EURL-AP NEWSLETTER N°38
July 2025

Dear NRL Colleagues,

We are pleased to diffuse this new EURL-AP Newsletter summarizing our discussions during the 1st session of the annual workshop held on Teams on the 15th May this year.

We thank the participants and the speakers for their active contributions.

Enjoy the reading of the minutes and looking forward to hear from you soon!

The whole EURL-AP team

18th EURL-AP workshop agenda

15th May
(1st online session)

09:00	1	Welcome and presentation of the agenda	O. Fumière
09:15	2	EURL-AP workprogram 2025-2027	O. Fumière, M.-C. Lecrenier, P. Veys
09:45	3	Open discussion	All
10:15	Coffee break		
10:30	4	Combined Proficiency test 2024: results and discussion	O. Fumière, P. Veys
11:15	5	PT 2024 results by MS	M.-C. Lecrenier
11:30	6	New batch of ruminant calibrant	O. Fumière
11:40	7	PCR mastermixes	A. Marien
12:00	8	Conclusion and end	O. Fumière

Welcome and presentation of the agenda

Olivier Fumière welcome the participants for the 18th EURL-AP workshop. This year the workshop is done on-line. A second session should take place during the Autumn. The date is still to be fixed but it should be in October or November.

The agenda of the session is then briefly presented. The rules to follow during the meeting are also reminded.

EURL-AP workprogram 2025-2027

The EURL-AP work program 2025-2027 is still not officially adopted but the grant is in preparation (at the moment of writing this newsletter, the grant is signed). The major activities are presented by techniques:

Light microscopy

Improvement of the double PE/TCE sedimentation is the priority for the submitted work programme. An internal study on the values of the different fractions collected by PE/TCE sedimentation will be undertaken. The purpose of this study is to simplify the observation work by possibly investigating only on the sediment and the final flotate. Since these two fractions are concentrating the heaviest fragments (bones and minerals) and the lightest ones (muscles, cuticles, etc…). One of the outcomes of this study will be a simplification of the SOP on the combination of methods or a revision of Annex VI of EC/152/209.

PCR

A plasmid containing simultaneously the inserts with the target DNA sequences of the three official methods will be built.

This plasmid will be used by JRC to produce a new ERM (European Reference Material) able to be used as a single calibrant to set the cut-offs of the PCR methods for the detection of ruminant, pig and poultry DNA respectively.

Besides this activity, an effort will be done to continue the assessment of PCR mastermixes from different companies and to enlarge the list of recommended mastermixes.

Mass spectrometry

The MS development over the next three years was summarized through three main steps. Each step depends on the results obtained in the previous one.

The first step is the organisation of an interlaboratory study (ILS). This study is ongoing. Five laboratories of the MS expert group are participating. All results have been submitted. Conclusions will be diffused during the second half of this year.
Based on the conclusions of the ILS, the next step will be the drafting of a SOP.
This method will then be transferred to a larger group of laboratories to proceed to the validation study.

Specific requests from DG SANTE were also included in the work programme:

- light microscopy, PCR, mass spectrometry as well as infrared spectroscopy will be tested to determine whether industrial used cooking oils were used to cook animal materials (e.g. burgers) or not (e.g. chips production).

- EURL-AP with the support of its HP Expert group and under the coordination of the Italian NRL will continue the assessment of a protocol (Povolo *et al.*, 2022) for the quality control of hydrolysed proteins before its eventual validation through an interlaboratory study.

Besides these activities, EURL-AP will continue the "basic" ones namely PTs, workshops, newsletters, scientific support to the Commission as well as to the NRLs, counter analyses, micrograph collection, audits, sample bank, website/intranet and trainings.

Discussion:

- *The aim of the work that will be done concerning the hydrolysed proteins is to determine if the HPLC-SEC protocol proposed by an Italian company and described by Povolo et al. (2022) is relevant for the control of hydrolysed proteins in general and to determine what is still needed before to go eventually to a validation study. Samples from different companies (in the Netherlands, Germany, Belgium and Norway) should be provided soon.*

***It is requested to the network to communicate the points of contact of the companies producing hydrolysed proteins to the Italian NRL** which centralize these activities and to the EURL-AP.*

*It is also agreed that **the German NRL joins the expert group** already gathering the NRLs from the Netherlands, Poland and France. **This expert group remains open to new members meanwhile they have expertise in the topic.***

- *Concerning the MS, the final goal is a validation of the method and its introduction in the legislation as an official and complementary method to light microscopy and PCR. For the moment, it is not clear if the Commission will ask to implement the method in all Member States or if it will be limited to few reference labs able to perform the analysis. It will also depend of future lifting steps of the feed ban.*

Open discussion

For this open discussion, topics were asked to the network. The topics collected during the registration to the workshop were organised according to techniques. Some of them were answered more briefly as they are addressed during presentations.

PCR

- *The mastermix from Diagenode (ref DDML-D2-D600) is still present in the list of recommended mastermixes. Some participants still used this mastermix during the PT 2024. A mastermix is provided by LACAR with the same reference number and has probably the same composition. Contacts were initiated to have more information.*
- *Pig and poultry PCR tests are slightly different concerning the reaction volume. An optimisation to have the possibility to perform the two tests on a same plate is proposed. The possibility to perform the 2 tests on the same plate already exists without changing the reaction volumes. EURL-AP will contact the Dutch NRL after checking the data sent previously.*
- *The development of multiplex PCR methods is proposed. The production of a single calibrant for the three PCR methods does not mean that EURL-AP envisages to work on multiplex PCR methods. Multiplexing can have an impact on the sensitivity of the methods and induces problems of competition for the reagents.*

Mass spectrometry

- *Main information about plan for MS development were given in the work programme presentation or will be discussed in the conclusions of PT analyses by MS. The focus will continue on ruminant peptides as request by DG Sante.*
- *Uta Herfurth (German NRL) gave an overview of her work on mass weight (MW) distribution of hydrolysates and on the differentiation of porcine PAP and blood products.*
  - *About MW distribution, tricin SDS-PAGE protocols are ready and BfR agrees to share sample collection, protocols and about what not to do on a size exclusion.*
  - *About blood/PAP distinction, Uta highlighted the fact that manufacturers didn't indicate whether it is a PAP or blood product. The proposed method is based on the detection of post translational modifications of the proteins, named "processing makers". Two papers describing the method are under preparation/submitted. More information will be given during the next workshop.*

Light microscopy

- *The use of other complementary reagents such as choral hydrate or chlorazol black or the use of paraffin oil was discussed. Chlorazol black reacts with krill teguments and is not*

specific to insect. Chloral hydrate is not accessible and dangerous and has never been tested. About the use of paraffin oil, no objection is raised. As well using the expensive immersion oil might not be interesting since no benefits is known.

- *Questions on dairy product identification are replied. Sometimes milk is not always visible, e.g. in piglet feed granules containing milk, Due to the process of incorporating. From former proficiency tests, where dairy products were introduced, the intention was to obtain positive detection by light microscopy. The quantities that were used were always a few percents. No other data are available in terms of "below this percentage dairy products are no longer visible".*
- *Problems of recovery of the intermediate flotate are reported. The small opening of the stopcock is responsible for that. A metallic rod may be used to push the material through the hole. Delicate pouring of the final flotate is an alternative to. After that the main intermediate flotate may be recovered easier.*

The Irish NRLs has purchased custom-made separation funnel with extra large stopcock (6-8 mm from Lennoks Laboratory Supplies – https://lennox.ie/
Contact with the customer service may be useful customerservice@lennox.ie).

- *A video on the implementation of the double PE/TCE sedimentation was also recorded by the EURL-AP: it will be placed on the intranet.*

Combined Proficiency test 2024: results and discussion

The 2024 edition of this test focused on pig, poultry and fish feeds adulterated with feather meals, egg powder, salmon PAP, insect meal, bovine PAP and haemoglobin powder. This patchwork of samples' adulteration allowed to test 3 parameters (terrestrial vertebrates, terrestrial invertebrates and fish) by microscopy and 3 by PCR (ruminant, pig and poultry).

The results obtained by light microscopy by the NRLs were plenty satisfactory: the total error rate was of a few 2 %. One point of attention was revealed, namely problems of specificity issues (with 8 % of false positive results) for terrestrial vertebrates in the sample adulterated with the salmon PAP. During the presentation, attention of the participants was drawn to the peculiar features of some salmonid bones features making them possibly confusing with terrestrial vertebrates' ones. A list of papers on salmonid bones was delivered to the NRLs for improvement of their knowledge.

The PCR results of the network were still improved compared to the PT 2023 with only 1 deviation out of 364 results (0.27 %)! This deviation was obtained with the poultry test (1 deviation out of 130 results representing 0.77%).

Having such excellent results at the level of the network, the individual performances of the NRLs were also highly satisfactory. Twenty-five NRLs were excellent and had no deviation. The remaining NRL was satisfying with only one positive deviation with the poultry test.

One result was kept out of assessment because of divergences during the homogeneity study. Nevertheless, all the participants would have return the expected results and came to the same conclusion as the organizers.

Concerning the cut-off quality criteria, they were successfully reached with 2 exceptions: for the ruminant test, 1 NRL set its cut-off at 8.98 copies instead of 9 ; for the poultry test, 1 NRL obtained a quality criterion of 7.92 copies instead of 9. Nevertheless, this had no impact on the results.

A point of attention concerns the periodical re-setting of the cut-offs. Five NRLs used at least 1 cut-off already in place in 2022 whereas the EURL-AP recommends setting all the cut-off once a year.

At last, the satisfaction inquiry after the publication of the final report showed that the answering participants are quite satisfied with the content as well as with the organisation but it is a pity to receive only 7 filled forms.

Discussion:

Was egg powder detected by light microscopy during the homogeneity study ? No that was not possible, since egg powder is lacking any structural features making it recognizable. There is also no legal need to look for it. It is reminded that the egg powder was strictly used for obtaining positive poultry DNA results.

In the future communication issues should be solved by either improving the mail exchange system, or a dedicated web interface, or a Google form option.

Proficiency Test 2024: results by MS

As in previous years, some laboratories have analysed PT samples by mass spectrometry: the Italian, Austrian and German NRLs + CER Groupe (Belgium). Norwegian NRL was not able this year to participate.

Regarding the instructions, laboratories were free to use the method of their choice. The only obligation was that they had to conclude on the presence of haemoglobin, connective tissues or milk of ruminant origin based on the detection of at least 2 peptides of the targeted proteins. Participants had also the possibility to send an alternative conclusion sheet applying their internal threshold.

Methods used are now relatively similar. Most of the peptide markers are common thanks to the addition by the participant of novel peptides to their method. This harmonisation is really helpful as the final goal is to have a common method to transfer to other labs.

Homogeneity test was performed by EURL-AP on feed (5 replicates) and on sediment (3 replicates) when terrestrial particles were detected by light microscopy.

Regarding the participant results, false positive results were only linked to the detection of milk by one lab in the 4 samples free of milk. The use of an alternative threshold consisting to declare a sample positive as soon as 1 peptide is detected, has of course no impact in this case.

Concerning the false negative results, one lab did not detect the presence of milk in sample 2, another lab did not detect the presence of connective tissues in sample 5 and no lab detected haemoglobin in sample 5. By applying the alternative threshold, the conclusion is different for the misdetection of milk and connective tissues as only one peptide was detected. However the use of the alternative threshold did not help for the detection of haemoglobin as no peptide was detected.

This study highlighted several points of interest:

- The harmonisation of the applied methods,
- Milk detection is still challenging due to the risk of carry over or cross-contamination,
- Haemoglobin detection in presence of PAP is also challenging at low level of PAP. The use of sedimentation can help.

Discussion:

The question on the threshold to be applied is a critical point. This is the main objective of the interlaboratory study. The two peptides threshold is applied in two standard ISO proteomic methods. So it will be a good think to use the same.

Conclusions will be discussed with the MS expert group and diffused to the network during the next workshop.

New batch of ruminant calibrant

ERM-AD482 is a batch of ruminant calibrant in use for almost 10 years and coming out of stock. A new batch named ERM-AD482k has been produced at JRC Geel and 550 kits are now available for distribution.

The instructions for use are slightly adapted to this new material. It is recommended to read carefully these instructions prior to use.

New kit of ruminant calibrant ERM-AD482k

The major change is that once thawed the material cannot be frozen again. Nevertheless, the material can be stored for 3 days at 4°C. During this period, it can be used to perform PCR and set the cut-off of thermocyclers.

EURL-AP will conduct a study to extend the storage period at 4°C up to 3 months.

Discussion:

The extension of the storage period at 4°C will be done in concertation with the JRC. Some labs used the previous ruminant calibrant as a positive control. They will have to adapt their procedure to this new material.

PCR mastermixes

Seven mastermixes are under assessment in view to enlarge the list of recommended mastermixes: qPCR™ Mastermix Plus without UNG and Takyon™ Low Rox Probe MasterMix dTTP Blue (Eurogentec), PerfeCta® qPCR ToughMix® (Quantabio), TaqMan® Universal PCR Master Mix (Applied Biosystems by Life technologies), Xpert Fast PROBE Mastermix (Uni) (GRISP research solutions), RealQ Plus Master mix for probe (Ampliqon) and SensiFAST Probe No-ROX kit (Meridian Bioscience).

Mastermixes under assessment

All of them successfully passed the tests concerning the possible presence of ruminant DNA in their composition and succeed also to obtain cut-off reaching the quality criterion fixed in the SOP.

Two additional parameters were also assessed (the sensitivity obtained with each mastermix and their susceptibility to PCR inhibition) and compared to the performances obtained with the Brilliant II QPCR Low Rox Master mix (Agilent) considered as the reference mastermix.

At this stage, the conclusion is that only 2 mastermixes show comparable performances to the one used as the reference : RealQ Plus Master mix for probe (Ampliqon) and Takyon™ Low Rox Probe MasterMix dTTP Blue (Eurogentec). The use of other mastermixes could cause "false" results with respect to a common analytical approach.

These results show the difficulty to manage the ruminant PCR test. All the mastermixes will continue to be evaluated with the other PCR tests.

Discussion:

A majority of the mastermixes assessed are not fit for the ruminant PCR test. Nevertheless, the EURL-AP will continue the evaluation of all the mastermixes for the pig and the poultry tests. The mastermixes will or won't be recommended for each test independently.

Conclusion and end

Olivier Fumière comes to the conclusions of the workshop:

- He reminds that the minutes of this session will be in the next newsletter and all the presentations will be available soon on the EURL-AP intranet ;
- Dutch NRL will be contacted directly in the next few days concerning the possibility to perform poultry and pig PCR tests on the same plate ;
- Concerning the development of the MS method, a meeting will be organised to discuss the results of the last interlaboratory study and take position on the next steps ;
- Hydrolysed proteins: the method under evaluation is described in "Development and validation of an analytical method to ensure quality requirements of hydrolysed proteins intended for agricultural use as biostimulants" (2022). C. Povolo, R. Avolio, E. Doria, A. Marra, M. Neresini. *Talanta Open*, Volume 5, August 2022. https://doi.org/10.1016/j.talo.2022.100082.

  The German NRL is welcome in the dedicated expert group.
- EURL-AP would like to receive more answers to the annual PT satisfaction survey.

Next coming conference

A very interesting international conference on feed will take place on 15-16 October 2025 in Novi Sad (Serbia). Topics addressed during the conference will be: circular economy and sustainability in the feed and the food chain, animal nutrition and feed additives, feed technology, quality and safety of feed and animal products.

More info on the programme, the deadlines and the registration at https://feed.uns.ac.rs/

List of the participants (from EURL-AP network)

Austria	Roland Weiss
	Sonja Axmann
Belgium	Jeroen Vancutsem
	Daniella Daou
	Ruth Vanhoof
Bulgaria	Sofiya Ivanova-Kirilova
Croatia	Manuela Zadravec
	Mislav Unger
Cyprus	Panagiotis Konstantinou
Czech Republic	Tomas Brychta
	Bronislav Simek
Denmark	Lotte Hougs
Estonia	Siiri Põldma
Finland	Maria Aarnio
France	Marilyne Bescond
	Danielle Huppert
Germany	Hermann Broll
	Uta Maria Herfurth
	Christiane Kamp
	Laura Draack
	Ralf Winter
Greece	Christos Chatzineiros
Hungary	Adél Szabó
Iceland	Halldóra Viðarsdóttir
Ireland	Michele Della Bartola
	Danielle Mc Auley
	Gabrielle Barrett
	Mandy Tracey
	Niamh Kelly
Italy	Giuseppina Buonincontro
	Daniela Marchis
Latvia	Inga Piginka-Vjaceslavova
Lithuania	Maskaliovas Viktoras
	Indre Jonauskaite Sedurskiene
	Šeškutė Gertrūda
Netherlands	Theo Prins
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Poland	Anna Weiner
Portugal	Hugo Guedes
	Gabriela Assis Teixeira
	Maria João Lino
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Serbia	Ksenija Nesic
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Slovenia	Igor Ujčič Vrhovnik
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