

**The European Union Reference Laboratory for the detection of
animal proteins in feedingstuffs**

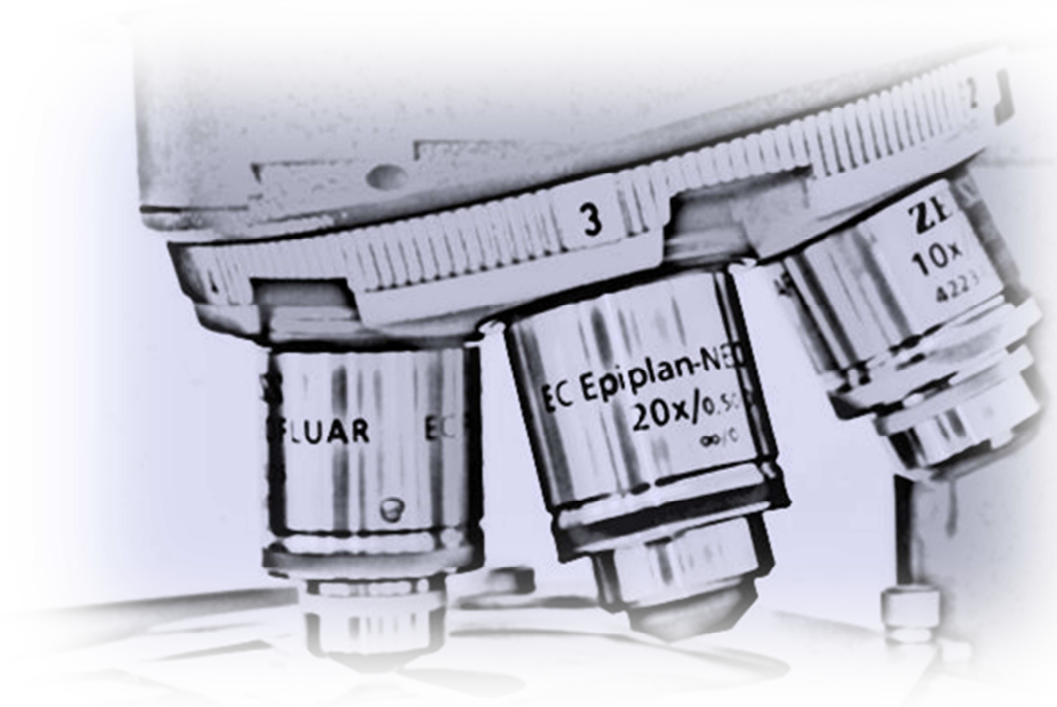


Walloon Agricultural Research Centre – CRA-W (Belgium)



Annual report on the 2012 activities

Public Version



1 Scientific advice and support to the European Commission (492 days)

1.1 Provide scientific and technical assistance to the European Commission in relation to the development of EC feed legislation.

To update the technical annex giving guidance on analytical techniques to detect processed animal proteins and to identify their specific origin.

- ***Meetings of 7th of March 2012, 29th of June 2012 and 12th of October 2012 in Brussels with DG-Sanco to make the point on the state of art of the analytical techniques to support the partial release of the feed ban.***
- ***Throughout 2012, the EURL-AP helped in the redaction of Annex VI of the upcoming regulation amending Regulation (EC) N°152/2009 as regards the methods of analysis for the determination of constituents of animal origin for the official control of feed.***
- ***The EURL-AP edited 8 draft versions of Standardized Operating Procedures (SOPs) to support the modification of the feed ban and the reintroduction of non-ruminant processed proteins in aquafeed. The EURL-AP initiated a PCR working group gathering experts from the Danish, Dutch, German, Italian and Luxemburg NRLs. The 1st meeting of the WG took place the 1st of October 2012 in Gembloux. Based on the comments of its members, the texts of the SOPs are under revision before a second round in the NRLs. The versions 1.0 of these documents will be publicly available on the EURL-AP website.***

1.2 Upon the request of the European Commission or in order to fulfil his role as EURL, participate in 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 EURL-AP activities.

- ***SCoFAH and TSE Working group meetings (Brussels, 14th of March, 10th of May, 18th of June and 17th of September 2012): the EURL-AP presented during the meetings updated overviews of the situation regarding the validation of***

the PCR test for the detection of processed ruminant proteins and its implementation in the NRLs as well as the developments/modifications of the microscopic protocol, of the Annex VI and of the Standard Operating Protocols (SOPs).

- *In January 2012, the EURL-AP participated to a TAIEX on the feed ban of animal proteins in Sarajevo, Bosnia-Herzegovina.*
- *The EURL-AP team was involved in the organisation and participation of the BTSF (Better training for safer food) platform on TSEs as coordinated by JVL Consulting. The EURL-AP was requested to prepare lectures (1) on the light microscopic detection of PAP according current legislation, (2) on the PCR method implementation and (3) on the alternative methods for future improvement of the existing methods. The involvement of the EURL-AP in these BTSF workshops initiated in 2011 continues in 2012. In 2012, the EURL-AP presented these lectures once in Gembloux, Belgium (February), once in Manchester, United Kingdom (July) and did the same exercise twice in Nicosia, Cyprus (March and November). The total number of participants – majorly official control veterinarians from EU and foreign countries – for these 2012 sessions was reaching 100 people.*
- *The EURL-AP participated to two international conferences: 1) The 4th International Feed Safety Conference took place in Beijing, China from the 11th till the 13th of September 2012; 2) The XV International Feed Technology Symposium joined to a workshop of the COST program “Feed for Health” took place from the 3rd till the 05th of October 2012 in Novi Sad, Serbia. For both conferences, two lectures were presented: “Reintroduction of processed animal proteins in feed: filled gaps and gaps to be filled” and “Implementation of the real-time PCR as official method of detection of processed animal proteins in the European Union reference laboratory network”.*
- *The EURL-AP was invited to participate to the VIIth meeting of the EFSA scientific network on BSE-TSE risk assessment held in Parma on 10-11 October 2012. On demand of the EFSA biohazard panel, a lecture was given by the EURL-AP about the state of art of testing feedingstuffs for the presence of*

animal proteins of animal origin.

- *In the framework of a bilateral project between Belgium and China (WBI/MOST project), the possibility to implement EC protocol in the official control has been discussed.*
- *Several meetings with JRC-IRMM (Dr Christoph von Holst and Dr Ana Boix) were held to discuss the development of alternative methods to support the partial ban of PAP. One join paper on the validation of the NIRM paper has been published.*

1.3 Upon the request of the European Commission or in order to fulfil his role as EURL, 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 EURL-AP activities.

Among the possible activities in 2012 there might be the starting of a CEN working group (within CEN TC327) defining guidelines for PCR in detection of PAP in feed.

- *The EURL-AP was present in the IAG annual meeting, held in Freising, Germany from the 12th till the 14th of June 2012. Two lectures about the results of the EURL-AP Proficiency Test Microscopy 2011 and the latest decisions regarding the TSE Roadmap 2 were presented by the EURL-AP.*
- *On 26 and 27 of September 2012, the EURL-AP attended the IAG autumn meeting in Hamburg, Germany. A lecture on the latest development concerning the microscopic part of the revised annex VI was presented by the EURL-AP.*
- *Representatives of CCL and EFRA visited the EURL-AP in December 2012 with as main goal to discuss on the position of the EURL-AP on immunological assays. The situation was thoroughly explained. EURL-AP is not against immunological assays which industry can use if they want for autocontrol but it is impossible – and this is not the position of EURL-AP but of DG-Sanco fully supported however by EURL-AP – that an immunological method which in some cases is much less sensitive would be recognized as an official method*

because this might lead to situations of conflicting results between PCR and immunological assays. A second topic handled was the use of the ruminant PCR assay validated by the EURL-AP that gave some strange results at CCL. A possibility of bad reagents was highlighted and small quantities of reagents of the EURL-AP were provided to allow the lab to test the hypothesis that was raised. Finally, CCL decided to use the same provider of primers and probe than EURL-AP.

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.

1.5 To keep at EURL-AP 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 EURL-AP lab.

1.5.1 To maintain scientific awareness in general about techniques that might be helpful in relation to topics of interest of the EURL-AP. In this context the use of digital PCR seems essential for the calibrants to be used in PCR.

1.5.2 To maintain the accreditation scope.

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

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

1.5.5 Submission of the dossier for the accreditation of the organisation of interlaboratory studies.

- ***In January 2012, Dr Marie-Caroline Lecrenier, veterinarian, started working for the EURL-AP in replacement of Camino Belinchon Crespo. She brings her expertise in animal physiology and nutrition and contributes to the development of complementary analytical methods.***
- ***People (Aline Marien, Julie Hulin) from the EURL-AP were trained to use the***

digital PCR platform by the team of the JRC-IRMM and prepared the calibrants used for the validation study of the ruminant PCR method and the PCR implementation study organised during the first half of 2012.

- *EURL-AP subscribed to a proficiency test (PT) scheme for the detection of animal proteins in animal feed by PCR organised by the AHVLA (Luddington, UK): 4 sets of 5 samples to be analysed per year. The sets of samples distributed in January, April, July and October were successfully analysed.*
- *EURL-AP subscribed also to proficiency tests for the detection of animal proteins in animal feed by classical microscopy organised by the AHVLA. Sets of 4 samples were received in March, June, September and December 2012. The results obtained by classical microscopy were successful.*
- *EURL-AP also participated to the IAG ring test in 2012 for animal protein detection in feed by light microscopy. A set of 4 samples was received and analysed in March 2012. The results obtained were successful.*
- *After the successful validation of a PCR ruminant probe, the EURL-AP decided to extend its accreditation scope to this new target. Experiments and analyses were initiated to complete the accreditation file under redaction for the audit planned during the autumn by the Belgian accreditation body, BELAC. The accreditation dossier introduced for this was accepted and the scope was extended to the use of the ruminant PCR assay.*

1.6 On the request of DG SANCO or the NRLs, to perform analyses on samples with disputed results.

- *In 2012, several requests of official counter analysis were submitted by NRLs:*
 - *2 samples from feedingstuffs, February 2012*
 - *1 sample from krill meal, February 2012*
 - *1 sample from feedingstuff, May 2012*
 - *1 sample from greaves, May 2012*
 - *2 samples from blood meals, July 2012*
- *Some analyses also performed for helping NRLs were not subjected to official reports (e.g. 3 samples submitted by a NRL for PCR ruminant analysis).*

2 Coordination of activities of NRL network (283 days)

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

2.1.1 *Information collection and validation.*

2.1.2 *Maintenance of the website.*

2.1.3 *Update and test of the information system and validation.*

- ***A new version of the EURL-AP Website has been prepared by the CRAW ICT team. This new website has a content which is largely identical to the existing one but is developed on a new platform totally owned by the CRAW. It will offer the following advantages:***

- *Compatible with mobile platforms,*
- *More user-friendly navigation,*
- *Easiness of content management by the EURL-AP,*
- *Straight access to the legal texts and related SOPs,*
- *Direct access to the intranet.*

Furthermore the old version of the intranet will be abandoned for a new interface integrated in the public website. The intranet pages are only accessible, according to the user rights and password, to the members of the NRL and IAG. The micrograph collection has been revised to offer better visualisation and sorting of the pictures. A search engine was also developed allowing users to make requests on the micrograph database.

The new integrated website – intranet will be launched by begin 2013.

- ***During the 12 months period, 7256 web pages from the public site were visited through 2493 visits by 1355 unique visitors coming from 78 countries and 468 network locations throughout the world.***

Regarding the intranet, 12161 hits were recorded during this period. 7 new NRL members, 3 EC members, 2 IAG members and 1 CRA-W staff member joined the network. 6 NRL members left the network.

For the public website and the intranet, all those statistics are increasing against the same period in 2011. Monthly updates were carried out. Maintenance tasks were provided to maintain security and confidentiality

systems and to operate backup.

2.2 Prepare and send a six-months newsletter for NRLs.

- **First year newsletter was prepared and posted on the intranet on June 2012. The 13th newsletter's content consists in the minutes of the 6th EURL-AP annual workshop held in Berlin from the 17th till the 19th of April 2012. This newsletter represents a workshop summary, including comments, decisions and brief abstract of the lectures. Newsletter N° 13 is attached to this report.**
- **A second newsletter was published on December 2012. This newsletter 14 announced the 7th EURL-AP Annual Workshop which will be held in Ljubljana in 2013. Some other important subjects were also pointed out as the forthcoming legislative changes, the last microscopic proficiency test and the planned program for 2013 regarding the SOP redaction, the development of complementary methods, the ongoing PCR proficiency test, the NRLs network support and the new version of the EURL-AP website. Several other announcements for conferences and meeting were also included. (Newsletters 13 and 14 are attached to this report).**

2.3 Organisation of the annual EURL-AP meeting/workshop.

2.3.1 Organisation of the 6th annual EURL-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.

- **As already mentioned at point 2.2, the 6th EURL-AP annual workshop was held from the 17th till the 19th of April 2012 in Berlin, Germany. Once again, as in 2011, the EURL-AP team didn't have to deal with the logistic organisation (meeting room renting, catering, hotel, etc), it was taken in charge by the Bundesinstitut für Risikobewertung (BfR).**
 - **The programme of the first day of meeting included the following items of presentation and discussions: BfR presentation and German NRL network organisation and activities and a presentation of the**

Commission legislation on TSE. The day concluded with the presentation of the Microscopy revised method and PCR method of the annex VI of EC/152/2009.

- *The second day of the workshop, the agenda dealt with: a summary of the 2011 activity report of the EURL-AP, the results of the 2011 EURL-AP Proficiency Test Microscopy 2011 and a presentation about the monitoring of lab cross-contamination. The morning continued with a presentation about the harmonisation of analytical methods for determining insoluble impurities in rendered animal fat, the presentation of the Croatian NRL and the activities presentation of the Institute for Hygiene and Veterinary Public Health of Bucharest. The afternoon was focused on PCR with the following items: Assessment of the ruminant PCR method of TNO Triskelion, the validation of the ruminant PCR method of TNO Triskelion, the presentation of at that time upcoming PCR implementation Test, the production of calibrants, the PCR analysis of sediments and a presentation of the Polish NRL about a conventional PCR method for the detection and species identification of processed animal proteins.*
- *The third day lectures included the following items: A presentation of RIKILT about the immuno-assay methods and the integration of LM and PCR methods. The day and the workshop finished with the PCR program for 2012, and the workshop conclusions. The localisation of the workshop 2013 will be in Ljubljana (Slovenia).*
- *This year again representatives of both, the microscopy method and the PCR method were invited to the workshop 2013. In most cases, 2 persons from each Member State were present, in other cases, the representative of the two methods turned out to be the same person.*
All the NRLs were present at the workshop.
- *Minutes of the workshop were recorded in the 13th EURL-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.

This task has been merged with task 3.3.

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

2.6 Prepare the six months and annual reports of activities according to the report guidelines transmitted by DG SANCO.

Finalizing the annual report of 2011 in the beginning of 2012.

Redaction of the first 6-months report (January 2012 - June 2012) done.

3 Interlaboratory studies and quality assurance. (558 days)

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. Preparation, production, calibration and distribution of plasmids as calibrants for the PCR methods. This task includes the preparation of the samples for the interlaboratory studies.

3.1.1 *Definition of the needs.*

3.1.2 *Collection of the raw materials.*

3.1.3 *Control of the raw materials.*

3.1.4 *Production of the samples or plasmids.*

3.1.5 *Test of the homogeneity of the samples produced / quantification of the plasmid calibrants.*

3.1.6 *Report on the produced sample / plasmid calibrants.*

3.1.7 *Distribution of the samples / plasmid calibrants.*

- ***The validation study of the TNO Triskelion bv ruminant PCR method initiated at the end of 2011 with the invitation letter was continued and finalised in 2012. All the necessary material was prepared by the EURL-AP and sent to the 12 participants the 31st of January 2012: sets of 3 ruminant calibrants at well-***

determined copy numbers of the target, sample sets constituted of DNA samples at the 4 concentrations of ruminant PAP – 0.1, 0.0250, 0.0125 and 0 % w/w of ruminant PAP – and the mastermix. The calibrants were prepared starting from a suspension with known copy numbers of the target thanks to use of the digital PCR platform of JRC-IRMM (Biomark™, Fluidigm). After appropriate dilutions, each calibrants was aliquoted in plastic tubes containing 450 µl of the plasmid solution. The final copy number of each calibrant and their homogeneity were determined on 10 vials taken in a random way using the same digital PCR platform. The DNA samples were prepared by dilutions from one compound feed spiked at 10 % w/w of cattle PAP while the negative samples (blanks) were DNAs extracted from 4 different non ruminant materials (vegetal ingredients or fishmeal). In addition, the participants received an Excel file for the automatic determination of the cut-off value of their PCR platform and for the reporting of the results. The deadline for the return of the results was the 23rd of February 2012 but the last participants sent back his results the 1st of March 2012. The method was officially declared validated the 9th of March 2012 on the EURL-AP website and a report was edited in June on the website in draft version (attached to this report).

- *A meeting was held the 8th of May 2012 at the JRC-IRMM (Geel, Belgium) with Drs Emons, Trapmann and Corbisier to discuss about the production and the distribution of plasmid calibrants. The discussion were very fruitful and the JRC-IRMM responsables showed a great interest for this project but the public release of the ruminant PCR method will be an important step before to go further.*

The 30th of November 2012, a second meeting was held in the EURL-AP facilities (Gembloux, Belgium) with Dr Corbisier to discuss how to continue the collaboration. The transfer to the IRMM of the material necessary to launch the production of the calibrants was agreed. Until the calibrants will become certified reference materials (CRM), the EURL-AP will be in charge of the distribution of the calibrants that will be only reference materials and there will be no mention of the IRMM on the labels.

- ***The plasmid containing the pig target was prepared and the production of the calibrants for the detection of pig DNA was initiated. Unexpected problems occurred during the quantification of the calibrants. These problems are most probably due to a bacterial contamination with a nucleic acid degradation as consequence. The production of a new batch of calibrants was decided.***

3.2 Organization of two interlaboratory studies for the determination of PAPs in feed using classical microscopy and PCR respectively.

3.2.1 *Redaction of the report of the EURL-AP microscopy interlaboratory study of autumn 2011.*

3.2.2 *Definition (with the collaboration of the DG-Sanco) of the objectives of the ring trials to perform at the end of 2012.*

3.2.3 *Preparation of the interlaboratory studies 2012.*

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.*

- ***The results of the EURL-AP Proficiency Test Microscopy 2011 were analysed and compiled in a report that was published in March 2012 in working document version for the NRL network. In this way all NRLs received the report before the 2012 workshop in Berlin. During the workshop the results were presented and NRLs were allowed to react. After the workshop a final version of this report (attached to this report) was prepared and posted on the EURL-AP website in May 2012. Results from the NRLs indicated a very good global performance of the network. The disclosure of animal particles in fat according to an optional protocol from Annex VI happened satisfactorily. Further analysis of the results revealed a serious lack of sensitivity for the detection of hydrolysed feather meal within fish feed. Corrective actions and request for improvement were asked for two NRLs that revealed to be underperforming. Both NRLs solved this issue rapidly and satisfyingly.***
- ***In February 2012, the EURL-AP sent to the NRLs a PCR roadmap letter to warn the labs of following steps/actions for the PCR implementation and to prepare the network to the upcoming PCR interlaboratory study (firstly called***

“proficiency test” and renamed “implementation test” on the request of the NRLs). Sets of the 3 ruminant calibrants prepared by the EURL-AP for the validation study of the ruminant PCR method were sent the 19th of March 2012 to the 26 NRLs of the EURL-AP network. In between, the NRLs had to sign a non disclosure agreement (NDA) with TNO Triskelion bv to have access to the PCR protocol. The NRLs received also a SOP describing the way to determine the cut-off value of their PCR platform using calibrants and an Excel file determining the cut-off on basis of the data provided. With the calibrants and as the PCR implementation involves also the DNA extraction, the NRLs received also 1 gram of a sample at 0.05 % w/w of bovine PAP in feed used by the EURL-AP as DNA extraction positive control.

The sample sets prepared by the EURL-AP were constituted of 4 feed samples (samples to extract the DNA according to the protocol of EURL-AP) and 6 DNA samples (ready to be used for the PCR). Each mix was aliquoted per 10 grams. For the DNA samples, 3 levels in ruminant PAP were used: 0, 0.1 and 0.2 % in weight of bovine or sheep PAP in blank feed.

A homogeneity test was realized on the feed samples containing ruminant PAP that were intended for the implementation test. Ten aliquots of 10 grams were taken randomly, 2 weight portions were taken per aliquot and the DNA was extracted according the EURL-AP protocol. These extracts were analysed by PCR with the ruminant target. For the blank feed sample, the analysis was realised on 2 aliquots of 10 grams.

On 3rd of April 2012, EURL-AP sent a samples set of the implementation study to each of the 26 NRLs of the EURL-AP network.

EURL-AP sent a sample set of the implementation study to 2 additional laboratories outside the EU (Croatia and Norway).

As part of a corrective action, the EURL-AP sent in June 2012, a new sample set constituted of 4 feed samples (meals to extract the DNA according to the EURL-AP protocol) to 6 NRLs having had bad results on the feed samples of implementation test. The 6 NRLs sent back correct results and their cases were closed.

Concerning the 4 remaining NRLs having no results, two of them sent finally

correct results in September and in November. The two others being unable to provide any result, they were invited to realise the study in the EURL-AP facilities in January 2013.

A report (attached to this report) was prepared and posted on the EURL-AP website in May 2012.

- *In autumn 2012 the EURL-AP team designed and prepared the proficiency test for microscopy. The official announcement was made on the 4th of September. The preparation of the samples as well as the homogeneity study took place in September and October 2012. Homogeneity study was fulfilled before sending of the sample set on the 5th of November 2012. The sample set used was totally free of any fish material as it was designed to investigate on the specificity problems for fish. Two terrestrial containing samples were added to the sample set which was consisting in 7 blind samples. All NRLs were included in the study (including for the first time the new Croatian NRL). As for previous years some non-European countries were invited to participate (Australia, Argentina, China, Japan, Norway and Serbia). Results of the analyses were collected by end of November and study of the results initiated in December. The report will be issued in 2013.*
- *The work regarding the development of the NIR Line Scan Imaging analysis of the feed ingredients and the compound feeds has been continued. The NIR Line Scan Imaging technique will be used to test the homogeneity of the samples prepared for the ILS organised in the framework of the EURL-AP activities.*

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 EURL-AP activities. 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.

3.3.1 Definition of the needs of the NRLs.

3.3.2 *Provide the requested help to the NRLs.*

3.3.3 *Preparation of syllabi including all the information needed for an appropriate detection of processed animal proteins in feedingstuffs.*

- ***According to the results from the EURL-AP Proficiency Test Microscopy 2011, two NRLs were classified as underperforming. One NRL solved the issue and delivered the requested information explaining the case by email. The case was closed. The second NRL replied by a report and was asked to perform complementary test. This NRL also requested an on-site visit in order to investigate on possible ways to improve its performance. By end of June 2012 the EURL-AP visited the facilities hosting the NRL. Upon this visit, recommendations were made to the NRL and the case was closed.***
- ***A reply was provided in October to an official request of a Polish laboratory (Instytut Zootechniki, Balice) that wanted to participate to the PCR proficiency tests. The position of the EURL-AP is that if the laboratory is not the NRL, it cannot be added to the list of laboratories for the proficiency tests.***

3.4 Proficiency study for GTH detection (with the collaboration of JRC-IRMM)

3.4.1 *Redaction of the report of the EURL-AP Proficiency Test GTH of Autumn 2011.*

3.4.2 *Definition (with the collaboration of the DG-Sanco) of the objectives of the GTH ring trial to perform at the end of 2012.*

3.4.3 *Preparation of a proficiency test in 2012.*

3.4.4 *Invitation of the NRLs to participate.*

3.4.5 *Packaging and sending of the samples.*

3.4.6 *Collection of the results.*

- ***This activity was no longer a priority for DG Sanco and thus cancelled.***

4 Development of analytical methods and tools. (296 days)

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

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 EURL-AP to these initiatives.

4.1.3 Establishment of the needs in the development of methods.

4.1.4 Reporting harmonisation of analytical methods for determining insoluble impurities in rendered animal fat ('tallow') (Methods ISO 663 and AOCS Ca 3a-46).

- ***The tests of specificity with pig targets under evaluation were completed during the first half of 2012.***
- ***The tests of specificity with poultry targets also continued during the first half of 2012.***
- ***As a consequence of the work of the PCR working group, a quality criterion of the cut-off for the detection of ruminant DNA was defined in the SOP. A new version of the Excel file for the determination of the cut-off was developed. It mentions automatically on the first sheet the number of copies at the Ct values corresponding to the cut-off.***
- ***EURL-AP participated to an interlaboratory study conducted by RIKILT for the validation of 2 immunoassays: Reveal for Ruminant in Feed and MELISA-TEK Ruminant kit. The study consisted in 3 rounds: a training set of 7 samples delivered in February, an entrance set of 7 samples provided in March and qualifying for the final validation set of 42 samples analysed in April.***
- ***The report on the assessment of the TNO Triskelion ruminant PCR assay with a battery of performance parameters was presented to TNO Triskelion b.v. on the 11th of May 2012. The full report exists already as a draft and may become public after the 09th of September 2012.***
- ***During the first half year the study on the analytical methods for determining insoluble impurities in rendered animal fat ('tallow') (Methods ISO 663 and AOCS Ca 3a-46) was finalised. Statistical tests were performed and subsequently allowed to write the final report (attached to this report) by May 2012. The report was sent the 29th of May 2012 to DG Sanco. The outcome of the study revealed major difference between methods. ISO 663 method associated with petroleum ether and filter crucible was evaluated as***

the best method within the context of assessment of residual BSE risk in tallow by a best PAP recovery rate. However, critical point remains sampling of such heterogeneous materials which is essential to provide an efficient method adapted to the laboratory.

- *The CRA-W supported the acquisition of a new NIR microscope intended to the EURL-AP microscopy team. This new equipment was delivered in May 2012 and formation of the personnel started in June. Time was also spent to adapt the EURL-AP microscopy lab to host this equipment. NIR microscopy will help to analyze and identify the origin of animal particles in feed, especially particles from the sediment in combination with the PCR analysis.*

4.2 Contribute to the development of complementary analytical methods necessary to assure the correct implementation of official methods and explorative or alternative methods

4.2.1 *Test of methods for the detection of plasma vs dairy products.*

- *Complementary methods for differentiating blood products vs milk products are necessary in case of PCR positive reaction to ruminant DNA. The EURL-AP started investigating on possible alternative methods in 2012. Contacts were established with the Veterinary Faculty of the University of Liège and with the CER group (Centre d'Economie Rurale) in Marloie, Belgium. Investigations are based on specific immunological interactions against proteins or other constituents that are exclusively present in blood products, plasma and milk. During the second half of 2012, the target of the immuno-assay was determined and fibrinogen was selected as it is a large glycoprotein specific for plasma. The CER started the production of the antibodies against bovine and porcine fibrinogen which required about 3 months. The purification and the first tests of these antibodies will occur during 2013.*
- *Some contacts were also established with the Dr. Yun-Hwa Peggy Hsieh from the Department of Nutrition, Food & Exercise Sciences, Florida State University - Tallahassee, USA, regarding a monoclonal antibody raised against an antigenic protein of the ruminant globins which was developed by her laboratory and that might be able to specifically detect ruminant blood in*

heat-processed animal feed by a competitive enzyme-linked immunosorbent assay (cELISA). Blind samples have been sent to this laboratory in September 2012 to evaluate the methods on EURL-AP test samples. The first results are not satisfactory, perhaps due to the low globins concentration in the samples sent or of a different blood meal processing in Europe. Further tests on new samples are envisaged for 2013.

- *The EURL-AP also investigated on the PCR analysis on particles from the sediment. Effectively in case of a positive reaction for ruminant DNA in a fish feed fortified by animal by-products such as porcine processed animal proteins which generates microscopic detection of terrestrial bones, it has to be controlled that the ruminant signal does not originate from the bone particles from the sediment as it may arise from other authorised ruminant product (dairy products, or bakery by-products). The main challenge is to obtain sediments with clean particles i.e. free from any environmental – or matrix bound - DNA presence. Development and research conducted by the EURL-AP during 2012 intends to develop a cleaning protocol of the sediment. Various cleaning protocols and extraction methods were tested. For the moment, none of them are totally satisfactory because sometimes the cleaning has not completely eliminated the DNA of the authorized products while reversely sometimes the treatment is too harsh and the DNA of the authorized product has disappeared but also the one that should have been kept in the sediment.*
- *During the second half of 2012, another complementary method, the fluorescence in situ hybridization (FISH) was investigated. The aim was the development of a method which combine the advantages of light microscopy and PCR to give a “tissue and species-specific” method. FISH is a powerful technique for detecting and localizing by fluorescence microscopy a specific DNA sequence in a specific tissue. In our context, this method should be used to determine if a specific PCR signal has or not a bone origin. The first step was to obtain an overview of the method in literature. Unfortunately, the method was never applied on large processed bone particles as PAPs. Therefore, the second step was to develop a protocol and to calibrate it as*

regards the buffer, the hybridization temperature, the probe, the labelling... Of note to highlight the fact that FISH technique also requires an efficient and accurate cleaning of particles before submitting the latter to the hybridisation itself. The benefits of the development of cleaning methods (cf previous point) will thus help to achieve results more quickly. The first results are encouraging but further improvements and an assessment study in the EURL-AP laboratory will be done during the first half of 2013.

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.

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 former interlaboratory studies regarding PCR methods, define the strategy for the optimum implementation in the NRL.*

4.3.3 *Preparation of EURL-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.*

- ***All the NRLs signed a Non Disclosure Agreement (NDA) to have access to the PCR ruminant protocol and participate to the PCR implementation study conducted by the EURL-AP. The SOP was diffused to the NRLs the 19th of March 2012. Since the announcement of the validation of the TNO PCR ruminant method, any public control lab having signed the NDA can have access to the protocol; it is already the case with 3 Italian labs and one Norwegian lab.***
- ***On 10 September 2012, six months after the short report stating the TNO ruminant PCR assay was validated, the TNO PCR ruminant method was made public on the website of the EURL-AP (this was a consequence of the non-disclosure agreement signed between TNO and the EURL-AP).***

4.4 Performing EURL-AP available methods or adapting them on outbreak material

to make them available for the NRLs network.

4.5 Extension of the samples bank with a special focus on specific animal material. 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 EURL-AP samples bank.

4.5.1 *Establishment of the specification for the EURL-AP 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 and maintenance of the samples bank.*

- ***During the first half year efforts to get new samples were continued. According to the wishes and the priorities of the Commission regarding a relaxation of the feed ban for the aquaculture, contacts were taken with FEFAC in April to organise a fish feed sampling campaign for the enrichment of the sample bank. Samples were delivered by major fish feed producers. Up to 30 fish feed samples were collected allowing the EURL-AP to get a representative view of the fish feeds used in the EU. Analyses of the samples by light microscopy, PCR, NIR microscopy as well as by other methods in development were started. This was continued in the second half year of 2012.***
- ***Samples collected by the FASFC (Belgian Federal Agency for the Safety of the Food Chain) in 2011 were still analysed in 2012 by complementary methods. These complementary tests allowed building up a complete synthetic table for development and research purposes.***
- ***Tasks related to an improved storage and recording management of the sample collection were undertaken by the EURL-AP technicians in May 2012. Elimination of old samples was carried out in order to increase storage potential of the sample bank.***

- ***The implementation of the NIR method to check rapidly the composition of the EURL-AP samples has been concluded. CRA-W is performing analysis of compound feeds by NIR under ISO17025 accreditation scope. The control can be done at the reception stage of the sample and before to include the new sample in the sample bank.***

5 Workshops/trainings. (140 days)

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.

5.1.1 Light microscopy training

5.1.2 PCR training

- ***Two training sessions for light microscopy were organised in the first half year of 2012 at the EURL-AP facilities. A first one held place in February 2012 for NRL collaborators from France and Ireland. A second training session was organized in June 2012 for the Swiss, Czech and Croatian NRLs. On this last occasion a full support was given to the new Croatian NRL: training samples, access to the EURL-AP intranet as well as the DVD on the microscopic method implementation.***
- ***No general PCR training session was planned during 2012. However, on the request of the Croatian NRL representative who visited the EURL-AP facilities for light microscopy training, an additional afternoon was dedicated to PCR explanations. Mrs Zadravec received the same PCR training manual with the EURL-AP protocols as the one distributed to all the other participants attending the previous PCR trainings organized by the EURL-AP. Furthermore, on the request of the Italian NRL, Mrs Beatrice Brusa came in the EURL-AP facilities from the 8th till the 12th of October 2012 to receive a training on the PCR. She observed the technicians of the EURL-AP all along the analysis of a sample and she also had the opportunity to manipulate.***

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.

Croatia could be selected in 2013.

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.

- ***Additional DVD copies on the PAP detection by PCR analysis were provided on request to NRLs (Italy and Greece), to control labs from EU (LTZ Augustenberg – Karlsruhe, Germany; IRTA – Barcelona, Spain), to the Croatian NRL that will become officially member of the EURL-AP network in 2013, to the Norwegian NRL (NIFES- Bergen, Norway) and to the Institute of Veterinary Medicine (Belgrade, Serbia).***
- ***Following the development of the EURL-AP online micrographs collection, an article on the “Definition of key parameters for constructing an online reference micrographs collection of processed animal particles in feed” produced in August 2011 was accepted and published in April 2012 in the journal BASE (Biotechnologie, Agronomie, Société et Environnement). The article is attached to this report***
- ***In November, EURL-AP provided 7 sets of ruminant calibrants to the German NRL. The material was used by the participants of a training.***