Implementation study on the detection of insect PAP in feed by double sedimentation method PE/TCE followed by light microscopy identification

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Introduction

In July 2017, the European authorities agreed to introduce insects for feeding aquaculture animals (European Commission, 2017). A list of seven insect species was established for rearing and for use in fish feeding: black soldier fly (*Hermetia illucens*), common housefly (*Musca domestica*), yellow mealworm (*Tenebrio molitor*), lesser mealworm (*Alphitobius diaperinus*), house cricket (*Acheta domesticus*), banded cricket (*Gryllodes sigillatus*) and field cricket (*Gryllus assimilis*). Recently the use of farmed insect processed animal proteins (PAPs) was extended to the feeding of poultry and pigs (European Commission, 2021). This introduction of farmed insects and their PAPs conducted to the development of a proper protocol for their isolation and identification (Veys & Baeten, 2018) as a complement to the official methods from Annex VI of Regulation EU/152/2009 for PAP detection in feed by light microscopy and polymerase chain reaction (PCR) used for quality control and fraud detection (European Commission, 2009, 2013, 2020). This protocol, based on a double PE/TCE (petroleum ether/tetrachloroethylene) sedimentation with a mixture of 30% PE of and 70% of TCE, to achieve a density of 1.26 $g.cm^{-3}$, succeeded in concentrating insect fragments simplifying their subsequent detection by microscopic analysis. The protocol was validated through a validation study (Veys et al., 2019) performed on various feed matrices fortified with insect PAPs at levels (w/w) of 0.5 %, 0.1 % and lower. The validation study nevertheless revealed some specificity issues on blanks mainly due to lack of participants' knowledge and experience on this new type of PAP. It was therefore concluded that a larger scale implementation test involving all National Reference Laboratories (NRLs) of the member states before translating the new protocol into the legislation was highly recommended. An implementation test was thus initiated as a part of the annual programme of the EURL-AP for the 2021-2022 period as granted by DG SANTE. Its objective was to assess the skills of laboratories at implementing the new protocol and their ability of identifying insect particles in feed matrices. This report presents the results of this implementation test to which the 26 NRLs were invited to participate.

Material and method

Material

Different compound feed matrices were used: feed for ruminant (RF), poultry feed (PF1), feed for broiler (PF2), pig feed (PGF) and fishfeed (FF). These matrices were selected to obtain a diversity of feed into which some insect PAPs are authorised (fishfeed, poultry feeds and pig feed), while in others insect PAPs are unauthorised (ruminant feed). The feed for broiler (PF2) was a batch accidentally contaminated by *Trilobium castaneum* at < 0.05 % (calculated w/w) and used by Veys and Baeten (2018) in their study to obtain an estimate of a limit of detection, as well as during the validation study (Veys et al., 2019).

Light microscopic analyses of these matrices, on the exception of PF2, showed them free from any traces of insects.

Three industrially produced insect PAPs were used: from *Hermetia illucens* (black soldier fly), from *Tenebrio molitor* (yellow mealworm) and from *Alphitobius diaperinus* (lesser mealworm).

Sample preparation

The feed for ruminant (RF) and fishfeed (FF) used as blanks were conditioned first in order to avoid contamination.

Addition of insect meals occurred by spiking to achieve levels of 1 % and 0.5 % for *H. illucens*, of 1 % for *T. molitor* and of 0.5% for *A. diaperinus* PAPs. After spiking all samples were ground at 2 mm by a rotor mill (Retsch ZM 200) to ensure homogeneity and optimal size reduction for microscopic observations.

From each sample, aliquots of 50 g were taken and transferred into hermetically closed vials composing the sample set.

Sample set

Each participant received a sample set composed of 8 vials (Table 1).

Table 1: Composition of the sample set, and homogeneity study.

Sample type	Expected result for the presence of insects	Homogeneity study	
		Nb of slides	Details
RF (blank)	negative	60	absence of insects
RF + 0.5 % *H. illucens*	positive	60	> 10 insect part./slide
PF1 + 1 % *H. illucens*	positive	60	> 10 insect part./slide
PF1 + 1 % T. molitor	positive	60	> 10 insect part./slide
PGF + 0.5 % *A. diaperinus*	positive	60	> 10 insect part./slide
FF + 1 % *H. illucens*	positive	60	> 10 insect part./slide
FF (blank)	negative	60	absence of insects
PF2 contaminated (< 0.05 % *T. castaneum*)	positive	60	5 ≤ 10 insect part./slide

Samples were blind for the participants; the only information was the sample numbers mentioned on the vials. Each number on the vial was unique and the code was only known by the organiser. All samples had to be submitted in duplicates to the PE/TCE sedimentation and subsequently analysed by light microscopy.

Homogeneity study

From each sample 10 aliquots were submitted to the PE/TCE sedimentation. From each final flotate obtained, 6 slides were prepared and analysed by light microscopy for the presence or absence of insect particles (Table 1). The blanks were free from any traces of insects. For the samples fortified at 0.5 % and 1 %, on each slide more than 10 insect particles were counted. For PF2 the number of insect particles per slide was lower (between 5 and 10 particles) although systematically present.

The type of insect particles observed were cuticular fragments, setae, stigmata, spiracles and tracheal structures, muscle fibres. In addition, for *T. molitor* and *T. castaneum* leg and mouth parts were also recorded.

Study organisation

The study started with a training period. Three months before the sending of the sample sets, each NRL received a set of permanent slides from pure insect PAPs, including the three species used in the study, in order to become familiar with the recognition of insect particles as well as one ruminant feed material fortified with a mix of insect PAPs to train in the PE/TCE sedimentation. In addition, the EURL-AP micrograph collection (available at https://www.eurl.craw.eu/publications/micrograph-collection/) was significantly enriched with insect micrographs for direct online consultation.

The official announcement for the implementation study was sent by mid-May 2021 to the NRLs. The shipment of the samples occurred by the begin of June and the deadline for the return of the results was fixed at the begin of September to allow participants to have time enough for the analyses.

Concomitantly with the sending of the samples a protected file containing detailed instructions and a report form was sent to the participants. Among the instructions:

- a responsibility agreement to commit following strictly the instructions and the protocols to use,
- mention that additional grinding was proscribed,
- mention that each sample had to be submitted in duplicate to the PE/TCE sedimentation and determination;
- a detailed protocol for the double PE/TCE sedimentation steps to be performed in closed sedimentation funnels,
- each microscopic analysis had to be performed on 6 slides made from the final flotate (< 1.26 $g.cm^{-3}$) prepared according EURL-AP SOP,
- only insect fragments strictly identified without any ambiguities had to be considered.

Records that had to be encoded were:

- total number of identified insect fragments for each of the 6 slides per determination, by selecting values or ranges from picklists (1, 2, 3, 4, 5 particles, ranges of particles from 6 to 10, from 11 to 15, from 16 to 20, and more than 20 particles),
- the weight of the obtained final flotate and the weight of remaining final flotate after the slide preparation. The weight of the final flotate used for slide preparation was automatically calculated.

From the 26 invited participants, 22 delivered results. Acceptance criteria for participants' results was the completeness of the results. One participant was rejected due to incomplete data. The study was thus based on the analysis of 21 result sets.

Data treatment and statistics

Assessment of the ability of identification of insect particles is a binary data treatment (presence or absence) which was expressed in terms of sensitivity (the probability of positive test given the real presence of insect into a matrix) and specificity (the probability of negative test given the real absence

of insect into a matrix). The influence of the number of slides to analyse on the sensitivities and specificities was investigated by comparing determinations from the whole set of 6 slides (one combination) with determinations based on only 3 slides (20 combinations). The latter slide number corresponds to the legal minimum slide number upon which terrestrial vertebrates and fish detection is allowed in current official light microscopic detection (European Commission, 2009 - consolidated). The impact of the '5 particles rule' on which the limit of decision is applied (EURL-AP, 2020) was also simulated.

The implementation of the PE/TCE sedimentation was assessed by analysing the recovery weights of the final flotates. Classical descriptive statistics were first applied. Normality of distribution of weights' data was tested for each sample by one-sample Kolmogorov-Smirnov tests as recommended by Sokal and Rohlf (1995). According to ISO 13528:2015, Kernel density estimation was applied to estimate probability density functions and robust statistics were used (ISO, 2015).

Results and discussion

Insect detection

From the data collected the very first point of attention was to estimate the abundancy of insect fragments identified by the participants per slide, a parameter reflecting a probability of occurrence. Therefore, a minimum mean number of identified insect particles per slide ($\bar{X}_{min}/slide$) was computed (Table 2 next page) by taking the exact numbers of particles reported (from 0 to 5) and selecting the lowest values or the ranges (6, 11, 16 and 20) from the picklists. The calculation of this parameter was therefore intentionally pessimistic since it is underestimated. For instance, for PF1 fortified at 1% with *H. illucens* 85 slides on 252 refer to more than 20 particles of insect origin without referring to an exact numerical value above this threshold. This minimum mean number per slide is thus a semi-quantitative parameter. The highest $\bar{X}_{min}/slide$ were observed into the PF1 fortified at 1 % with *H. illucens* or *T. molitor* (12.6 and 12.1 respectively). The two lowest $\bar{X}_{min}/slide$ in samples containing insects were found in the FF adulterated at 1 % with *H. illucens* and in the PF2 contaminated (6.4 and 2.4 respectively). In the two blanks used in the study this value was, as it could be expected, below one particle per slide (0.8 for RF and 0.03 for FF).

Consequently, for the prepared samples at 0.5 % and 1 % of insect PAPs, it could be hypothesised that limiting the observation to a single slide from the final flotate would readily lead to a positive result for the insect PAP presence because the value of $\bar{X}_{min}/slide$ is yet above the minimum of 5 particles upon which the limit of decision for declaring a sample as positive (for animal particles from terrestrial vertebrates and fish) is based. This possibility should however not be considered for the following reasons. First, at lower concentration such as demonstrated for PF2 contaminated, this threshold is not reached and thus the risk of false negative results would be important when only one slide would be observed. Secondly, due to the small subsample fraction from the flotate used for preparing slides there is a risk for heterogeneity among slides. This heterogeneity of slide content can be overcome by multiplying the number of slides to a reasonable number, to a minimum of 3 slides per fraction in accordance with the legal requirement existing for terrestrial vertebrates and fish detection (European Commission, 2009).

Table 2: Sensitivities and specificities for the sample set, with or without the application of the '5 particles rule' applied to the observation of 6 slides (42 data per sample) or 3 slides (840 data per sample).

	$\frac{\overline{X}_{min}}{slide}$	no 5-particles rule, over 6 slides		5-particles rule, over 6 slides		5-particles rule, over 3 slides	
		sensitivity	specificity	sensitivity	specificity	sensitivity	specificity
RF (blank)	0.8		0.810		0.833		0.882
RF + 0.5 % *HI*	11.5	1.000		1.000		0.976	
PF1 + 1 % *HI*	12.6	1.000		1.000		1.000	
PF1 + 1 % *TM*	12.1	0.952		0.929		0.892	
PGF + 0.5 % *AD*	10.6	0.929		0.929		0.920	
FF + 1 % *HI*	6.4	1.000		1.000		0.942	
FF (blank)	0.03		0.905		0.976		1.000
PF2 contaminated	2.4	0.714		0.571		0.523	

The rates of correct results were calculated by the sensitivities (for the samples containing insect materials) and specificities (for the samples were insect material was not present). Both rates were calculated on the full series of 6 slides prepared, and on only 3 slides.

A first series of results based on 6 slides without the application of the '5 particles rules' revealed already faultless scores of sensitivities for the three samples fortified with *H. illucens* and excellent results for the two other prepared samples, with *T. molitor* (only 2 false negative results from one participant only) and *A. diaperinus* (with 3 false negative results, from two participants). Regarding PF2 the sensitivity was lower due to numerous false negative results (12) explained by the lower mass fraction of insects into this matrix (estimated < 0.05 %). Concerning the specificities for the two blank matrices, the scores obtained were of 0.810 for RF (with 8 false positive results but limited to 4 participants, i.e. including both determinations) and of 0.905 for FF (with 4 false positive results from 2 participants, i.e. including both determinations to).

When applying the '5 particles rule' to the same series of 6 slides, sensitivities were hardly affected on the exception of PF2 for which the value was decrease 0.571. In other words, the initial number of 12 false negative results was increased to 18 because from the results 6 sets of slides from this sample had no more than 5 particles and thus, consistent with the rule, had to be declared as negative. Regarding the specificities, the value for RF barely increased from 0.810 to 0.833 for RF, reflecting a decrease of only one false positive result, whereas for FF the improvement was more significant with an increase from 0.905 to 0.976. This improvement is linked to the low numbers of particles classified as from insect origin per slide which lead to a reduction of the number of false positive case from 4 to 1 when applying the rule.

Simulations on the reduction from 6 to 3 slides for the light microscopic analysis, combined with the application of the '5 particles rule', showed to have a limited effect on both parameters, sensitivity and specificity. Only two samples were impacted by a decrease of more than 5 % of the sensitivity values. It concerned FF fortified with 1 % *H. illucens* with a sensitivity of 0.942 due to some heterogeneity of slides from which only a limited number of insect particles were identified. The second sample impacted was PF2, with a sensitivity decrease to 0.523, for the same reasons explained in previous

section. A slight improvement for the specificities was also noted for both blanks, leading even to a perfect score for the FF. Nevertheless, those improvements are only logically expected and linked to the very low number of insect particles identified allowing to rank more slide combinations (for reminder 20 per determination) as negative or below the limit of decision according to the '5 particles rule'.

Misidentifications

Although good specificity scores were obtained for the two blank matrices, some particles were misidentified as from insects leading to false positive results: 10 particles within the FF and a minimum of 206 particles for the RF, both from a total of 252 slides prepared by the participants.

The identified insect particles in the FF are limited to only two participants and even not systematically on all slides from their series (Table 3), although the misidentification is probable it cannot be excluded, considering their scarce occurrence, that it may result also from carryover from other samples from the study sample set. A situation that would result to correctly identified insect particles but leading also to false positive results for this blank sample.

Table 3: Details of insect particles findings (numbers) leading to false positive results for the two blank matrices (RF and FF).

	Determination	Slide 1	Slide 2	Slide 3	Slide 4	Slide 5	Slide 6
(FF) Fishfeed							
Lab 5	1st	0	1	0	0	1	0
	2nd	0	0	1	0	0	1
Lab 8	1st	1	1	2	1	0	0
	2nd	0	0	0	1	0	0
(RF) Ruminant feed							
Lab 1	1st	4	0	2	1	0	0
	2nd	6 ≥ 10	6 ≥ 10	6 ≥ 10	5	4	5
Lab 26	1st	6 ≥ 10	6 ≥ 10	6 ≥ 10	6 ≥ 10	5	6 ≥ 10
	2nd	6 ≥ 10	6 ≥ 10	4	6 ≥ 10	6 ≥ 10	5
Lab 20	1st	11 ≥ 15	> 20	> 20	6 ≥ 10	11 ≥ 15	11 ≥ 15
	2nd	2	1	1	1	3	1
Lab 5	1st	0	1	0	2	1	2
	2nd	1	1	0	0	1	2

The situation that was observed on the RF is quite different. Both the occurrence of slides where problematic insect particles was reported as well as their numbers were higher (Table 3) although limited to four participants. While the preceding section showed that the ability of detecting properly insect fragments when present was very good, specificity issues have to be understood, especially in matrices into which insect PAPs are not authorised such as ruminant feed, to prevent positive deviations that would penalize producers and affect the performance reliability of official control laboratories. Table 3 points in particular to the situation of laboratories 26 and 20. Furthermore, for laboratory 20 the difference in insect detection capability in between the two determinations for a same sample is questionable. In this respect a request was sent to the four participants having false

positive results for the RF, to deliver micrographs of the particles identified as from insect origin. Three participants replied to this invitation by sending pictures. From the received micrographs it became clear that misidentifications occurred. Two situations happened. First some particles with undefined features, i.e. particles without typical plant, animal or mineral morphological microscopic markers were categorised as from insect origin [Annex 1 – Fig. I]. This type of situation can be avoided by strictly sticking to the rule of categorising particles only when the analyst is absolutely sure on the histological or microscopic feature of the observed structures – it was one of the conditions that was explained in the instructions. Secondly plant fragments from the aleurone layers of wheat covers were erroneously identified as from insect origin. This finding was corroborated by the composition of the ruminant feed mentioning as ingredient wheat and wheat middlings, a fraction rich in bran. The histological patterns of plant cells [Annex 1 – Fig. II, (Hourston et al., 2017)] with their polarised cell walls [Annex 1 – Fig. III] is a distinctive criterion to avoid confusion with insect cuticular fragments. As well a good knowledge of the histology of wheat grain layers (or bran), and in particular in the present context of the aleurone layer, a monolayer of regular polygonal cells attached to other layers of the pericarp, allows correct identification.

Implementation of PE/TCE sedimentation

Monitoring of the implementation of the PE/TCE sedimentation was assessed by analysing the recovery weights of the final flotates. From the data collected boxplots were computed for descriptive statistics on each sample (Fig. 1).

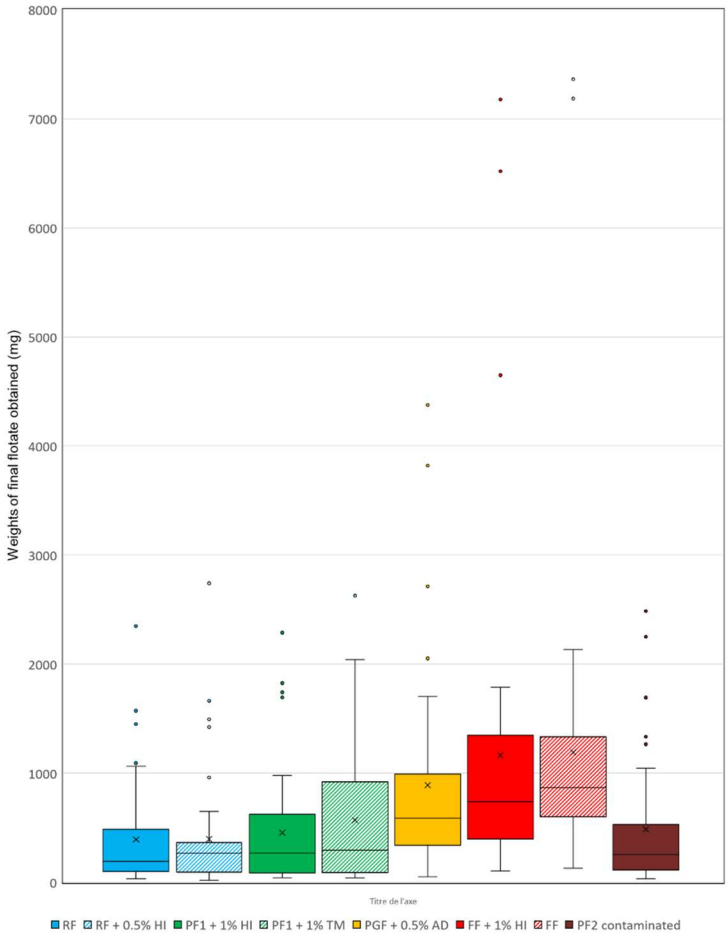

Fig. 1: Boxplots of the weights of final flotates per sample.

For all samples of the study, the weights of final flotates recovered after the PE/TCE sedimentation showed a distribution deviating from a normal one. This absence of normality for the data was confirmed by Kolmogorov-Smirnov tests. Strong positive skewing of the values is generalised. For each sample a number of outlying values (Q3 + 1.5IQR) were observed. The share of outlying values was of 31 on 336 (or 9%). For instance, a value of 7363 mg of final flotate was the maximum observed for the FF whereas the minimum obtained for this matrix was of 126 mg. Considering that the PE/TCE sedimentation had to be started from a 10 g fraction of sample, the recovery rate of final flotate for the FF had range varying from 1.2 % to 73.6 %. A similar situation of great variability among the quantities of final flotate was also observed for the other samples.

According to ISO 13528:2015, Kernel density estimation (Fig. 2) was applied to estimate probability density functions and verify potential multimodal distributions. For each sample distinctive peaks appeared separated from the principal bulk of data, they correspond to the outlying values responsible for the skewing. The principal kernel densities observed (outliers aside) were unimodal and roughly symmetric for all samples.

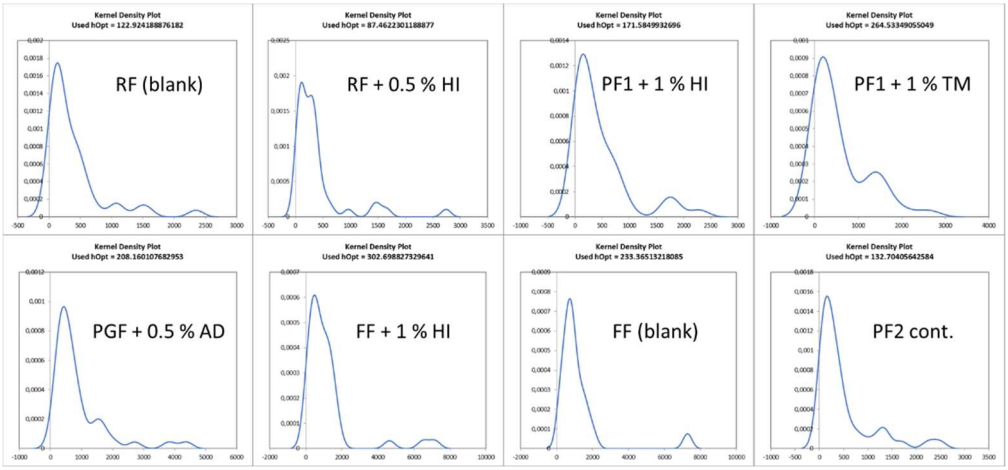

Fig. 2: Kernel density plots of the weight of the final flotates per sample.

These distributions being close to symmetric the use of robust means as assigned values was chosen as recommended by IUPAC (Thompson et al., 2006). The advantage of using robust statistic (Table 4 next page) is to consider all participants' data, including outlying values, by downweighting the impact of the values distant from the mean and thus allowing to estimate an assigned consensual value.

Values of robust means obtained for the feed matrices used for preparing two samples (the ruminant feed RF, the poultry feed PF1 and the fishfeed FF) showed that the weights of final flotates obtained are in a comparable range (robust mean ± 2 SEM): respectively 290 ± 77 mg and 265 ± 62 mg for RF, 351 ± 101 mg and 498 ± 167 mg for PF1, 850 ± 188 mg and 912 ± 172 mg for FF. It is linked to the dispersion of the mean values which is high. The relative standard deviations for the reproducibility go from 61% for the best reproducibility observed to 110% for the worst. None of values of RSD_R are satisfying and reflects a high variability of the obtained weights of final flotates among participating laboratories. In contrast, the within-laboratory variability, expressed by the relative standard deviations for the repeatability (RSD_r), shows that between replicates the weights of final flotates are more repeatable, with even yet acceptable ranges (< 30% in bold italics in Table 4) for the samples

prepared with the poultry feed PF1 and fishfeed FF matrices. However, for the other samples the repeatability needs to be improved.

Table 4: Robust statistics on the weights of final flotates (mg).

	Robust mean	SEM	S_r	RSD_r (%)	S_R	RSD_R (%)
RF (blank)	290,21	38,28	148,44	51%	248,11	85%
RF + 0.5 % *HI*	265,50	31,25	113,50	43%	202,49	76%
PF1 + 1 % *HI*	350,58	50,56	88,83	***25%***	327,64	93%
PF1 + 1 % *TM*	498,25	84,37	80,29	***16%***	546,80	110%
PGF + 0.5 % *AD*	710,66	79,92	226,64	32%	517,97	73%
FF + 1 % *HI*	850,35	94,24	225,31	***26%***	610,73	72%
FF (blank)	912,13	85,78	185,56	***20%***	555,95	61%
PF2 contaminated	347,02	48,07	174,15	50%	311,54	90%

S_r : repeatability standard deviation, S_R : reproducibility standard deviation, SEM : standard error around the mean (= $S_R/\sqrt{n}$), RSD_r : relative standard deviation for the repeatability, RSD_R : relative standard deviation for the reproducibility

From the monitoring of the implementation of the PE/TCE sedimentation, it appears that technical improvement is essential to obtain both a better repeatable and reproducible recovery of final flotates. This is possibly linked to a lack of mastery on the technical aspects of the double PE/TCE sedimentation with the use of closed sedimentation funnels among the different laboratories. Several factors may explain this situation:

- Although a three months training period was organised, with the sending of a ruminant feed material fortified with a mix of insect PAPs to train in the PE/TCE sedimentation, it cannot be guaranteed that each NRL took profit of this period to become familiar with the technical aspects of the protocol. Some NRLs are used to perform the usual TCE sedimentation on a routine way with conical open beakers, as legally allowed. Nevertheless, switching from open beakers to close sedimentation funnels requires some new technical skills from operators. The validation of the PE/TCE sedimentation has only been done, for technical reasons (easier separation of the different fractions obtained and limitation of carry-over from one fraction to another) on closed sedimentation funnels.
- The presence of 9% of outlying values, as revealed from the boxplot constructions, were often limited to some few laboratories (Lab 14: 12/31, Lab 26: 8/31 and lab 1: 4/31) which is indicative of repetitive excessive recoveries of final flotate.
- The ratio between the maximal and minimal weights of final flotate between two repetitions of the PE/TCE sedimentation on each sample is often higher than a factor two. An illustration of this is on Table 5 (next page) for the ruminant feed RF for which the lowest repeatability was noted. Considering that repeatability conditions were respected, i.e. the same equipment, the same protocol and the same solvent mixture, the same sample and usually a same operator, the discrepancy among the weight points likely out manipulation inaccuracies as an explanatory factor.

Table 5: Final flotate weights from the ruminant feed (RF).

	Weights of final flotates (mg) from the two repetitions		
	1st	2nd	$R_{(max/min)}$
Lab 1	479	**2348**	4,9
Lab 2	43	69	1,6
Lab 3	50	32	1,6
Lab 5	380	385	1,0
Lab 7	749	441	1,7
Lab 8	206	185	1,1
Lab 9	59	48	1,2
Lab 11	414	146	2,8
Lab 12	162	172	1,1
Lab 13	**1065**	377	2,8
Lab 14	**1092**	376	2,9
Lab 15	278	588	2,1
Lab 16	190	74	2,6
Lab 17	110	80	1,4
Lab 18	198	138	1,4
Lab 20	221	73	3,0
Lab 21	504	516	1,0
Lab 22	597	187	3,2
Lab 23	119	79	1,5
Lab 24	161	105	1,5
Lab 26	**1451**	**1572**	1,1

Values underlined and bold are outliers. Greyish cells refer to values of the ratio $R_{(max/min)}$ above 2.

Regarding the protocol used for the study, all parameters were fixed as far as possible: the starting amount of material, durations of each step, specific gravities of the solvents to use, volumes of solvents and even the mention of a final flush of PE for rinsing the walls of the sedimentation funnel in order to collect whole of the final flotate. In absence of concrete feedback on its use on a routine way, possible improvements are difficult to plan. Nonetheless a first recommendation can be made of verifying the specific gravities (or densities) of the TCE and PE (bp 40-60 °C) respectively at 1.62 $g.cm^{-3}$ and 1.26 $g.cm^{-3}$ since it impacts on the density of the 30% PE/ 70% TCE mixture. Another possibility for improvement would be to submit directly the matrix to a single step sedimentation by a prepared 30% PE/ 70% TCE mixture of which the density of 1.26 $g.cm^{-3}$ would be checked prior to use. By doing so the dissolution of the fats from the matrix after the first TCE step and possibly altering the remaining TCE density would be circumvented. This would however impact the organisation of the routine analyses on ruminant feed when insect presence would have to be checked in accordance with the SOP on the combination of methods, and most likely increase the workload since the PE/TCE sedimentation would no longer be a complement to the first step but instead to be performed from a new sub-portion of 10 g of raw sample. This deserves further discussion prior hand.

Conclusions

The present study enables to draw conclusions on both the ability of the NRL network to detect insect fragments from PAPs and the implementation of the protocol.

This large-scale implementation study reveals that sensitivity and specificity scores obtained by the participants are good. The scores obtained are in a range all in all comparable to those observed from other regular proficiency tests on the detection of animal proteins in feed by light microscopy. Even the application of the '5 particles rule' to a series of only 3 slides used to declare a sample as positive and above the limit of decision of the method did not decrease significantly the scores on the sensitivities nor improve substantially the specificities obtained by the study. It confirms that the proposal made during the validation study (Veys et al., 2019) of limiting the number of slides is a valid option. The minimum mean numbers of identified insect particles per slide, for the levels of adulteration used, account for this. Investigations on the few specificity issues allowed to demonstrate that training on the histological pattern allowing to differentiate insect fragments from other fragments (as from plant origin, such as aleurone layers) is of upmost importance to avoid misidentifications. Even the lowest level of insect contamination showed values of sensitivities that are sometimes obtained for terrestrial vertebrates' detection (Fumière et al., 2017 and 2021) by the same network of participants.

The implementation of the double PE/TCE sedimentation showed that amounts of final flotates recovered are still highly variable. Improvements are expected for the reproducibility or in-between laboratory variability and for the repeatability inside laboratories. The evidenced lack of mastery on the method should be overcome by gaining experience through more frequent usage of the PE/TCE sedimentation method. However, considering the present infrequent use of insect PAPs in feed, this gain of experience will only be achieved when such ingredients will be commercially attractive and spread among feed producers. Though, the analytical relevance of such gain of experience in the PE/TCE sedimentation should be considered as only a minor issue since it does not impede with the good performance scores yet achieved through this implementation study. Experience in the PE/TCE sedimentation could only be gained if a legal framework introducing this protocol in the official control analyses would lead laboratories to apply it on a routine way.

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Annex 1

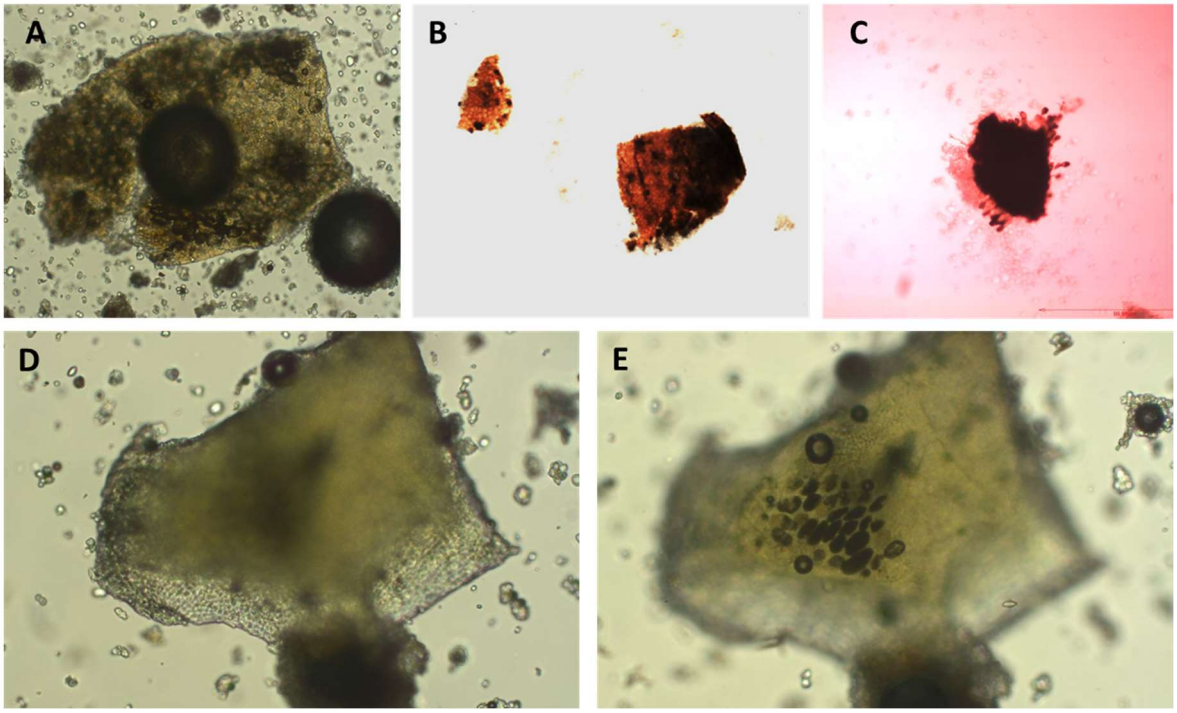

Fig. I: Pictures of structures erroneously reported as from insect origin (magnifications are unknown). A: plant origin, B: undefined, C: undefined, D and E: plant structure corresponding to wheat grain layers (different focus plans of the same structure). Pictures by courtesy of participants #1 and #5.

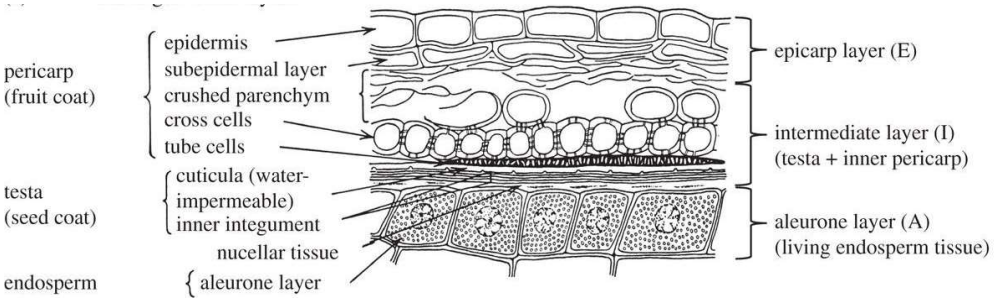

Fig. II: Scheme of wheat grain bran layers, transversal section. (adapted from Hourston et al., 2017).

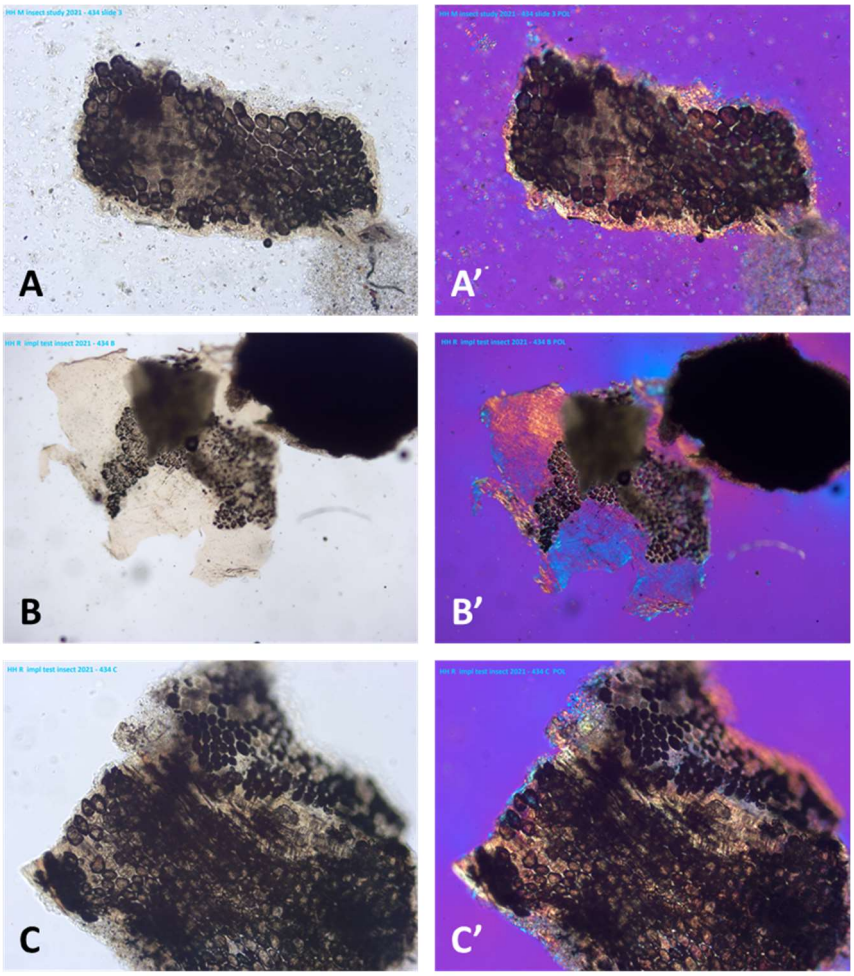

Fig. III: Wheat bran particles from the ruminant feed used. A & C: front view of aleurone layer, B: front of wheat bran with aleurone layer and more translucent intermediate layer (A,B,C = bright field, A',B',C' = polarised). Pictures by courtesy of participant #20.