SAFEED-PAP meeting held in Vilnius, Lithuania from 13th- 14th of May. The involvement of the CRL-AP team during the meeting concerned the future improvements and developments of new microscopic markers for a better detection of PAPs in feed and a more efficient screening method for the control laboratories. Its implication was also concerned by other method development such as the improvement and validation of a PCR based test kit, the alternative quantification method by NIRM, etc…

On 7th and 8th of September the 4th SAFEED-PAP meeting was held in Stratford-upon-Avon and the CRL-AP team participated. During the meeting results on the detection of natural contamination of feedingstuffs by rodents species based on the microscopic hair identification were presented. Results of this participation of the CRL-AP microscopy team will be included in the next release of ARIES planned in the SAFEED-PAP project.

First meeting in Gembloux on 2 September 2008 with EFPRA and CCL who both heard about the preliminary version of the report on the assessment of the Neogen dipsticks for detection of ruminant material in non-ruminant PAPs. Clarifications were made about what the main problem was found by the CRL-AP. Minutes of this meeting were made. Second meeting in Gembloux with EFPRA and CCL on 19 September 2008 with communication by CCL of the possible better potential of the Melisa-Tek ELISA kit for

ruminant detection according to the EFPRA proposal. It was therefore decided to do a preliminary test with it and make a small addendum to the report assessing the Neogen dipstick methods. To that purpose it was asked to DG-Sanco I to delay the deadline of delivery for final report of one month. Minutes of this meeting were made.

The CRL-AP also participated to a TRACE dissemination workshop about the Molecular Biology Methods for Traceability Purposes held in Berlin, Germany the 18th and 19th of December. A lecture on the status of DNA and protein based methods for the detection of animal species in feedingstuffs was presented by Prof Lampen (BfR housing also the German NRL in the NRL-network of the CRL-AP). The CRL-AP took the opportunity of the discussion to mention the future developments of the PCR method in 2009 : an interlaboratory study organized by the CRL-AP to validate a transferability protocol of the PCR method and a validation study of a PCR kit for the detection of cattle within the framework of the SAFEED-PAP project.

1.4 To actively participate to technical and scientific support of the European Commission in the context of incidents or crises linked to incorrect use of animal proteins. (12 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 2008:

- Analysis of 1 sample of feed (April 08)

- Analysis of 1 sample of fishmeal (April 08)

- Reception of 2 samples of fishmeal (January 08, analysed in July)

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

1.5.3 Preparation of the file for the organisation of interlaboratory studies

An extension of the accreditation scope to PCR tests for detection of bovine material in feed was asked to BELAC at the end of November 2008. Standard operation procedures and the validation dossier of the assay for which accreditation under ISO17025 is asked are being prepared

The molecular biology analyses done within the framework of the CRL-AP are integrated within the existing quality assurance system of the Department Quality of Agricultural products. The maintenance of the existing quality assurance system also benefits to the CRL-AP activities (e.g. balances are checked, micropipettes are checked and recalibrated if necessary, real-time PCR devices are checked,…) even if the PCR analyses done for the CRL-AP are not yet accredited as such.

CRL-AP 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 2008 were successfully analysed.

CRL-AP also subscribed to a similar proficiency test scheme for the detection of bone in animal feed by microscopy also organised by VLA. Sets of 4 samples were received in March, June, September and December 2008. The results obtained by classical microscopy were successful. The samples are analysed in parallel by PCR. The sets of samples distributed in March, June, September and December 2008 were also successfully analysed. The PCR results were in accordance with the expected results (presence of fish and/or terrestrial animals).

The CRL also participated to the IAG ring test on the detection of animal proteins in feed. The molecular biology team also analysed the three samples.

The CRL-AP continues to work on controls to standardize the PCR test for the detection of cattle MBM in feed in order to allow its in-house validation and its accreditation in 2009.

Analyses were performed in parallel on the former thermocycler (ABI 5700) and on the new thermocycler (LightCycler LC 480) installed in June '07 in order to accumulate results. The aim of these analyses is to provide as soon as possible results with equivalent level of confidence on both devices.

The data acquired with the LightCycler LC 480 are subjected to a statistical analysis conducted by the Unit of Biometry, Data processing and Agrometeorology of the CRA-W in order to determine the best way to set the crucial parameters of the analysis and to allow the development of a protocol for the transfer of the method on any platform (thermocycler + reagents)(see 4.2.2 and 4.4.2).

Finally a control system for the detection of possible air flux cross-contamination was organised in the second half year. The system is based on the regular observation of dust collected from watch glasses located at strategic places in the CRL-AP facilities (sample preparation lab, microscopy lab). The system is very efficient, and up to the date of writing no case of cross-contamination was noted.

1.6 On the request of DG SANCO, to perform analyses on samples with disputed results. (6 p/m)

1.6.1 Perform the requested analyses

1.6.2 Report on the analyses performed

- Analysis of 1 sample of fishmeal (February 2008)

- Analysis of 2 samples of feed (March 2008)

- Analysis of 1 sample of feed (April 2008)

1.7 Assist TAIEX with targeted assistance towards specific training/workshop for new member states or/and candidate countries (0.5 p/m)

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

2 Coordination of activities of NRL network (15 p/m)

2.1 Construction, development and maintenance 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 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, 3862 pages were visited through 2678 visits by 537 unique visitors coming from 60 countries and 271 network locations through the world.

Regarding the intranet, 8666 hits were recorded during this period. 4 new members joined the network and 2 members left.

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

Major enhancement of the intranet platform during the first half year of 2008 consists of its enrichment by the micrograph collection accessible to the NRL network (cf. 4.5.).

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

2.2.1 Preparation and sending of three newsletters in 2008

Three newsletters were prepared and diffused among the NRL during the reporting period: Newsletter 4 in February, 5 in May and 6 in December. Content of the fourth newsletter included the program for the 2nd annual workshop as well as an exhaustive listing of web based information sites on the PAP issue in feedingstuffs and other related topics in general concerning

the feed safety. The fifth newsletter content consisted of the minutes of the 2nd CRL-AP annual workshop that was held in Namur from the 15th – 17th of April. It included comments from all NRL delegations at this workshop but also all decisions taken even as the next planned initiatives and actions regarding the CRL-AP - NRL network duties. The sixth newsletter contained information on the micrographs collection development (rodent's hair identification), on the ongoing tests on quantification in collaboration with the NRL network and news on the December training sessions. In the last newsletter the CRL-AP also briefly reported on its different missions within EU, its collaboration with the Chinese Agricultural University and the last development on a NIR microscopic based method for the quantitative detection of animal proteins. Finally the sixth newsletter also informed on the 3rd CRL-AP Annual Workshop which will be held in March 2009. A request on the revision of the EC 126/2003 text and reflexion on potential improvement of the text was asked to the NRL community. Their suggestions will be discussed in 2009.

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 2nd 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

2.3.6 Participation to the Workshop

A 2nd CRL-AP annual workshop was organised in Namur from 15th to 17th of April 2008. The agenda of the workshop to which all NRLs participated was focused on the two collaborative studies organised in between the first workshop and the second one, i.e. the CRL-AP Interlaboratory Study 2007 on an revised quantification method and the CRL-AP Proficiency test 2007. Results from both studies were discussed, and subsequent additional

experiments (cf. 3.2) were decided in order to test the impact of different factors on the revised quantification method. Minutes of this workshop were included in the 5th Newsletter.

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)

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

A list of equipments as exhaustive as possible was drawn up in order to allow the Portuguese NRL to prepare the budget for a PCR lab dedicated to the detection of PAPs. The list mentioned the models and prices of equipments used in the CRL-AP as well as web sites of providers or distributors.

CRL-AP answered to questions of the French NRL on the potential of biochips for the detection of animal DNA in feed and the extraction of DNA from particles of the sediment fraction.

In February, a two-day PCR training was organized at CRA-W for 2 persons of the Dutch lab CCL Nutricontrol. The CRA-W PCR method (reagents and protocol) is already implemented in the CCL lab but more information on the organization of the analysis (steps, procedures, results interpretation rules,…) were needed to improve the quality of the results.

As a decision from the 2nd CRL-AP annual workshop, a protocol for the submission of official counter analysis request was developed and communicated to the NRL network. The intention is to improve at the maximum level the sequence of actions and communication between the NRL and the CRL-AP for each request of counter analysis. The protocol defines the minimal quantity of material needed and improves also the identification of the sample material according to the respective codes used by the two laboratories or referring to the Rapid Alert System for Food and Feed. Finally it describes how official analyses report shall be communicated. The protocol is valid as from 22nd of April.

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. (1 p/m)

2.6.1 Prepare and submit the 6-months report (January 2008 – June 2008) and annual report (January 2008 – December 2008)

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

3 Interlaboratory studies and quality assurance (19 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. (3 p/m)

3.1.1 Definition of the needs

3.1.2 Set up of a planning of the samples to produce in the 2008-2009 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

3.1.9 Collecting the results

3.1.10 Preparation of the draft report

3.1.11 Integration of remarks in the final report

By end November 2007, the production of a 0.1% MBM adulterated mixed

compound feed was launched with the intention of delivering to each of the 26 NRL a batch of 100 vacuum bags of 12g each. The production itself, its sealed vacuum bag packing and shipment to all NRLs was still running at the beginning of 2008 and a first set of shipment boxes was ready by mid of January 2008. Unfortunately, the results from quality control for this first batch were not satisfying. CRL-AP decided not sending this batch and the whole process of preparation was started again with new material components. The production of a second batch was achieved by end of June. Intensive quality controls on this second batch revealed it fitted for distribution among the NRL network. The sending of the bags containing this training material was done at the beginning of July.

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 November 2007

3.2.2 Definition (with the collaboration of the DG-Sanco) of the objectives of the ring trial to perform at the end of 2008.

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.

Collection of the data from the CRL-AP Interlaboratory Study 2007 on quantification has been realised as from 21st of January which was the deadline postponed due to the non respect of the delivery schedules of the counting grids from the sole provider existing on the market and located in the USA. Analysis of the results was performed from mid February to beginning of April, so that a working document version of the report could be sent to the NRL network in order to prepare the discussions of the 2nd annual workshop. Of note the longer analysis and data treatment period required: this originates

from the fact that the most possibly exhaustive statistical treatments were required in order to determine what factors could impact the quantification. A final version including the comments from the NRLs was prepared afterwards and diffused on the CRL-AP Intranet, even as communicated to the European Commission, on the 17th of June.

Based on the results from the CRL-AP Interlaboratory Study 2007 on quantification and the decisions from the CRL-AP 2nd annual workshop, two extra collaborations of the NRL network have been asked. In this way, firstly all NRL were asked to reiterate quantifications on two samples with differing adulteration levels from the CRL-AP ILS 2007 but this time without any staining in order to investigate on a possible bias effect introduced by the Alizarin Red staining process on the quantification. Results had to be returned to CRL-AP by mid of June 2008. This extra collaboration on a free base revealed to be successful and reflects the motivation of the whole NRL network for the improvement of the quantification method. Secondly, a selected panels of 12 NRLs was chosen, to realize quantifications on two sets of permanent slides in order to analyze the variability of results on exactly the same slides and same particles and hence to determine the influence of the human skills. The two sets of slides start traveling from one NRL to the other on the 14th of May. Results from this first array of 12 NRLs were collected mid August. The results obtained were still presenting a high variability, therefore a second panel of 12 NRls was asked to do the same exercise on the same set of slides during the second half year. The slide sets were traveling between NRLs from September till end November. The CRL-AP had also to check the slide quality in between 3 or 4 sending's to NRLs : some damages were noted and slides were replaced when needed. From the global analysis on the quantification on same slides it appeared that the global overestimation was no longer observed but nevertheless the interlaboratory variability was still present although slightly improved. Conclusions on those results will be discussed with the NRL network during the 3rd CRL-AP Annual Workshop in March 2009.

Because of this extra charge of work, based on a voluntary choice, only one

proficiency test was organized during autumn 2008. Nevertheless the participation of the NRLs to this extra collaboration will be qualitatively evaluated.

The CRL-AP Proficiency Test 2008 was announced beginning of September. On the 7th November 2008 a set of 10 blind samples was sent to the NRL network for analysis. Results were returned on the 1st December. The data analysis took place in January 2009 and the working document version of the report will be distributed among the NRL network before the next coming CRL-AP annual workshop. Of note this year, for the second time, some foreign participants were allowed to subscribe to the CRL-AP Proficiency Test 2008. Those outside-EU participants were the official control institutes from Canada (second participation), Croatia and Serbia. Invitations were also sent to Russia and Ukraine. Ukraine did not reply. Russia replied positively but finally was unable to participate (cf. 5.3).

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 (2 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

Two visits to NRLs were organised and realised during the period. Detailed reporting was communicated each time to DG Sanco..

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

4 Development of analytical methods and tools (31 p/m)

4.1 Contribute to the development of new methods of analysis and improvement of existing methods of analysis. (1 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

The CRL-AP Interlaboratory Study 2006 demonstrated the inapplicability of the quantitative method as described in EC/126/2003 Directive. Research for proposing a new method (or at least enhancement of actual method) has been initiated in 2007 and delivered a new method based on a stereological derived method (grid or point counting). The method applicability was confirmed by a CRL-AP internal test. The validation of this method was planned by the CRL-AP Interlaboratory Study 2007. By the end of November 2007, a set of 10 blind samples adulterated with different percentages of fish was sent to the participating NRLs. Results were asked to be returned to the CRL-AP by the 21st of January. Thus the results analyses and report were realised during the present half year. Final results from CRL-AP Interlaboratory Study 2007 were presented during the 2nd CRL-AP annual workshop but revealed to be not satisfying enough at least to be validated, even if the study demonstrated a twice as better reproducibility among participants. From the conclusions of the workshop, additional experimental work for checking some parameters that could influence and hence could improve the method implementation itself has been realised this first half year. Those experiments include the number of fields being required to observe, the density of particles, the slide preparation way and its homogeneity of particle distribution, the effect of a possible overestimation linked to the use of the Alizarin Red staining, as well

as other parameters. This work will still be in progress during the second half year.

The "Departamento de Nutrición, Bromatología y Tecnología de los Alimentos – Universidad Complutense de Madrid" (Spain) analyzed in April 2008 a set of 14 blind samples and 4 DNA references prepared by CRA-W. The samples were tested for the presence of cattle, pig and fish DNA. The results were promising with only one false negative result for a sample spiked with 0.1% of cattle PAP. The evaluation of the method will continue with the analysis of a second set of samples to estimate more accurately its efficiency to detect 0.1% of PAP.

Submission by the "Station de recherche Agroscope Liebefeld-Posieux ALP, Switzerland" (acting as NRL for the Luxemburg) of species-specific PCR methods in documentary form for review by the CRL-AP.

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

The study with the JRC-IRMM initiated in 2007 to transfer the real-time PCR method developed in CRA-W to other instruments and to the laboratory of IRMM-FSQ was followed in 2008. The analysis of a first sample set was completed in January 2008. Based on the results, the protocol was slightly modified to improve the statistical analysis of the data. It is a kind of holistic statistical approach and it differs from the research done within SAFEED-PAP for transfer where there the focus was done on how a cut-off value can be defined for any PCR patform (corresponding in fact to a combination of thermocycler and reagents). Of course there are interconnections between both approaches and discussions done during meetings held with IRMM were very fruitful for both approaches. A second set of samples was prepared in March 2008 and analysed in May 2008. The report of the study is in

preparation and a publication is foreseen.

A meeting of the CRL-AP PCR Working group gathering VLA, TNO, JRC-IRMM, CCL and CRA-W was held in Brussels (17 November 2008). It decided to organize in 2009 an inter-laboratory study to validate the PCR method transferability protocol proposed by CRA-W. Minutes of this meeting were made.

Research on the possibility to realise quantification by way of NIRM have been initiated recently : the main advantage of NIRM as compared by classical microscopy is the fact that it is almost not sensitive to human factors as revealed to occur during the CRL-AP Interlaboratory Study 2007 on the quantification. However any promising allegation would be too early, this research is presently in its developing phase and several technical problems have to be overcome. The development of a quantitative method based on NIRM technique implies to improve capacities and to fulfill requirements for an accurate determination of adulterating particles present in feed sample. The best way should not include the intermediary step of sedimentation as described in the official method (EC Directive 126/2003) in order to avoid the problem due to the unknown factor f which is one of the major drawbacks of the protocol. It is important to analyze a representative sampling taking into account particles from both gross (higher than 250 µm) and fine fraction (smaller than 250 µm). This is essential because, adulterant material (i.e. PAP) has not necessarily the same particle size distribution as the rest of the sample. The first results show that the mode of collection of spectra is associated to the size of particles. Fine fraction seems to be better analyzed in the transmission mode. Mapping protocols are being developed in order to automate the protocol of quantification and to measures spectra of larger number of particles and samples. The intervals, the number of scans, the aperture are being optimized in order to quantify accurately the rate of adulteration by PAPs. Works are still undertaken and trials to overcome technical problems continue to be performed.

The intralaboratory variability or repeatability among quantitative results, delivered by the revised method, was studied inside the CRL-AP. This analytical work was realised up to the limits and took 54 days. It was part of the so-called homogeneity study for the CRL-AP Interlaboratory Study 2007 and was realised during the first quarter of 2008. Results are part of the study report.

A simulation exercise on the significance of a "natural" cross-contamination such as it occurs by rodents was carried on beginning of April 2008. Based on maceration of entire rats, which allows obtaining pure bones, keratinised structures and hairs separately, a modelization based on the weight of the bones and the morphometric features of rats could be developed. The results, based on the finding of some few bone particles in a feed that could be interpreted as purely accidental from rats cross contamination, can be expressed in the form of number of rats present in one ton of feed. The same process should be investigated in the future but with mice, pigeons and seagulls, three other common sources of natural cross contamination in feed production plants.

During the same period, the difference of polarization according the age of bone spicules being found in adulterated feed was studied by CRL-AP microscopy team. The aim of this research was to estimate if any difference of polarized feature between processed animal bone fragments and bone fragments originating from soil contamination (dead small field animals) could be useful to discriminate between fraud and natural cross contamination. Results revealed that the polarized character of a bone particle could not be used.

On NRL requests, but also in collaboration with the SAFEED-PAP project and in order to enrich the micrograph collection for the NRL network, investigations on specific mammalian hair features have been started during the first half year. This focus mainly on hair identification in case of natural cross contamination and is therefore orientated for the recognition of rats,

mice, rabbits and other rodents rather than the identification for very scarce other farmed animal hairs (bovine, porcine,…) in case of sources of feed contamination. During the second half year, hairs of the following animal species were studied: bank vole, brown rat, black rat, house mouse, common vole, mountain hare, domestic rabbit, cows and pigs. The analysis was made by bright field and DIC microscopy on entire hairs and on hair prints in order to study the species specific differences of the cuticular pattern and organization of hair scales. This work was time consuming but allowed us by December this year to build up a new synthetic key to the exact identification of those rodents' hairs. The initiative of this new simplified key system will be diffused among the NRL network in 2009 after the realisation of a user friendly excel version of this key in relation with the micrograph collection available from the intranet platform.

On request of NRLs, such as the Finnish or Danish ones, the CRL-AP started collecting sea mammal's materials. A collaboration with the Mathematical Modelling Unit of the North Sea (c/o Dr Jauniaux from the University of Liège) have been started begin of June for getting materials from autopsies of different species of sea mammals. This type of material is intended to find features of such cetacean bones when they are found in fish meals in order to be able to differentiate them from terrestrial animals. The underlying hypothesis is that those animals are subject only to microgravity and hence the bone lacunae shape might be different. This has never been studied prior. Material from seals and harbour porpoise has been collected. This material (bones, muscle and skin) has been partly prepared by the CRL-AP team in order to obtain pure material for the production of meals. A protocol for maceration was defined. By beginning of 2009, bone meals will be analysed by microscopy. Next year continuation of collecting material from other sea mammal species is planned.

The fatty acids profiles of feed fats have been determined by gas chromatography in order to discriminate fats according to their "species"

origin. Experimentations have been fully achieved and we are now treating the data.

JRC-IRMM have developed a gas chromatography method to determine specificaly 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 is implementing the method and participates to the Collaborative study organized by JRC in that framework.

An HPLC method proposed by prof. Gianfranco Piva to control the Protein Molecular Weight of hydrolysed protein was investigated. The aim of this method is to detect protein fragments having a molecular weight above 10000 Daltons the basis of known reference standards ranging from 1355 to 45000 Daltons.

The principle of gel filtration to control the presence of proteic fragments whose size is higher than 10.000 daltons, it is imperative to control on the one hand, the composition of the mobile phase and its setting in solution and on the other hand, the linearization of the proteic fragments (unfolded proteins).

While implementing the method, several difficulties were encountered.

i) During the preparation of the phase, the setting in solution of the various components of the phase was particularly difficult. Heating the solution did not really help and we had some recrystallisation in the HPLC itself. After several trials we came to the conclusion that it could relate to the content of EDTA in the mobile phase and the form of EDTA used to carry out this phase. 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. In order to improve solubilization of the EDTA in the buffer, the disodic salt (Na_2EDTA) will be tested. The method does not specify the type of EDTA.

The denaturation of proteins is taking place before the injection or during

elution on the column. The protocol of the studied method does not envisage denaturing the proteins present in the samples before chromatographic separation: the SDS (denaturing agent) is not mixed with the extracts but is placed in the mobile phase. Are the reaction time, reaction temperature and SDS concentration sufficient for a complete unfolding of the protein fragments?

Some more trials will be conducted before giving conclusions over the suitability of this method.

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. (8 p/m)

4.3.1 Transfer of validated PCR methods to the CRL

The last difficult issue before a validation study of a PCR method remains the transfer from a PCR platform (device and reagent) to another one. Taking advantage of different initiatives (collaboration with JRC-IRMM and European project SAFEED-PAP), CRL-AP accumulated useful data to determine the most efficient transfer strategy. A protocol was set up to determine on based on statistical data how to define for give platform was is the cut-off value of the PCR assay. The protocol that was set up is still under investigation to assess it is fit for purpose to be proposed for the transfer of the method to other platforms.

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 NRLs network

4.4.2 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

The PCR method transferability protocol developed by CRA-W will be evaluated in 2009 through an inter-laboratory study (cf 4.2.2.).

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, 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 samples bank. (14 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 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

Discussion with the Belgian feed sector (BEMEFA-APFACA) to receive some representative feed samples (e.g. feed samples containing dairy products or fish meal, samples from units where no PAPs was ever used,…) resulted in the delivery of over thirty samples of commercial feed matrix and fish meals. BEMEFA-APFACA delivered the samples by mid July. The blank matrices were carefully analysed during summer period by classical microscopy and PCR in order to ascertain their pure plant origin. The fish meals received by the CRL-AP were sedimented in order to study and define the present percentage of fishbones (or defining a mean f factor) actually present on the market. This was possible as the geographical origin and the diversity of fish species was encountered through the different sample materials ordered. Those results revealed to be crucial for the establishment

of the limit of detection according to the present microscopic method.

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 efforts made by the microscopy team of the CRL-AP for developing an on-line collection of best quality micrographs accessible to each NRL have been appreciated by the network. After a first set of micrographs posted on the CRL-AP Intranet by end of December 2007, a lot of work has been realised and the micrograph collection was extended by a second release in March 2007. The second release contained a lot of pictures helping at recognizing blood and blood derivative by help of dedicated stains, milk products, fossils and a lot of atypical fishbone pictures. By the end of the first half year the total number of pictures taken reached over 1500 wherefrom over 300 selected and published on the CRL-AP Intranet. By end of 2008 more than 400 pictures were published with the third release of the micrograph collection posted in October. The third release included micrographs of reference for the proper identification of common rodent and lagomorph's species which are suspected to provoke natural cross contaminations of feed. It must be emphasized that the selection process occurs according very severe quality criteria: adequate colour of temperature, sharpness of correct focussing, the pertinence of the contained information, an annotated scale bar and a CRL-AP engraved logo. The achievement of high quality pictures is made possible by intensive use of the Carl Zeiss AxionVision software acquired by the CRL-AP. Asking from some NRLs for participating to the construction of the image collection becomes frequent. In this respect it can not be excluded that micrographic training should be developed. The growing interest of this unique collection of pictures has extended the limit of the proper NRL network. In this respect access to the CRL-AP micrograph collection will be granted to the

IAG feed microscopy section members on next year.

During the period, the centralized and integrated management tool called Sample Management Suite (SMS) has been developed for the collection of samples, sediments, slides and micrographs. The 2007 release was only limited to the encoding interfaces. The 2008 release includes now the consultation screens, the search screens, the interfaces for listings creations such as the automatic updates of the micrographs collection files for the NRL network. The use of SMS has effectively increased the reliability of the sample traceability and the facilitated the sample collection consultation that previously revealed to be time consuming. The efficiency of the system is demonstrated and further developments are still in progress for its optimization. But reliability has a price and this was experienced during this first 2008 semester: it requires time for encoding rigorously all information of the samples in the SMS software and a labelling system had to be developed for the slide collection (based on a Brady LabXpert printer). This later labelling system has been developed during the second half year.

5 Workshops/trainings (2,5 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 quantification of PAP 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 asking of the Romanian authorities a dedicated on-site training on microscopy was realised mid January after agreement was received from DG SANCO. This training was organised for 3 persons (one from the Romanian NRL and two from other important accredited labs in Romania).

The selection of participant was made by the Romanian NRL in order to support their national network of accredited labs. The results of the training revealed to be utmost profitable for the participants who revealed to be extremely motivated. Certificates of participation were given to each participant.

An on-site training session was also realised in October for the Bulgarian authorities at the Bulgarian NRL in Sofia. The training was intended for 4 persons from different Regional Veterinary Services selected by the NRL. All participants were successful in the training. At the end of the training, they were all able to determine the blind samples. Certificates of participation were provided to each participant.

Continuation of training sessions for all NRLs as planned was set up during the second half year of 2008. In December, three training sessions of three days on microscopy were organised at the CRL-AP in Gembloux for NRLs. Participating NRL personal came from Denmark, Estonia, France, Austria, Ireland, Sweden, Finland, Luxemburg and Germany. The total number of person trained was of 9. In February 2009 the remaining member states' NRLs will have the same training so that by mid march all 26 NRLs will have followed at least one training session.
Request for training are still numerous and comes also from countries outside EU : Croatia, China, Serbia.

In the scope of the SAFEED-PAP project, the CRL-AP was asked to organised a training session on microscopic detection of PAP in feed intended for the former Eastern Block countries. This training was held in Vilnius and was part of the SAFEED-PAP workshop held on the 15th-16th of May. Participants to this training session were coming from Czech Republic (NRL), Hungary (NRL), Latvia (NRL), Lithuania (NRL), Poland (NRL), Romania (NRL), Slovakia (NRL), Croatia, Russia and Ukraine. The total number of participants was of 15 among them 11 were from NRLs.

In 2008, the training course notes were extended to new fields of knowledge on the microscopic detection of PAPs in feed : a new presentation on the identification of rodent hair species was introduced even as some new staining processes complementary to the present EC 126/2003 directive. Those amendments are profitable to the training sessions and hence contribute to the achievement of an complete course book on the detection of animal proteins that could be delivered to the NRLs. Reflexion on a larger diffusion of this tutorial support through the respective national accredited labs network via each NRLs is ongoing. In 2008, the CRL-AP also developed sets of permanent training slides based on the utilization a new resin. This permanent set of slides has been greatly appreciated by the NRL trainees who considered it as a great support allowing discussions among different participants. Several NRLs asked if in the future the CRL-AP could deliver a similar set of training slides for maintenance of their competences and home training of their personal.

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 2008

5.3 Provide specific workshop of experts from canditate 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. (0.5 p/m)

5.3.1 Organisation of a workshop training for Croatia and Turkey for the correct application of 126/2003/EC directive

Ms Zadravec and Ms Jaki from the Croatian Veterinary Institute participated to the training organised in the framework of the SAFEED-PAP project in Vilnius mid May. From the contact initiated at the IAG meetings and the

SAFEED-PAP workshop in Vilnius, the Croatian Veterinary Institute, the Institute of Veterinary Medicine of Serbia, the Russian Central Veterinary Lab were invited, after agreement from the DG-Sanco, to participate to the CRL-AP Proficiency Test 2008. Only the Russian institute could not participate due to custom problems on the samples. Croatian and Serbian institutes appreciated their participation.

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.
No activities forecasted in 2008.

The 5th of October 2008, The Walloon Agricultural Research Centre, Quality Department of agricultural products, that hosts the CRL-AP, received the visit of 560 persons. The CRL-AP team had the opportunity to give information to a wide public on its mission as well as to explain the BSE European problematic, its work and introduce people into the world of microscopy.