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Laboratory experience with the microscopic method for the detection of insects in poultry feeds

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Abstract

Introduction: The use of insects and their processed animal proteins (PAPs) for animal nutrition creates the need for research into methods useful for routine surveillance for their presence. The aim of this study was to evaluate a modified microscopic method for the detection of particles of insects in poultry feed. **Material and Methods:** A total of 90 samples including PAP of insects (*Hermetia illucens* and *Tenebrio molitor*), poultry feeds produced with different levels (0–27%) of insect PAP content, and other poultry feeds spiked with insect PAP at 1% were investigated using a modified microscopic method with a double sedimentation protocol. **Results:** Characteristic features of insects including cuticulae, muscles, bristles and tracheoles were determined in the microscopic images obtained. In all spiked samples, characteristic fragments of insects were detected. The fragments of muscle and tracheoles only indicated the presence of material from members of the insect class but could not facilitate identification of organisms to species level. **Conclusion:** The results obtained with this double sedimentation protocol for the isolation of insect PAP from feed for poultry have shown that the method can be used in routine analysis.

Keywords: microscopic method, insects, sedimentation, *Hermetia illucens*, *Tenebrio molitor*.

Introduction

Currently, one of the world's most serious economic problems is the depletion of protein sources. Between 1988 and 2002, the percentage of fishmeal used for aquaculture feed production increased from 10% to 45%. Because of the rising demand for fishmeal without the scaling up of its availability, the price of this material has increased sharply and, consequently, the cost of aquaculture production has increased (2). The world's population growth entails the need to search for alternative protein sources.

Insects are the most ubiquitous and diverse group of organisms in the world. More than 2,000 species of insect are considered edible, and generally the members of the insect class are rich in lipids and total protein, which recommends their exploitation as substitute protein sources. Fish, amphibians, reptiles, birds and mammals consume insects in nature. Thus, it can be supposed that insect proteins may be an important source of protein in commercial feed (18, 22, 23).

The European Commission approved Regulation 2017/893 of 24 May 2017, which allows the breeding of the following species of insects and their use in aquaculture nutrition: the 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*). In this regulation, the safety requirements for production of insect processed animal protein (PAP) are also detailed.

These species were selected after considering the national risk assessments and the European Food Safety Authority opinion of 8 October 2015 (9). Insect species to process for protein should not have any adverse impact on the health of people, animals or plants and should not transmit pathogens; neither should they be protected or invasive alien species.

The level of nutrients derivable from insects depends on their species, life stage and/or the nutrition used during their breeding. Most insect species are

marked by high content of proteins (CP) of between 40 and 60%, which is similar to soy meal levels (50% CP) but lower than fishmeal (73% CP) (3, 17, 20, 27). It should be noted that insect proteins are characterised by a high level of exogenous amino acids, *e.g.* threonine, valine, histidine, phenylalanine and tyrosine (21).

Fat is the second largest nutrient in the insects described. The fat content of insects is highly variable. It is higher in the larval stages than in adults, e.g. 37.1% of dry matter (DM) for larvae of mealworm but only 14.41% DM for mealworm imago. This content and fatty acid composition differ depending on the diet of insects, but generally insect meal contains more polyunsaturated fatty acids than fish or poultry meal (1, 25).

It is generally accepted that chitin is one of the factors limiting the use of insects in feed. The chitinase activity was observed in several fish species in an attempt to understand enzymatic degradation of chitinous substances (13). Alternatively, before being added to fish diets chitin may be degraded by chemical or enzymatic methods such as chitooligosaccharides (COS), N-acetylglucosamine or chitosan (11). Low level addition of chitin and its metabolites have an immunostimulatory effect on fish. However, this process significantly increases the cost of producing insect meal (10, 13, 15, 16, 26).

The use of insects and their processed animal protein (PAP) for animal nutrition requires the development of a method for the surveillance of contamination and dishonest practice in the formulation of feedstuffs. Presently, there are two methods authorised for the detection of animal proteins: light microscopy and real-time PCR (5, 6, 7, 8). The microscopic method allows observation of the different structures of animals, vegetables and minerals. Lighter fragments of insect cuticulae are concentrated in the flotate with plant particles. This fraction is very large, and the detection of elements of insects is difficult. For this purpose, a modified microscopic method with a double sedimentation protocol (24) was used. This article describes experimental work to assess the usefulness of the modified microscopic method with a double sedimentation protocol.

Material and Methods

Samples were divided into two groups. The first group (n = 45) comprised 5 samples of black soldier fly (*Hermetia illucens* – HI), 30 samples of poultry compound feed (10 samples of starter, 10 samples of grower and 10 samples of finisher) with known levels of insect PAP of 0%, 13%, 20% and 27%, and 10 samples of poultry feed spiked with 1% HI PAP. The second group (n = 45) had the same composition except for the inclusion of yellow mealworm (*Tenebrio molitor* – TM) PAP instead of HI PAP.

For the identification of insect PAP, the microscopic method described in Commission Implementing Regulation (EU) 2020/1560 was applied with modifications (24). This regulation provides guidelines for the detection and identification of animal particles. In the first step, samples of insect proteins were examined. Microscopic images showed characteristic features of *Hermetia illucens* and *Tenebrio molitor*. In the second step, samples of poultry feed with insect PAPs were analysed.

Protocol of feed samples sedimentation. A 50 g mass of a feed sample was ground using a knife mill and 10 g was transferred into a closed sedimentation funnel of 250 mL capacity. Next, 100 mL of tetrachloroethylene (TCE) with a density of 1.62 g/cm^3 was added. After at least 3 min, the sediment on the filter paper was collected. This step was mandatory under the regulation cited above.

Approximately 30 mL of the TCE was drained, the same volume of petroleum ether (PE) with a boiling point of 40–60°C and density of 0.65 g/cm^3 was added to the sedimentation funnel, and the funnel contents were thoroughly mixed. After 10 min, the second sediment and flotate were collected on Petri dishes. Three fractions were dried in a fume cupboard. The entire flotate or a part thereof was examined for specific insect particles under a biological microscope.

The microscopic identification of insects by characteristic features was performed using paraffin oil as the embedding medium. In routine analysis, paraffin oil serves for the identification of bone fragments in sediment. The samples were observed using an Olympus BX53 light microscope under a transmitted bright field at different magnifications. With regard to the spiked samples, one slide with matter from the flotate was analysed.

Results

Characteristic features of insects were determined in the microscopic images obtained. During the analysis, the appearance of the exoskeleton was explored. Figures 1–5 present the irregular shape and cellular structures of the constituents of the HI exoskeleton (cuticula). In these structures, five or six walls with a lighter centre were visible and they resembled a honeycomb (Figs 1–3). In some structures, a central darker dot was detected (Fig. 3). The colour of these materials was observed to range from from greyish-cream to dark brown.

In addition, fragments of HI exoskeletons with numerous long bristles ranging in colour from yellow to brown were observed (Figs 4–5).

In the material from TM exoskeletons, only sporadic black dots were observed (Fig. 6). In some dots, very short, dark bristles were visible (Fig. 7). The fragments of the exoskeletons were bright greyish-yellow to deep greyish-amber in colour. Irregular light spots were observed in the exoskeletons of TM larvae (Fig. 8).

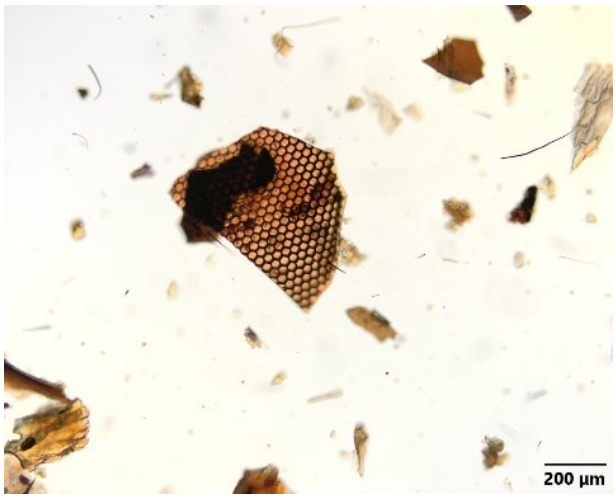

Fig. 1. Fragment of *Hermetia illucens* cuticula with visible honeycomb-like structure

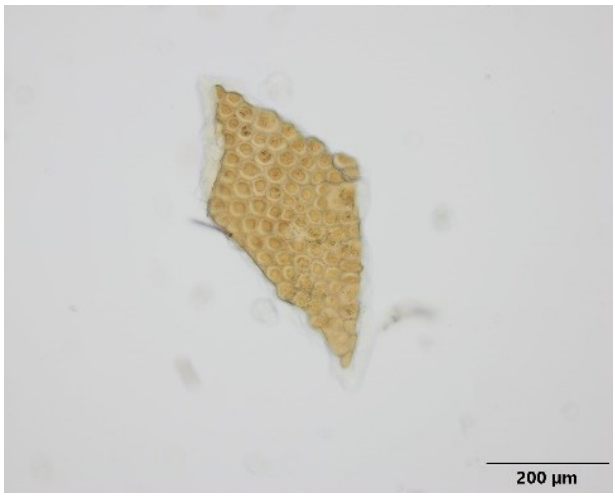

Fig. 2. Typical fragment of *Hermetia illucens* cuticula with honeycomb-like structure

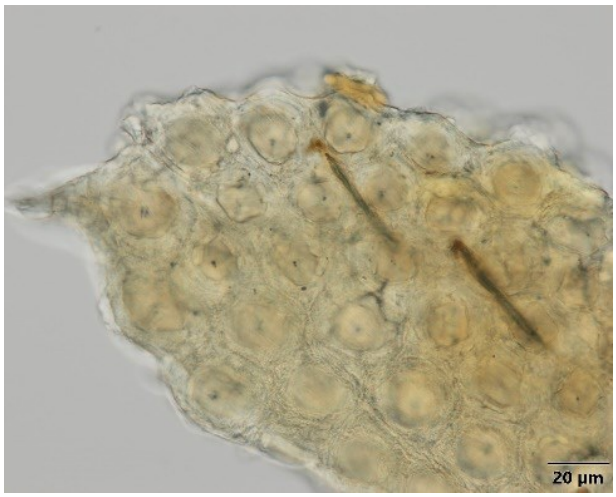

Fig. 3. Fragment of *Hermetia illucens* cuticula with visible dark dots in the centre of cells in the honeycomb-like structure

Fig. 4. Fragment of *Hermetia illucens* cuticula with characteristic bristles

Fig. 5. Fragment of *Hermetia illucens* wing with characteristic bristles

Fig. 6. Fragment of *Tenebrio molitor* cuticula with characteristic black dots

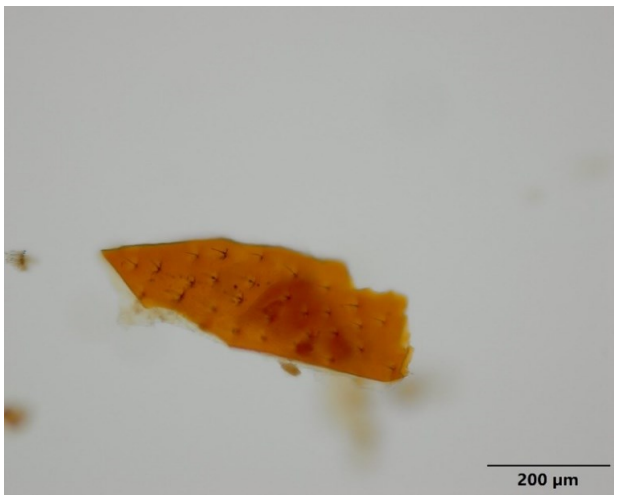

Fig. 7. Fragment of *Tenebrio molitor* cuticula with black dots and short, dark bristles

Fig. 8. Fragment of *Tenebrio molitor* cuticula with irregular light spots

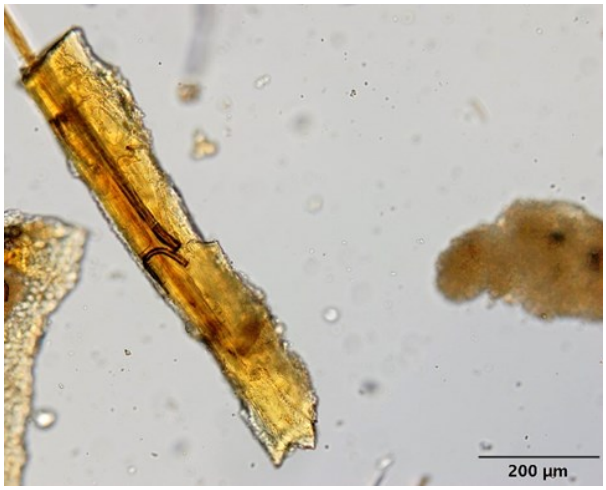

Fig. 9. Fragments of muscles with visible tracheoles

In the material of both species of insect, elements of muscle fibres were found and fragments of tracheoles as respiratory system particles were noted (Figs 9–12). Fragments of muscle as square or rectangular structures were also discovered. These were bright yellow in colour and sometimes transparent. In the fragments of muscle sarcomeres, zigzag striation was visible (Fig. 10).

At higher magnification, a spiral, transversal thickening was observed in the tracheoles (Figs 10–12).

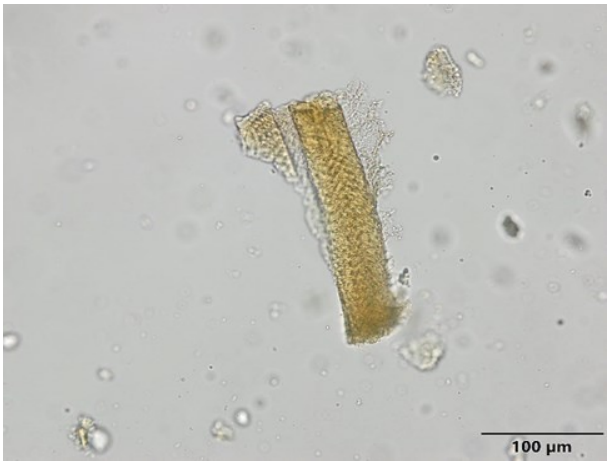

Fig. 10. Fragments of muscle with zigzag striation

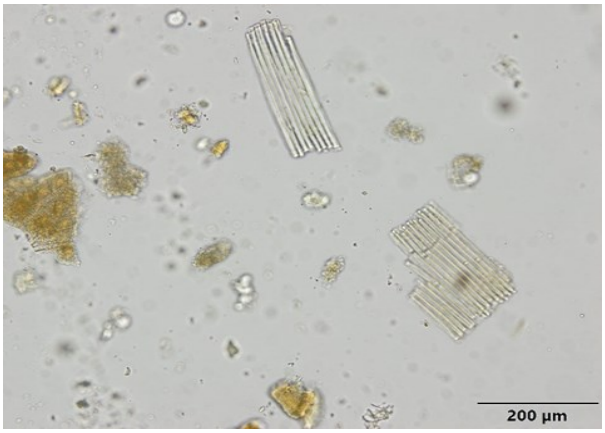

Fig. 11. Fragments of a tracheole with circumferential thickening

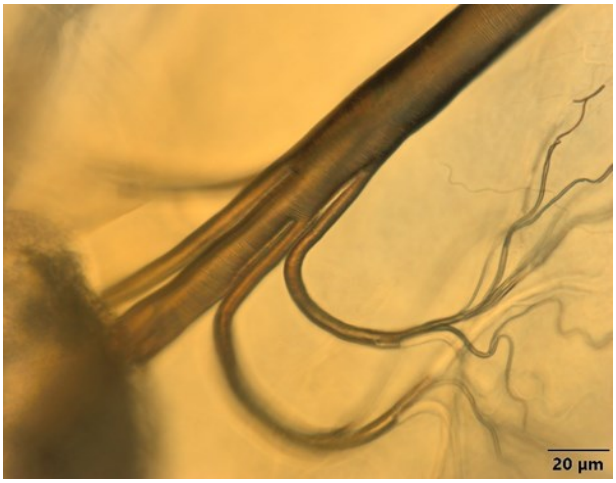

Fig. 12. Tracheoles with circumferential thickening of the cuticle inside

In the next stage of the research, samples of compound feed for poultry spiked with insect PAP were examined. Characteristic fragments of insects were detected in all spiked samples. The most often observed fragments were of muscles, tracheoles, bristles and cuticula.

No particles were observed in the samples without PAP from insects. Figure 13 shows the mean amount of characteristic particles. Readily identifiable HI and TM elements were observed in all poultry feed samples with insect PAP content (whether as produced or added as spiking), as Figs 13–15 show. In the samples contaminated to a level of 1%, more than five typical particles were detected: nine particles in samples with HI and seven in samples with TM.

In the samples with HI PAP content level above 13%, on average 27 particles were observed, and 51 particles were detected in the samples with 27% PAP.

In the samples with a TM PAP constituent of above 13%, an average of 21 particles were observed, and 36 were visible in the samples with 27% PAP. Only one fragment of bristle was observed in the samples with 27% TM PAP and no such particles were identified in samples with lower TM PAP content.

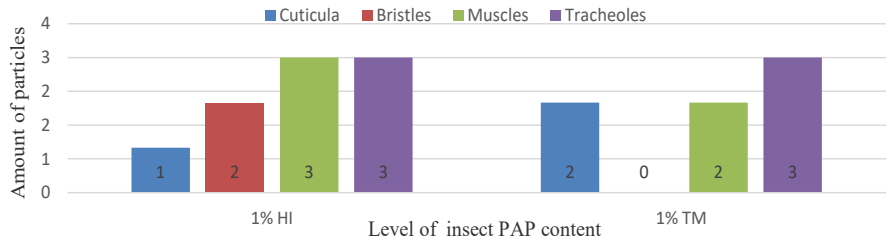

Fig. 13. Comparison of the mean numbers of insect particles of *Hermetia illucens* (HI) and *Tenebrio molitor* (TM) in the samples of poultry feed spiked with 1% of insect processed animal protein (PAP) content

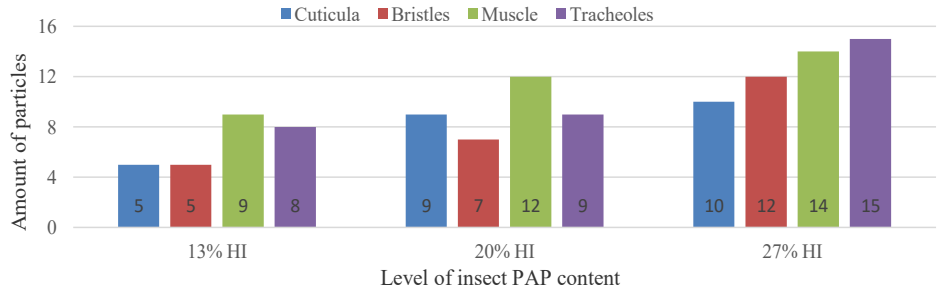

Fig. 14. Correlation of the mean numbers of *Hermetia illucens* (HI) particles per slide with the level of insect processed animal protein (PAP) content in poultry feed

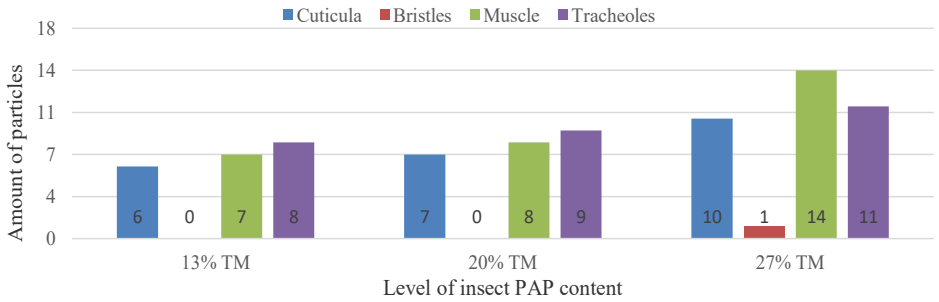

Fig. 15. Correlation of the mean numbers of *Tenebrio molitor* (TM) particles per slide with the level of insect processed animal protein (PAP) content in poultry feed

It should be noted that in samples with every proportion of PAP content, fragments of muscles and tracheoles were observed most frequently.

Discussion

Currently, the microscopic method is one of the methods authorised for the detection of animal protein in feed. This method has routinely been used in the official monitoring of feed since 2004. The method is characterised by high sensitivity: the limit of detection has been determined to be 0.1%. The choice of this method for the identification of particles from insects is appropriate because of its simplicity and the modest level of experience needed in analysts. No specific laboratory equipment is required for the examination; this method is technically easy.

Modification of the PAP isolation protocol in the microscopic method is a very important element that facilitates the detection of insect particles. Only the flotate obtained after double sedimentation was used for the examination. Veys and Baeten (24) confirmed the coefficient for the separation of insect particles from other fragments of feed for aquaculture. Tetrachloroethylene with a density of 1.62 g/cm^3 was used in the routine analysis, and petroleum ether with a boiling point of 40–60℃ and density of 0.65 g/cm^3 was applied in the experiment. The mixture of 30% PE and 70% TCE had a density of about 1.26 g/cm^3. Particles from insects were obtained from flotate in the range of 40 to 69%, approximately 25 times higher than is possible by using the method described in the Commission Implementing Regulation (8).

In the first step, the characteristics of the insect fragments were determined. The pre-existing literature describes possible applications of microscopy for differentiating the fragments of insects in fish feed. Ottoboni *et al*. (19) first described the characteristic features of insects. More details were described by Veys and Baeten (24). The appearance of cuticle fragments is quite specific to insects and such fragments present no great challenge in identification. However, in this study new elements of the HI and TM cuticula were observed. Furthermore, some of the dark browns of the cuticula fragments of HI were similar to the husks of rape and therefore these particles were harder to classify. In husks of rape, the centre of the structures was darker than in the insect fragments (14). The bristles on the surface of cuticula fragments of HI were the most noticeable characteristic (19, 24). However, the cuticula fragments of TM were difficult to recognise because the dots were not always observed.

The most typical fragments of insect PAP are fragments of muscle and tracheoles. In insect muscles, zigzag structures are commonly observed as the result of a specific striation of sarcomeres. Frequently, the ratio of myosin:actin is 6:1 in insect muscle (12). Some limited taxonomic attribution of muscles is possible because for the flight muscles of *G. assimilis*, the ratio of myosin:actin is 3:1 (4). Fragments of tracheoles with taenidia were visible and were frequently observed in the muscles. The presence of the fragments of muscle and tracheoles indicates only that the material is of insect origin and cannot assist in classification to lower taxons.

In routine analysis of feed in the first determination, four slides of sediment and two slides of flotate are examined. In this study, only one slide of flotate from the feed samples was inspected. This reduction confirmed that double sedimentation complements the microscopic method.

The obtained results confirm that it was possible to detect insect fragments in the feed. Typical insect particles were detected in all insect PAP–containing samples. The range of particles from the insects widened proportionally to the level of insect PAP in the samples. In all samples with 1% insect PAP, particles of muscles and tracheoles were detected most frequently. Only in samples with 20% of HI PAP and 27% of TM PAP were fewer fragments of tracheoles than muscles detected.

It should be pointed out that the fragments are not species specific to the extent to which they could be observed in this study. The presence of these fragments can only be an indicator of the presence of insect material. The appearance of cuticulae of the examined insects allows their characteristic features to be determined. However, in routine analysis, it is possible that only fragments of muscle or tracheoles are observed. For this reason, it is necessary to seek a rapid method for the identification of the insect's species. More specific methods for determining the insect species would be used in the second step of feed monitoring. Similarly, the official surveillance of aquafeeds with no declared PAP content is now being undertaken in the first analysis using the microscopic method. When terrestrial PAP is detected, the particular terrestrial sources from which it originates are established using real-time PCR for the identification of ruminant DNA.

In conclusion, the results obtained using the protocol of double sedimentation for the isolation of insect PAP from feed for poultry have shown that the method can be used in routine analysis. The presence of muscle or tracheole fragments can only be a coarse indicator of the presence of insect material. To determine the insect species, more specific methods must be used, *e.g.* PCR.

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