DOI: 10.2478/fv-2026-0028

FOLIA VETERINARIA, 70, 3: 62—68, 2026

ORIGINAL ARTICLE

MICROSCOPIC EXAMINATION OF FOODS CONTAINING INSECTS

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OPEN ACCESS

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Citation: Javůrková, Z., Homolková, R., Pospiech, M., Bartlová, M., Havlová, L., Dobeš, J., Tremlová, B., 2026: Microscopic examination of foods containing insects. Folia Veterinaria, 70, 3, 62–68.

Received: June 24, 2026

Accepted: July 31, 2026

Published: September 15, 2026

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Ethical considerations: When reporting experiments on animals Observation of the ARRIVE guidelines 2.0: Updated guidelines for reporting animal research, published on July 14, 2020 (DOI: 10.1371/journal.pbio.3000410), is applied. The authors ensure that all procedures were performed in compliance with the guidelines for animal care of their institutions or with national/international guidelines.

ABSTRACT

Edible insects have traditionally been consumed in many regions of the world and are currently reconsidered as a sustainable alternative to conventional animal protein sources. In food products, insects are commonly used in powdered form, which complicates their identification in final products. This study aimed to evaluate the applicability of microscopic methods for the detection of insect structures in foods and to compare the effectiveness of the histochemical staining methods PAS-Calleja and Pasini. A total of 14 commercially available insect-based food products were analysed using standard histological techniques. In all samples, the presence of insect structures, particularly fragments of cuticle and muscle tissue, was confirmed. In addition to their detection, the size of individual fragments was evaluated, providing supplementary information on the degree of technological processing and the preservation of insect structures in the final products. The PAS-Calleja staining method provided a stronger contrast of chitinized structures compared to the Pasini method. The results indicate that microscopy, combined with appropriate histochemical staining and evaluation of fragment size, represents a suitable and practical method for the detection and assessment of insect components in foods.

Keywords: cuticle fragments; food control; light microscopy

INTRODUCTION

The consumption of insects has a long tradition and is still common today in many parts of the world, where insects represent an important source of protein and other nutrients. In recent years, interest in entomophagy has been increasing again, primarily in connection with the search for more sustainable alternatives to conventional sources of animal protein [1]. There are currently four species of insect authorised for human consumption in the European Union: *Tenebrio molitor* [2], *Locusta migratoria* [3], *Acheta domesticus* [4], and *Alphitobius diaperinus* [5]. However, the authorisation does not apply solely to specific insect species but also to specific forms and processing methods in which these products may be placed on the market. Insects can be consumed whole; however, espe-

cially in European countries, they are more often technologically processed, for example, into powders or protein extracts that are subsequently added to foods. The significance of insects is in their nutritional value and production efficiency. Insects are also considered an alternative source of animal protein with potential use in the future food system [6, 7]. In addition to their nutritional value, insects also contain bioactive compounds with antioxidant, antimicrobial, and anti-inflammatory effects, which support their use in functional foods [8].

The range of foods containing insect ingredients has expanded significantly in recent years and includes both whole edible insects and products enriched with insect powder, such as crisps, protein bars, pasta, breakfast cereals, and confectionery [9]. Edible insects are most commonly used in bakery and cereal products, particularly in bread, biscuits, crackers, muffins, and pasta. Consumers generally accept the addition of insect meal at levels of up to 10 per cent in bread and around 5 per cent in biscuits and muffins [10], while concentrations of 2–10 per cent have been successfully tested in pasta [11]. Higher levels have also been investigated in pasta (up to 30 per cent) and biscuits (up to 20 per cent), with some of these products retaining acceptable technological and sensory properties [12, 13].

The growing use of insects in food is increasing the need for reliable analytical methods to verify authenticity and determine the content of insect-derived ingredients in products. The microscopic methods we use are important tools for quality control and the identification of ingredients of insect origin [14]. Microscopic analysis is one of the methods used for the detection of insects or their fragments in food. The principle of this method is based on the direct observation of insect morphological structures in food samples using microscopic techniques [15]. Among the most commonly observed structures are fragments of the cuticle, setae (hairs), and parts of the respiratory system, such as tracheoles. Cuticular fragments are relatively well recognizable even after technological processing of food, as chitin is chemically and mechanically stable [16].

The aim of this study was to analyse the applicability of microscopic methods for insect structures detection in selected insect-containing foods and to compare the histochemical staining methods PAS–Calleja and Pasini.

MATERIALS AND METHODS

Food samples (n = 14, with cricket powder) containing insects as an ingredient were obtained from retail markets in the Czech Republic. These were commercially available products declaring the presence of insects (e.g., protein bars or snacks with the addition of insect powder).

The food samples were processed by histological techniques before microscopic examination. An AFA fixative (alcohol–formalin–acetic acid) solution was used for fixation [17]. From each sample, four paraffin blocks were prepared and subsequently cut on a rotary microtome RM2255 (Leica, GER) into 4 µm thick sections. From all samples, two sections from each block were prepared for each staining method. For each sample, eight sections were randomly selected and stained using the Pasini histochemical staining method [18]. The remaining eight sections were stained using the PAS–Calleja (PC) method modified according to Lukášková et al. [19]. The samples were then mounted using Solacryl (Penta, CZE). For each sample, eight sections per staining method were examined at a magnification of 100× and higher using a Nikon Eclipse Ci-L microscope (Nikon, JAP) equipped with a Proscan III motorized table (Prior, USA).

During sample examination, attention was primarily focused on the presence of characteristic morphological characters of insects, especially fragments of the cuticle (chitinous exoskeleton) and muscle tissue. These structures were identified in terms of their characteristic appearance after staining and were used as indicators of the presence of an insect component in the analysed food samples. The size of the fragments was evaluated using NIS Elements software (Laboratory Imaging, CZE) based on the image analysis.

RESULTS AND DISCUSSION

Of the samples analysed, the packaging of two products (pasta and granola) did not specify the type of insect used or its proportion in the product. For the other products, powder or flour made from the house cricket (*Acheta domesticus*) was declared as the insect ingredient. Commission Implementing Regulation (EU) 2022/188 [4] lists the approved forms of *Acheta domesticus* as frozen, dried, and powdered forms. In three cases, the protein bars contained

10 per cent cricket powder and in two cases 17 per cent, which complies with the legal requirements. In all cases, the crisps and triangular snacks analysed were labelled as containing 7 per cent cricket powder, which contravenes the aforementioned regulation, which permits a maximum amount of 5.0 g per 100 g. The bars contained 5 per cent, which is in accordance with the legislation. Two samples of protein bars incorrectly listed "cricket flour"; according to current European Union legislation, the correct designation for this ingredient is "house cricket (*Acheta domesticus*) powder" or "powdered form," not "cricket flour" [4]. The declared concentrations of the insect component in the analysed products correspond to the ranges reported in the scientific literature for baked goods, cereal products, and snack products containing edible insects [10, 11]. The inclusion of 10–17% cricket powder in protein bars confirms the trend toward using insects as a protein source in functional foods, while concentrations of up to 10% are more commonly used in pasta and other cereal products [11, 12]. These results highlight the importance of proper labeling and control of products containing insect ingredients in accordance with novel food requirements.

Microscopic examination of samples stained using the histochemical methods PAS–Calleja and Pasini confirmed the presence of insect-derived structures in all analysed samples. In most samples, these structures were present as larger fragments, which could be readily distinguished from other components of the food matrix under microscopic observation. These findings are generally consistent with results reported in the literature, which describe microscopy as a reliable method for detecting insect particles in food and feed [1, 15]. Our results are consistent with published studies reporting that the most commonly identified insect structures in food include the cuticle, parts of the tracheal system, muscle fibres, setae, and other morphological features characteristic of insect bodies [20, 15, 21]. Microscopic analysis is often used to detect contamination by insects and foreign particles in grains, flours, animal feed, and food [22]. However, its usefulness decreases when applied to finely ground insect powders and technologically processed products, where diagnostic morphological features are lost and the risk of raw material substitution or adulteration increases [23, 14]. Histochemical staining can enhance the contrast of insect structures and, according to Pečová et al. [21], enable detection of insect fragments in food even at concentrations as low as 1% of the insect component.

With respect to the size of insect muscle fragments, the largest were observed in protein bars, whereas the smallest were found in insect-based triangles. Based on the size measurements of insect cuticle fragments, the largest fragments were detected in chips, while the smallest fragments were measured in the sample of protein bars (Table 1). Particle size measurement is important for foods containing edible insects, because this matrix is highly heterogeneous due to the presence of the chitinous exoskeleton. Schiel et al. [24] demonstrated that particle size after milling significantly affects sample homogeneity and the reproducibility of subsequent analytical results. Similarly, Lisboa et al. [25] emphasize that technological processes, particularly drying and grinding, significantly affect the structure of insect powders and their functional properties in food matrices.

Table 1. Size of cuticle and muscle fragments identified by microscopic examination

	Muscle tissue				Cuticle fragments			
	Minimum size		Maximum size		Minimum size		Maximum size	
Staining Group (number of samples)	PC	Pasini	PC	Pasini	PC	Pasini	PC	Pasini
Bars (2)	279.83	336.20	955.94	951.25	313.68	290.78	626.49	631.27
Chips (4)	387.43	389.55	1207.72	1080.49	383.27	311.57	1440.59	963.04
Granola (1)	350.32	264.90	698.02	640.74	371.20	462.61	942.38	620.92
Triangles (1)	340.50	230.68	549.26	571.23	383.27	359.04	617.27	680.27
Protein bars (5)	279.86	418.82	1029.58	1335.11	356.07	228.24	1016.35	856.02
Pasta (1)	273.95	432.41	724.85	694.34	471.83	237.31	713.81	549.71

PC - PAS–Calleja staining method

Differences in fragment size are likely related to the intensity of grinding the insect raw material and the processing methods used for individual products, as particle size and morphology significantly influence the structure and properties of the final food product [26].

The presence of larger cuticle fragments can lead to greater heterogeneity in the food, and it has been shown that the particle size of insect powders affects not only the homogeneity of the mixture but also the texture, sensory properties, and stability of the final products [27].

Using the PAS–Calleja method, both chitinized parts of the exoskeleton (cuticle) and fragments of muscle tissue were determined. The PAS–Calleja staining showed high intensity and provided strong contrast between the cuticle and other sample structures. The cuticle fragments were elongated, without distinct segmentation, and were mostly still attached to the muscle tissue. They exhibited an intense blue coloration, occasionally with a slight greenish tint (Figure 1). The muscle tissue appeared light to dark brown in color (Figure 2).

Comparable results were also obtained using the Pasini staining method, in which structures corresponding to insect tissues were likewise observed. Microscopic slides most frequently contained fragments of muscle tissue and parts of chitinized structures. The insect cuticle exhibited an intense blue color (Figure 3), while the muscle tissue appeared pink (Figure 4).

PAS–Calleja staining provided higher contrast between cuticle, muscle tissue, and surrounding matrix, particularly for the cuticle. In contrast, the Pasini staining method showed weaker contrast. Similar results were reported by Pečová et al. [21], who described the PAS–Calleja staining method

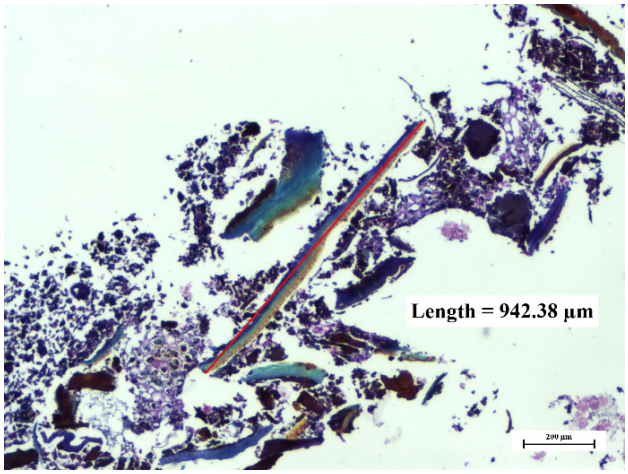

Fig. 1. Chocolate granola with cricket powder, magnification 40×, PC (cuticle)
PC - PAS–Calleja staining method

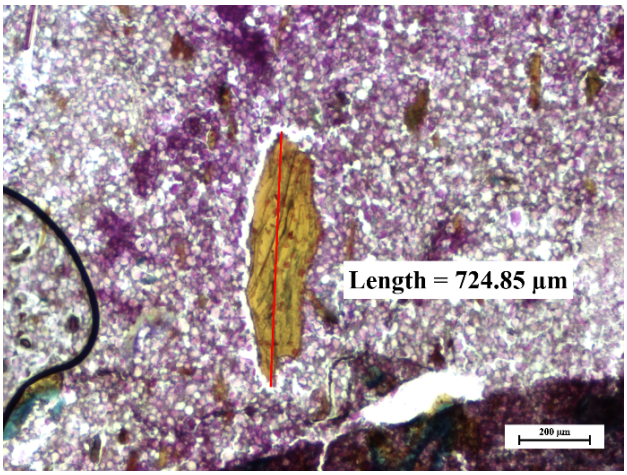

Fig. 2. Wheat egg pasta with cricket powder, magnification 40×, PC (muscle tissue)
PC - PAS–Calleja staining method

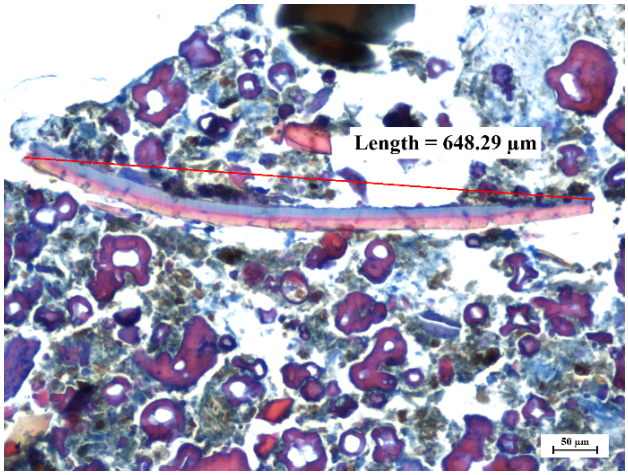

Fig. 3. Protein bar containing insects, magnification 100×, Pasini (cuticle)

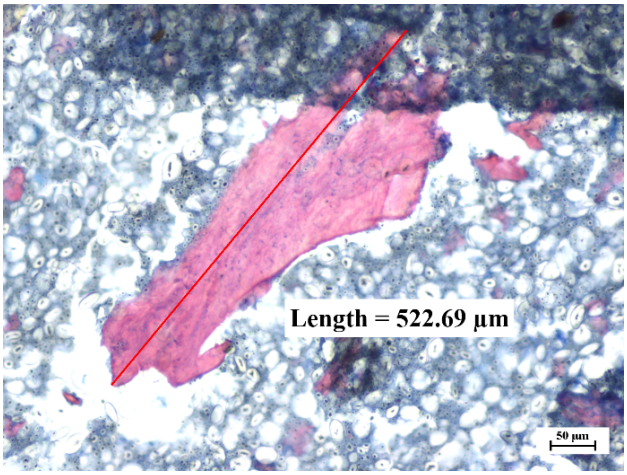

Fig. 4. Wheat egg pasta with cricket powder, magnification 100×, Pasini (muscle tissue)

as more sensitive. Ottoboni et al. [28] used light microscopy in combination with differential staining in their study. They also demonstrated that this combination allows for more reliable differentiation of insect fragments from marine arthropod material by highlighting chitinized structures.

In addition to microscopy, other methods suitable for the detection of insects have been described, including molecular and spectroscopic approaches. Although these methods can be highly sensitive, they do not allow direct visualization of structures. In contrast, an advantage of microscopy is that it enables the observation of specific insect fragments, which may be more practical in certain cases [15, 29, 30].

Some authors also report that microscopy represents a relatively simple and cost-effective method of analysis that can be applied in routine laboratory practice [15]. This is particularly advantageous compared to molecular methods, which, although more precise, are more demanding in terms of equipment and sample processing.

CONCLUSION

The presence of insect-derived structures was confirmed in all tested samples, most commonly in the form of cuticle fragments and muscle tissue, which could be clearly distinguished from other components of the examined samples. Both staining methods proved suitable for the detection of insect-derived structures. However, the PAS–Calleja method produced clearer, more distinct staining, particularly of chitinized structures.

It was also observed that the size of the fragments depends on the degree of technological processing of the food, although the structures remain preserved despite processing. The measurement of fragment size represents an additional important parameter that contributes not only to a more objective assessment of the presence of insects in food but also to a better understanding of the effect of technological processing on other food properties.

The results indicate that microscopy, in combination with appropriate histochemical staining and fragment size measurement, represents a suitable tool for the control of food products containing insects.

Ethical Statement

No ethical approval was necessary for this study.

Conflict of Interest

None of the authors has any conflicts of interest.

Data Availability Statement

The raw data of this article will be made available by the authors, without undue reservation.

Acknowledgement

This work was financially supported by the Institutional Support for the Long-Term Conceptual Development of Research Organizations provided to the Department of Plant Origin Food Sciences, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno.

Generative AI Statement

This manuscript was not written using generative AI.

Authors' Contributions

Z.J. contributed to the study design, provided methodological supervision, assisted with data interpretation, and critically revised the manuscript; R.H. performed the laboratory analyses; M.P. contributed to the methodology, sample processing, and critically revised the manuscript; M.B. performed the laboratory analyses; L.H. conceived and designed the study; J.D. carried out sampling; B.T. critically revised the manuscript.

All authors have read and approved the final published version of the manuscript.

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