Validation study on the isolation of insect PAP in feed by double sedimentation method PE/TCE and subsequent detection by light microscopy

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Introduction

In July 2017, based on the recommendation of the EFSA, the European authorities agreed to introduce insects for feeding aquaculture animals [1]. A closed list of seven insect species authorized to be reared and used in aquaculture was established: 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*). From this list, and according to IPIFF, the two most promising species likely to be adopted in practice for feed purpose are the black soldier fly and the yellow mealworm. This introduction of farmed insects and their processed animal proteins (PAPs) raised some questions about the methods to be used for quality control and fraud detection. Annex VI of regulation EU/152/2009 [2] official methods for PAP detection in feed are light microscopy and polymerase chain reaction (PCR) as amended by regulation EU/51/2013 [3]. Light microscopy is perfectly adequate for the detection of particles of fish and terrestrial (grouping all other vertebrates than fish) PAPs. It relies principally on the categorization of the bone fragments into those two groups, while other types of animal particles are minor. Bone isolation is facilitated by a sedimentation step using tetrachloroethylene (TCE), by which all material of a density higher than 1.62 $g.cm^{-3}$ are concentrated. However insects only have an exoskeleton of lighter density and lack bones. Therefore an alternative protocol based on a double sedimentation where next to the classical TCE sedimentation step, the flotate of this first step is submitted to a second sedimentation with a mixture of 30 % of petroleum ether (PE) and 70 % of TCE, to achieve a density of 1.26 $g.cm^{-3}$, was developed [4]. The proposed protocol succeeded in concentrating insect fragments in the flotate of the second sedimentation step simplifying their subsequent detection by microscopic analysis. The objective of this study was to validate this protocol by its transfer and implementation in several laboratories and by comparing the obtained results to the official single TCE sedimentation which was also to be applied by the participating laboratories.

Material and method

Material

Different compound feed matrices were used : feed for salmon (SA1), feed for fry (SF2), feed for marine fish (FF), feed for poultry (PF1), feed for broiler (PF2), feed for ruminant (RF). These matrices were selected to obtain a diversity of feed into which some insect PAPs are authorised (fishfeed), while in others insect PAPs are unauthorised at the time of publishing this report. The feed for broiler (PF2) was a batch accidentally contaminated by *Trilobium castaneum* at 0.015 % (calculated w/w) and used by Veys and Baeten [4] in their study to obtain an estimate of a limit of detection and to stress the sensitivity of the protocol.

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

Two industrially produced insect PAPs were used: one being produced from *H. illucens* (black soldier fly) and another from *T. molitor* (yellow mealworm). Both are authorised species according to European legislation and are the most widely produced according to IPIFF.

Sample preparation

The fishfeed (FF) used for the blank was conditioned first in order to avoid contamination.

Addition of insect meals occurred by spiking to achieve levels of 0.1 % and 0.25 % for both *H. illucens* and *T. molitor* 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.

The sample fortified at 0.5 % with *T. molitor* was prepared during a past study organised by the EURL-AP team [5].

From each sample, aliquots of 25 g were taken and transferred into hermetically closed vials composing the sample set.

Sample set

Each participant received a sample set composed of 17 vials (Table 1).

Table 1 : Composition of the sample set.

Sample type	Sample nb	Type of sedimentation
SF2 + 0.1 % *T. molitor*	1	TCE
	4	double
SF2 + 0.1 % *H. illucens*	11	double
	17	TCE
SA1 + 0.25 % T. molitor	6	double
	13	TCE
SA1 + 0.25 % H. illucens	3	double
	15	TCE
PF2 contaminated at 0.015 % *T. castaneum*	7	TCE
	14	double
RF + 0.1 % *H. illucens*	10	TCE
	12	double
FF + 0.5 % *T. molitor*	5	TCE
	9	double
PF1 + 0.1 % *T. molitor*	2	TCE
	16	double
Blank FF	8	double

Samples were blind for the participants; the only information was the sample numbers mentioned on the vials. Sample numbers were the same for all participants but this was unknown to them. Except for the blank fishfeed which was only to be double sedimented, all other samples were in duplicates and had to be submitted to the two sedimentation processes.

Study organisation

Laboratories to which the invitation was sent to join the study had one of their staff people being trained at the EURL-AP facilities for invertebrate's recognition by light microscopy. These training sessions were organised over the period June 2017 – March 2018. Fifteen participating laboratories enrolled on voluntary base.

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,
- a mention that additional grinding was proscribed,
- a mention of the type of sedimentation to perform for each sample number as well as a detailed protocol for the double PE/TCE sedimentation,
- each microscopic analysis had to be performed on 3 slides of the flotates (< 1.62 $g.cm^{-3}$ and < 1.26 $g.cm^{-3}$) prepared according EURL-AP SOP,
- only insect fragments strictly identified without any ambiguities had to be taken into account.

Records that had to be encoded were: total number of identified insect fragments from each of the 3 slides, the weight of the obtained flotate and the weight of remaining flotate after the slide preparation. The weight of the flotate used for slide preparation was automatically calculated.

Data treatment and statistics

Acceptance criteria for participants' results were (1) the accurate implementation of the protocol in the framework of a validation study, (2) completeness of the results and (3) the absence of false positive results for insects in the blank. Participants missing one of the criteria were rejected.

Normality of distribution of data was tested for each insect fragment count by one-sample Kolmogorov-Smirnov tests as recommended by Sokal and Rohlf [6]. Due the lack of normality data were square root transformed. Means of insect fragment counts identified from both TCE and PE/TCE sedimentation protocols were compared by t-test.

Results and discussion

Rejection of data sets

From the 15 participating laboratories, only 8 sets of data were accepted and treated. Seven laboratories' results had to be excluded from the study. The justification of their rejection was:

- One participant did not follow the protocol of the validation study, and had in addition > 10 insect particles erroneously identified in the blank.
- Five other participants obtained false positive results in the blank (with a range of erroneously identified insect particles from 2 to > 300)
- One participant did not use a filter paper to collect the flotate with consequently the recovery of a sticky flotate due to condensed fat. This resulted in abnormal values in the

weight of this fraction used for slide preparation (range of weights from 2 mg to > 9000 mg declared to be used on the filled report form)

The details of the false positive insect findings are presented in Annex 1. Investigation on the particles that were erroneously identified as from insect origin was undertaken. Contact was taken with these participants to ask them to send picture records of these particles. They are illustrated on Fig. 1.

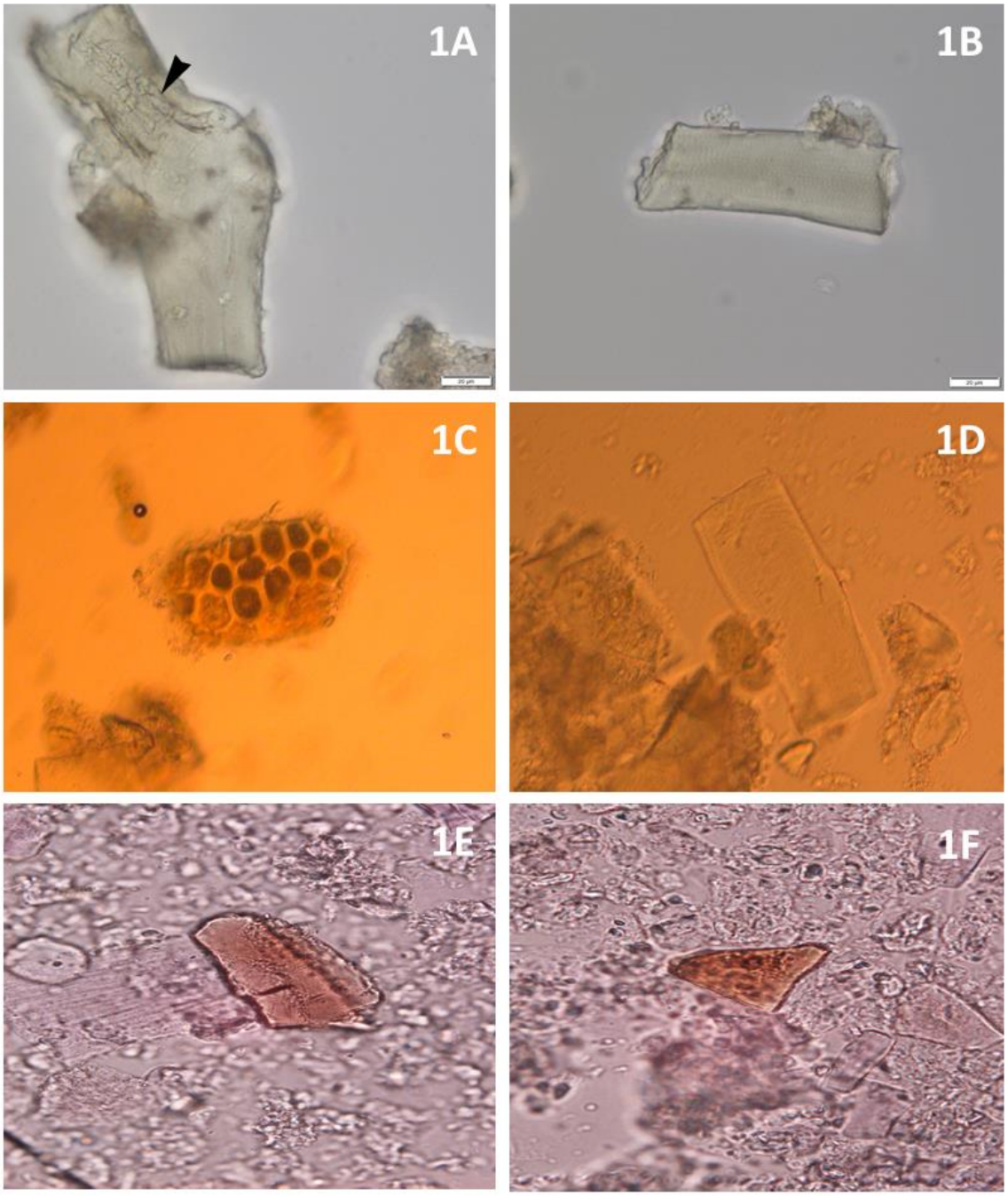

Fig. 1 : Erroneously identified insect particles from the blank fishfeed (FF). A, muscle fibre with air filled cracks (arrowhead); B, muscle fibre; C, plant structure; D, muscle fibre; E, unknown structure; F, unknown structure. Mounting media A and B : paraffin oil, C and D : no information, E and F : Fehling reagent.

The major source of confusion is originating from muscle fibres (Fig 1A, 1B and 1D) that are categorised as from insect origin. Analysis of the pictures sent revealed that some features like an undulating pattern of the sarcomers, some slight birefringence (not illustrated) and structures resembling to a network of tracheae (arrowhead from Fig. 1A) were misleading. Only the presence of tracheal structures into muscle fibres allows a confirmatory ranking of insect muscles [4]. Other structural patterns are informative but not confirmatory. Particularly interesting is Fig. 1A which is presenting a faint network of air filled cracks resembling to a tracheal network. The mounting media used, paraffin oil, which is legally not authorised could explain its origin. However accurate visualisation, helped by DIC as advocated by Veys and Baeten [4], would have revealed the absence of taenidia confirming tracheal structure. Other confusions were generated by plant fragments (Fig. 1C) and other uncharacterised structures (Fig. 1E and 1F) both interpreted as insect cuticle fragments.

Collaborative study

On the hypothesis that a satisfactory homogeneity of the flotate is reached, then quite logically the more material from the flotate is used for slide preparation, the higher the probability to detect insect fragments if present. Under this assumption, comparisons of the number of insect fragments can only be made if the amount of flotate used to prepare the requested 3 slides is the same.

According to the SOP it is recommended to use about 10 mg of sediment per slide, this amount can be extended to other fractions such as the flotates. It was thus decided to report all insect fragment counts on a same standard amount of 30 mg of flotate. This transformation is similar to that from past studies [7]. Because of the lack of normality of their distribution, the counts of insect fragments from each treatment were 'square root' transformed.

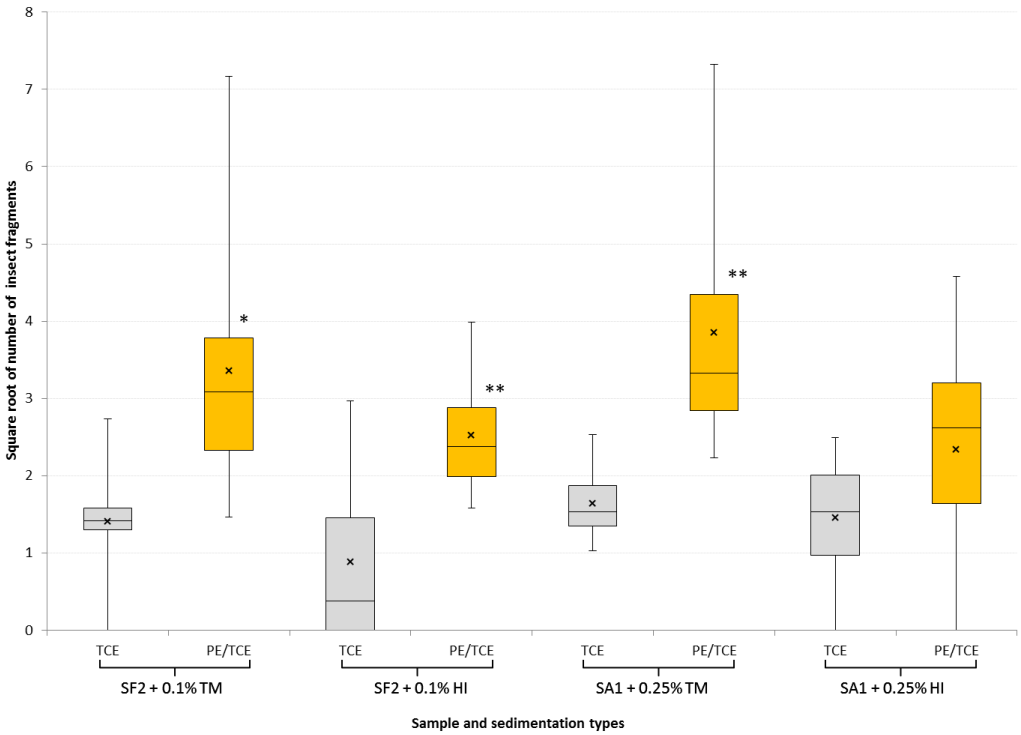

Fig. 2 : Boxplots of number of insect particles after square root transformation and standardization to 30 mg of flotate – group 1. Legend: TCE: single TCE sedimentation; PE/TCE: double sedimentation; TM: *T. molitor*; HI: *H. illucens*; *: significant at P < 0.05; **: significant at P < 0.01.

A first group of results is presented together (Fig. 2). It consists of data obtained from fish feeds which may legally contain insect PAPs. It includes the following samples: the feed for salmon (SA1) fortified at 0.25 % with either *T. molitor* or *H. illucens*, and the feed for fry (SF2) fortified at 0.1 % with again one of the two common insect PAPs. In all four samples the application of the double sedimentation resulted in an increase of the mean number of insect fragments detected compared to what was obtained by the single TCE sedimentation. This increase was significant (at $p < 0.05$) in one occasion and highly significant (at $p < 0.01$) for two other cases. For the SA1 feed fortified at 0.25 % with *H. illucens* the increase in number was only a trend resulting from the variability of the counting (including two false negative results). Concerning the failure at detecting insect from this first group of results, a total of 8 false negative results were noted whereof 6 out of them were delivered by using the single TCE sedimentation. The sample delivering the highest number of false negative results (4) was the SF2 adulterated at 0.1 % with *H. illucens*. A more detailed discussion on the emergence of false negative results is presented further in the study.

A second group of results (Fig. 3) included feed matrices into which insect PAPs are legally prohibited and a fishfeed. This group contained two poultry feeds, one (PF2) naturally contaminated with *T. castaneum* and another (PF1) fortified at 0.1 % with *T. molitor*, a ruminant feed (RF) fortified at 0.1 % with *H. illucens*. The fourth sample of this group was a fishfeed (FF) fortified with 0.5 % of *T. molitor* and submitted to the NRL network in a past study [5].

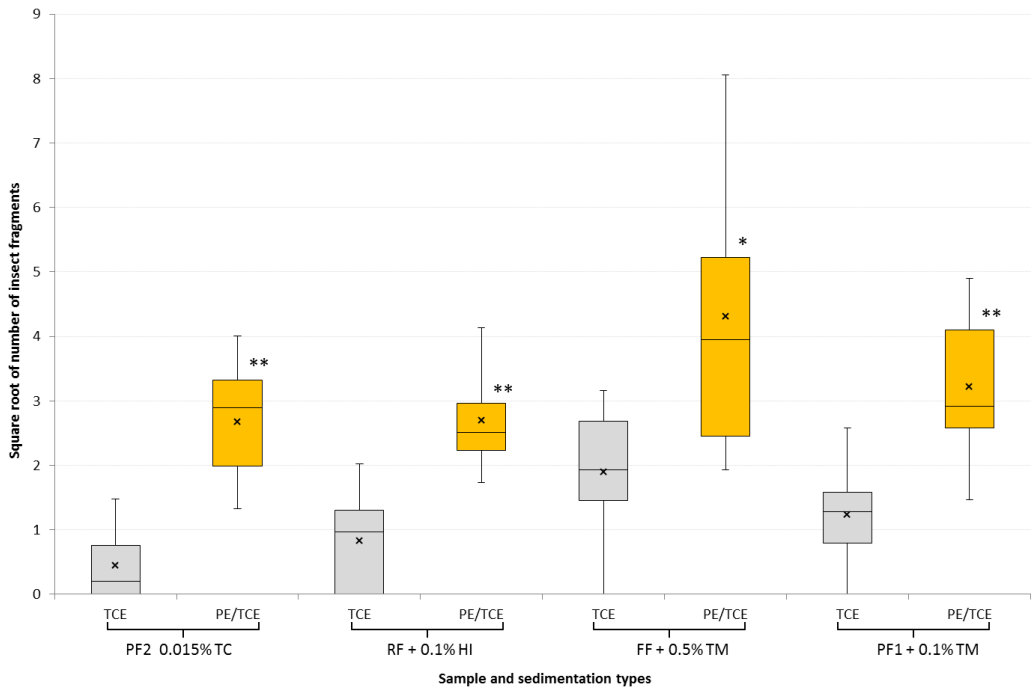

Fig. 3 : Boxplots of number of insect particles after square root transformation and standardization to 30 mg of flotate – group 2. Legend: TCE: single TCE sedimentation; PE/TCE: double sedimentation; TC: *T. castaneum*; TM: *T. molitor*; HI: *H. illucens*; *: significant at P < 0.05; **: significant at P < 0.01.

In this second group an increase in the number of identified insect particles following the double sedimentation was always found compared to the records from single TCE sedimentation. The raises in mean numbers were all significant (at $p < 0.05$) or highly significant (at $p < 0.01$) whatever the matrix, the insect species or the percentage of insect PAP. No false negative was found when the double sedimentation was used even at the lowest level of insect presence, for the naturally contaminated PF2. On the contrary 9 false negative results occurred when the single TCE sedimentation was performed (4 for PF2 contaminated at 0.015 % with *T. castaneum*, 3 for RF adulterated at 0.1 % with *H. illucens*, and 1 for each of the two other samples from this group).

Regarding the emergence of false negative results after the application of the double sedimentation, the question of a minimum number of slides to observe was addressed. According to the instructions all participants had to prepare 3 slides and to report for each of them the number of insect fragments identified. Based on these detailed counts, simulations were made by combining participants results based on two or three slides and by applying the legal decision rule ('5 particles') as prescribed by Annex VI of EU/152/2009 [2]. Results are summarised in Table 2. This table is however based on raw insect fragments data thus not reported on a standard 30 mg material.

Table 2 : Calculated percentages of negative, <LOD and positive results with the application of the '5 particles' rule applied to the observation a minimum of two or three slides after double sedimentation. Percentages in red refer to false negative results.

	2 slides *(set of 24 combinations)*			3 slides *(set of 8 combinations)*		
	negative	**<LOD**	**Positive**	**negative**	**<LOD**	**positive**
FF + 0.5 % TM		58 %	42 %		62 %	38 %
SA1 + 0.25 % TM		71 %	29 %		62 %	38 %
SF2 + 0.1 % TM	**4 %**	63 %	33 %		62 %	38 %
PF1 + 0.1 % TM		75 %	25 %		75 %	25 %
SA1 + 0.25 % HI	**25 %**	71 %	4 %	**25 %**	75 %	
SF2 + 0.1 % HI	**13 %**	87 %			100 %	
RF + 0.1 % HI	**4 %**	96 %			100 %	
PF2 0.015 % TC	**4 %**	92 %	4 %		100 %	
Grand total	**6 %**	77 %	17 %	**3 %**	80 %	17 %

By limiting the observations to only two slides, many false negative results (β errors) arise. These percentages of negative conclusions range from 4 to 25 % and imply five of the eight samples. Interestingly all samples fortified with *H. illucens* were concerned with β errors. By pooling all samples together the percentage of β error, over the whole study, is of 6 %. It has also to be noted that a majority (77 %) of the sample are declared as below the LOD considering the 5 particles rule application.

By increasing the observations to three slides, the number of false negative results was only restricted to one sample at a percentage of 25 %. This sample, Sa1 fortified at 0.25 % of *H. illucens* is also the only sample for which no significant increase in mean number of insect was found as it can be deduced from Fig. 2; the 2 false negative results (out of 8) account for it. Pooled together the

percentage of β error was of 3 % when three slides are observed. Considering the current legislation prohibiting the use of insect PAPs in feed, on the sole exception of fishfeed, no permitted limit is established. Therefore it is advised to observe a minimum of 3 slides from the flotate obtained by the double sedimentation to be sure that no more than 5 % of β error would occur in accordance with Commission Decision EC/2002/657 [8]. Moreover this minimum of 3 slides proved to be sufficient to allow a systematic disclosure of insect PAPs in matrices were they are not allowed: poultry feed (PF1 and PF2) and ruminant feed (RF).

Concerning the number of insect particles identified, the double sedimentation coupled with the observation of 3 slides reveals that these particles are found in 97 % of the cases. However the counts that would deliver a positive result, with reference to the legal expression of results as per Annex VI of EU/152/2009, are only found for the samples fortified with *T. molitor* and would only account for 17 %. This means that all other findings of insect fragments in the submitted sample set should, still according to the legal expression of results, be considered as <LOD. Two elements may explain this situation. At first, it is known that the share of flotate fraction obtained by double PE/TCE sedimentation from PAPs made of *H. illucens* is lower than the one originating from PAPs of *T. molitor* [4]. This results in less insect fragments from *H. illucens*. Secondly as discussed by Veys and Baeten [4] larvae from *T. molitor* are presenting more differentiated sclerotinized cuticle fragment which make them easier to distinguish compared to Diptera larvae such as from *H. illucens*. A third element which is known to impact on the detection of insect is the composition of the feed matrix. In the present study such effect could not be shown.

Results obtained from the fishfeed (FF) fortified with 0.5 % of *T. molitor*, submitted to the NRL network in a past study [5], deserves also comment. This sample was used for research purpose in a proficiency test; only 16 % of the participants reported the presence of insects at that time by applying the single TCE sedimentation and without training beforehand. In the present study, when the single sedimentation was used (see Annex 2) 87 % of the participants detected them while by using the double PE/TCE sedimentation this ratio raised up to 100 % (table 2), this however without taking into account the number of particles identified. When looking at the counts, reported on a standard 30 mg of flotate used, the observed means (± SEM) were of 23.4 ± 8.4 for the double sedimentation and of 4.5 ± 1.2 for the single sedimentation: a highly significant difference in favour of the new sedimentation protocol.

Finally the present results also demonstrated that the protocol enables at detecting natural low contamination levels. By using 3 slides, all participants succeeded at detecting *T. castaneum* at the level of 0.015 % w/w into a poultry feed. It supports previous statement [4] that the absence of synchronicity of developmental stages in case of natural contamination is facilitating the disclosure of insect fragments due to a higher number of fully differentiated cuticular fragments from imago's.

Conclusion

The present study enables to draw two conclusions.

A first conclusion is about the validation of the protocol based on the double PE/TCE sedimentation. The results demonstrated that this double sedimentation enables at obtaining a significantly better segregation of particles from insect PAPs than the one that can be obtained with the single TCE sedimentation used in current Annex VI of regulation EU/152/2009. The transfer of the method through a series of 8 participants occurred successfully. The microscopic analyses following the double PE/TCE sedimentation require at least the observation of 3 slides as a minimum to ensure a level of β error inferior to 5 %. This number of slide is even enough to detect insect PAPs in unauthorised matrices as demonstrated on poultry and ruminant feed under the current feed ban conditions. The study however revealed that applying the current rules of declaring positive a sample if at least 5 particles of such particle type, in the present case insect or terrestrial invertebrates, is found will lead to uncertain situations in terms of repeatability since numerous samples will be declared as below the LOD.

A second conclusion is related to the rejection of a large number of participants mainly due to erroneous interpretation of particles as from insect origin. This was demonstrated on the fishfeed free of insect PAP serving as a negative control for the study. Although one of the conditions for the participation of laboratories was to have one staff member having followed a specific training on the detection of invertebrate products in feed, many erroneous insect detection occurred. The frequency of use of this new type of feed ingredient is very low. Microscopists lack experience and proficiency in the recognition of insect particles. This was evidenced by the specificity issue on the blank. On the other hand since microscopic method relies on human expertise a better knowledge will also improve the sensitivity on insect detection. The need for in depth education pertains and the organisation of a single training session is not sufficient. There is a lack of reference micrographic records and literature on these new feed materials. Therefore it is highly recommended to organise a larger scale implementation test involving all NRLs after the present one before translating the new protocol into the legislation.

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Annex 1

Slides' details of the numbers of particles erroneously interpreted as from insect origin from the blank matrix, FF.

Participant ID	Slide 1	Slide 2	Slide 3	TOTAL
4	110	154	115	379
9*	1	1	0	2
12	38	41	33	112
13	16	15	18	49
11	1	1	1	3
6**	12	0	7	19

** Participant 9 also included in it weight measurements the weights of the filters used; ** participant 6 did not follow the prescribed protocol.*

Annex 2

Calculated percentages of negative, <LOD and positive results with the application of the '5 particles' rule applied to the observation a minimum of two or three slides after single TCE sedimentation. Percentages in red refer to false negative results.

	2 slides *(set of 24 combinations)*			3 slides *(set of 8 combinations)*		
	negative	<LOD	positive	negative	<LOD	positive
FF + 0.5 % TM	**13 %**	75 %	12 %	**13 %**	75 %	12 %
SA1 + 0.25 % TM	**8 %**	92 %			100 %	
SF2 + 0.1 % TM	**17 %**	79 %	4 %	**13 %**	75 %	12 %
PF + 0.1 % TM	**17 %**	83 %		**13 %**	87 %	
SA1 + 0.25 % HI	**21 %**	79 %		**13 %**	87 %	
SF2 + 0.1 % HI	**54 %**	46 %		**50 %**	50 %	
RF + 0.1 % HI	**42 %**	58 %		**38 %**	62 %	
PF2 0.015 % TC	**54 %**	46 %		**50 %**	50 %	
Grand total	**28 %**	70 %	2 %	**24 %**	73 %	3 %