

Article

Species Discrimination of Edible and Feed Insects via Raman Spectroscopy of PBS-Extractable Components

Jana Čaloudová ¹, Dani Dordevič ¹, Martina Fikselová ², Bohuslava Tremlová ¹, Martin Kulma ³, Kateřina Šebelová ⁴, Zdeňka Javůrková ¹ and Matej Pospiech ^{1,*}

¹ Department of Plant Origin Food Sciences, Faculty of Veterinary Hygiene and Ecology, University of Veterinary Sciences Brno, Palackého tr. 1, 612 42 Brno, Czech Republic; h25489@vfu.cz (J.Č.); dordevicd@vfu.cz (D.D.); tremlovab@vfu.cz (B.T.); javurkovaz@vfu.cz (Z.J.)

² Institute of Food Sciences, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 94976 Nitra, Slovakia; martina.fikselova@uniag.sk

³ Department of Zoology and Fisheries, Faculty of Agrobiological Sciences, Food and Natural Resources, Czech University of Life Sciences Prague, 165 00 Prague, Czech Republic; kulma@af.czu.cz

⁴ Department of Food Analysis and Nutrition, Faculty of Food and Biochemical Technology, University of Chemistry and Technology, 166 28 Prague, Czech Republic; sebelovk@vscht.cz

* Correspondence: mpospiech@vfu.cz

Abstract

The growing market for insect-based food and feed ingredients raises concerns about authenticity, labelling transparency, and regulatory compliance, highlighting the need for rapid screening methods capable of distinguishing selected insect-derived materials. In this preliminary feasibility study, Raman spectroscopy combined with partial least squares discriminant analysis (PLS-DA) was evaluated for the fingerprinting and differentiation of PBS-extractable components from nine insect species. Extracts of 96 dried insect samples prepared in phosphate-buffered saline (PBS, pH 9.4) were analysed, yielding 358 insect spectra after quality control; PBS spectra were included as a technical control class. The overall correct classification rate (CCR) reached 61.6%, indicating moderate classification performance rather than validated species-level authentication. *Apis mellifera* drones achieved 100.0% CCR, while *Hermetia illucens* and *Tenebrio molitor* both reached 80.0%. Complete misclassification of *Shelfordella tartara* as *Blaptica dubia* indicated strong spectral similarity between closely related cockroach species. VIP score analysis identified the 1700–1800 cm^{−1} region, corresponding primarily to lipid ester carbonyl vibrations, as the most discriminatory spectral range. The results suggest that Raman spectroscopy can provide taxon-related biochemical fingerprints of PBS-extractable insect components, while also highlighting the need for expanded reference libraries, improved sampling strategies, and validation in complex food matrices to further develop its potential as a screening approach.

Keywords: Raman spectroscopy; edible insects; food authentication; PLS-DA; chemometrics; novel food



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1. Introduction

The increasing global demand for sustainable protein sources has led to growing interest in edible insects as an alternative to conventional livestock production. Insects are considered nutritionally valuable due to their high protein content and favourable amino acid composition, while their production is associated with reduced environmental impact compared to traditional animal farming systems [1,2]. In the European market, edible insects are typically available either as whole bodies for direct consumption or, more

frequently, processed into a powder form, which tends to be better accepted by consumers when incorporated into familiar food products, as it reduces the visual recognisability of insects and thus lowers the associated psychological barrier [3,4]. As a result, insect-based ingredients are increasingly incorporated into a wide range of food products, including protein powders, snacks and meat analogues. However, the rapid expansion of insect-based foods also raises concerns related to food safety, quality control, transparency of product labelling, and the potential risk of allergies [5].

In the European Union, edible insects are classified as novel foods under Regulation (EU) 2015/2283 [6], which requires mandatory pre-market safety assessment and authorisation before any insect species may be placed on the market for human consumption. To date, four species have been individually authorised for human consumption: *Tenebrio molitor* larvae [7], *Acheta domesticus* [8], *Alphitobius diaperinus* larvae [9], and *Locusta migratoria* [10]. Additionally, partially defatted *Acheta domesticus* powder has been authorised as a separate novel food application [11]. Other species, including *Gryllus assimilis* and *Hermetia illucens*, are not authorised for human consumption but are listed among insect species permitted for processed animal protein production for feed use under the EU framework considered here [12]. Species such as *Zophobas morio*, *Blaptica dubia*, and *Shelfordella tartara* are not authorised for human consumption in the EU and are not listed among the permitted farmed insect species for processed animal protein intended for feed for farmed animals; however, they may be commercially available as live feed for exotic or terrarium animals. Their unintended presence in insect-based food products would therefore indicate the presence of non-authorised insect material. This regulatory framework creates a clear need for reliable analytical methods capable of distinguishing authorised from non-authorised insect species, thereby supporting the enforcement of food labelling regulations and the detection of potential food fraud.

The shift towards highly processed insect-based foods creates significant analytical challenges. Since morphological features are lost during processing, verifying label claims and ensuring regulatory compliance become impossible through visual inspection alone [13]. This lack of transparency increases the risk of food fraud and poses a threat to food safety. Furthermore, the presence of insect proteins can trigger cross-reactivity in sensitive individuals, particularly those with shellfish or dust mite allergies [14]. Therefore, robust analytical approaches are required to enable rapid and accurate detection of insect components within complex food matrices [5,13].

Conventional methods for detecting insect material in food include molecular techniques such as polymerase chain reaction (PCR), chromatographic analysis, and light microscopy. PCR-based approaches offer high specificity and sensitivity but are often time-consuming, require extensive sample preparation, and involve destructive analysis [15,16]. Microscopic identification, while less destructive, is highly dependent on the presence of intact morphological fragments, which are typically absent in finely ground insect powders, and relies heavily on the expertise of trained analysts [16,17].

Similarly, chromatographic and immunochemical techniques often require complex and labor-intensive sample preparation. For instance, the indirect sandwich ELLA (Enzyme-Linked Lectin Assay) developed by Pospiech et al. [18] targets N-acetylglucosamine in chitin and chitosan polymers as insect-specific biomarkers, and while it shows promise for routine screening, its performance may be affected by interference from the food matrix. For the chromatographic detection of insect chitin, acid hydrolysis is necessary prior to analysis, a process that is inherently destructive to the sample [19]. Moreover, the applicability of these conventional tools may be limited in highly processed foods where target biomarkers can be degraded or masked by the food matrix [16,18]. These limitations highlight the need

for alternative analytical techniques that are rapid, cost-effective, and suitable for routine screening purposes.

Vibrational spectroscopy techniques, including near-infrared (NIR), mid-infrared (MIR), and Raman spectroscopy, have emerged as promising tools for food analysis. These methods are non-destructive, require minimal sample preparation, and allow rapid acquisition of chemical information. They have been successfully applied in the detection of food adulteration, authentication of raw materials, and classification of biological samples [20,21]. The integration of spectroscopic methods with chemometric analysis enables the extraction of relevant patterns from complex datasets, facilitating discrimination between different sample types and supporting their application in food quality control [20].

Among these techniques, Raman spectroscopy offers several advantages due to its sensitivity to molecular structure and chemical composition. Raman spectra provide detailed information on key biomolecular components such as proteins and lipids [22,23], as well as polysaccharides including chitin, which is a characteristic structural component of insect exoskeletons [24]. In contrast to infrared-based methods, Raman spectroscopy is less affected by water interference, making it particularly suitable for the analysis of aqueous and liquid samples [25]. Moreover, Raman spectroscopy has been successfully applied in food analysis for compositional characterisation and detection of adulteration [23,26]. These properties suggest that Raman spectroscopy has strong potential for rapid detection and characterisation of insect-derived materials.

Despite the growing interest in spectroscopic techniques, studies focusing specifically on the detection and differentiation of edible insects remain limited. Most existing research has primarily utilised NIR spectroscopy or ATR-FTIR techniques and has often focused on simplified model systems with known compositions or on a limited number of species [27–29]. In contrast, there is still a lack of studies investigating the application of Raman spectroscopy for distinguishing between insect species or detecting insect material in more complex matrices. Furthermore, many existing studies emphasise classification performance without providing detailed interpretation of the spectral features responsible for discrimination [28,29]. These gaps highlight the need for studies that combine robust classification with biochemically meaningful spectral interpretation across a wider range of insect species, including both authorised and non-authorised species.

Therefore, the aim of this study was to evaluate the potential of Raman spectroscopy combined with chemometric analysis for the detection and differentiation of selected edible and feed insect species. Specifically, the study aimed to determine whether Raman spectroscopy can successfully distinguish insect-derived extracts from the solvent matrix and discriminate between different insect species, while identifying the key spectral regions responsible for such classification. The study also explored the regulatory relevance of the method by including both EU-authorised edible insect species and non-authorised species used in animal feed applications.

2. Materials and Methods

2.1. Samples and Experimental Design

A total of 96 dried insect samples collected in 2023 and 2024 were analysed in this study. All samples were obtained from a private insect breeder based in the Czech Republic, where the insects were reared under standard breeding conditions. The dataset was designed to cover a representative range of insect species commonly used in food and feed applications, enabling evaluation of both taxonomic and compositional variability across different biological groups.

The analysed species included *Acheta domesticus* (AD), *Gryllus assimilis* (GA), *Tenebrio molitor* (TM), *Zophobas morio* (ZM), *Alphitobius diaperinus* larvae (ALD), *Blaptica dubia* (BD),

Shelfordella tartara (ST), *Apis mellifera* drones (AM), and *Hermetia illucens* (HI). These species represent different biological groups, including Orthoptera (crickets), Coleoptera (beetles and their larvae), Blattodea (cockroaches), Hymenoptera (bees), and Diptera (flies), enabling evaluation of both taxonomic and compositional variability.

The selection of insect species also reflected their regulatory status within the European Union. The dataset included species authorised for human consumption under the Novel Food Regulation (EU) 2015/2283 [6], as well as species that may be commercially available in different countries but are not authorised for human consumption in the EU. This approach enabled evaluation of the method’s potential for detecting food adulteration, where authorised edible insect ingredients may be substituted with non-authorised or feed-grade species.

Samples originated from two independent collection periods (2023 and 2024), allowing assessment of inter-annual variability and robustness of the analytical approach. The distribution of physical samples across species is shown in Table 1.

Table 1. Overview of analysed insect materials, EU status and sample distribution.

Abbr.	Species/Material	Order	Stage	EU Status	n Phys./n All/n Proc.
AD	<i>Acheta domesticus</i>	Orthoptera	Adult	NF/FEED	18/72/68
ALD	<i>Alphitobius diaperinus</i>	Coleoptera	Larvae	NF/FEED	8/32/30
AM	<i>Apis mellifera</i>	Hymenoptera	Drones	NF app.	4/16/15
BD	<i>Blaptica dubia</i>	Blattodea	Nymph	NA	22/88/83
GA	<i>Gryllus assimilis</i>	Orthoptera	Adult	FEED	12/48/45
HI	<i>Hermetia illucens</i>	Diptera	Larvae	FEED	4/16/15
ST	<i>Shelfordella tartara</i>	Blattodea	Nymph	NA	6/24/19
TM	<i>Tenebrio molitor</i>	Coleoptera	Larvae	NF/FEED	12/48/45
ZM	<i>Zophobas morio</i>	Coleoptera	Larvae	NA	10/40/38
Total insect dataset					96/384/358

Abbreviations: NF = authorised as a novel food for human consumption under Regulation (EU) 2015/2283 and species-specific implementing regulations; FEED = permitted farmed insect species for processed insect protein/feed use under Commission Regulation (EU) 2017/893; NF/FEED = species covered by both categories; NF app. = novel food application/transitional case without formal EU-wide authorisation during the period considered in this study; NA = not authorised for human food or feed use under the EU framework considered here. *Locusta migratoria* is authorised as a novel food under Commission Implementing Regulation (EU) 2021/1975 but was not included in this dataset. n phys. = number of physical samples; n all = total number of acquired Raman spectra; n proc. = number of spectra retained after quality control.

The differences in Table 1 between the numbers of physical samples, total acquired spectra, and processed spectra reflect the hierarchical sampling design and quality-control procedure employed in this study. Each physical insect sample was measured in four technical Raman replicates, obtained from two measurement positions with two repeated measurements per position, resulting in the total number of acquired spectra. Spectra showing excessive background, low signal intensity, or technical artefacts were excluded prior to chemometric analysis, with the remaining spectra reported as processed spectra. Therefore, the apparent numerical differences in Table 1 do not represent discrepancies in sample collection, but rather distinguish between physical biological samples, technical spectral replicates, and spectra retained after quality control.

Among insect species authorised as novel foods in the EU under Regulation (EU) 2015/2283, the present dataset included *Acheta domesticus*, *Tenebrio molitor* larvae, and *Alphitobius diaperinus* larvae. *Locusta migratoria* is also authorised as a novel food in the EU, but it was not included in the present dataset. These authorisations are specified in the respective Commission Implementing Regulations amending the Union list of novel foods. *Hermetia illucens* and *Gryllus assimilis* are not authorised for human consumption but are listed among the insect species permitted for the production of processed animal

protein from farmed insects for feed use under Commission Regulation (EU) 2017/893 [12]. *Apis mellifera* drone brood represents a special case, as although a Novel Food application was submitted under Regulation (EU) 2015/2283 [6] and the product may have been commercially available in some Member States under transitional provisions, it had not received formal EU-wide authorisation during the study period. The remaining species, namely *Zophobas morio*, *Blattica dubia*, and *Shelfordella tartara*, are not authorised for human consumption in the EU and are not listed among the insect species permitted for the production of processed animal protein from farmed insects for feed for farmed animals under the EU framework considered here; however, they may be commercially available as live feed for exotic or terrarium animals. A summary of the analysed species, regulatory status, developmental stage, and sample distribution is provided in Table 1.

For chemometric analysis, samples were also grouped according to biologically relevant categories, including larval versus adult stage, and broader taxonomic groupings, to evaluate the method's capacity for discrimination at different levels of biological organisation.

Importantly, the present dataset was not designed to isolate the effect of developmental stage within the same species. Species identity and developmental stage are therefore partly confounded, particularly when larval species are compared with adult insects. The purpose of the study was to evaluate Raman fingerprinting of commercially relevant dried insect-derived materials as supplied in powdered form, rather than to model ontogenetic changes. This limitation is acknowledged in the interpretation of the spectral differences and classification results.

2.2. Sample Preparation and Extraction

To exclude potential bias caused by feed residues in the insect digestive tract, the insects were starved for at least 24 h prior to processing. Euthanised insects were freeze-dried using an L10-55 PRO lyophiliser (Gregor Instruments s.r.o., Sázava, Czech Republic). The dried material was then cryogenically homogenised in the presence of liquid nitrogen using a laboratory knife mill (A11 Basic Analytical Mill, IKA Labortechnik, Staufen im Breisgau, Germany) to obtain a uniform powdered sample matrix prior to extraction.

PBS adjusted to pH 9.4 was selected as the extraction solvent based on preliminary method-development screening, in which this condition provided improved Raman signal intensity and clearer protein-associated spectral features compared with alternative tested extraction conditions. This preliminary screening should not be interpreted as a full systematic pH-gradient optimisation. Each physical sample was extracted once, while spectral acquisition repeatability was assessed through technical Raman replicate spectra acquired from each filtered extract. Filtration through 0.45 µm membrane filters was necessary to remove insoluble residues and particulate matter that could otherwise increase light scattering, elevate spectral background, and reduce the reproducibility of the Raman signal.

Extraction was performed at a solvent-to-sample ratio of 1:5 (2 g of homogenised insect material per 10 mL of solvent). The extraction process was carried out in 15 mL centrifuge tubes placed in an ultrasonic water bath (Bandelin, Berlin, Germany) for 15 min. After sonication, samples were centrifuged at 3000 rpm for 30 min (Centric 322A, Domel, Železniki, Slovenia) to separate the solid residue and lipid fraction from the soluble extract.

The supernatant was carefully collected and filtered through a 0.45 µm mixed cellulose ester syringe filter (FiltrateCH, Saran, France) into 4 mL glass vials. The filtered extracts were analysed immediately by Raman spectroscopy to minimise time-dependent changes in PBS-extractable biomolecules.

2.3. Raman Spectroscopy Data Acquisition

Raman spectra were recorded using a high-resolution HR-TEC-X2-785 spectrometer (StellarNet, Tampa, FL, USA) equipped with a stabilised diode laser (Ondax, Monrovia, CA, USA) operating at an excitation wavelength of 785 nm and an output power of 91 mW at 80% laser intensity. Spectral acquisition was performed over the wavenumber range of 200–2000 cm^{-1} , covering the fingerprint region relevant for protein-, lipid-, and carbohydrate-associated Raman features [22,24,30]. Spectral data were acquired using Enlighten™ software (version 2.0) (Wasatch Photonics, Logan, UT, USA). Instrument settings included MPLS correction enabled, boxcar smoothing of 3 pixels, and detector control configured according to the manufacturer's recommendations. The spectral axis was calibrated in wavenumbers with a reference value of 268.98 cm^{-1} .

An integration time of 2000 ms was used for all measurements included in the final dataset. Each individual measurement represented the average of eight consecutive scans acquired automatically by the spectrometer, which improved the signal-to-noise ratio and reduced random measurement noise.

For each filtered liquid extract, spectra were collected at two measurement positions within the cuvette. At each position, two repeated measurements were acquired, resulting in four technical Raman replicate spectra per physical sample. This approach differs from direct point measurement of dry powders because the analysed signal represents PBS-extractable components obtained from a defined mass of homogenised insect material rather than from a single localised powder particle or surface region. A total of 384 spectra were acquired from insect samples. Prior to chemometric analysis, all spectra were visually inspected, and spectra showing substantial deviation from the typical spectral profile of the respective taxon, such as excessive background, low signal intensity, or technical artefacts, were excluded. After quality control, 358 insect spectra were retained for further analysis (Table 1). PBS buffer measured under identical conditions ($n = 7$ spectra) was included as a technical control class in the classification model to assess spectral interference from the extraction medium.

2.4. Spectral Data Processing and Chemometric Analysis

2.4.1. Spectral Data Preprocessing

To minimise the influence of baseline shifts, overlapping bands, scatter-related spectral variation, and instrumental noise, preprocessing of the raw Raman spectra was performed prior to chemometric analysis. A second-order Savitzky–Golay derivative was applied using a second-order polynomial and a smoothing window of 15 points. Subsequently, the spectra were normalised using the Standard Normal Variate method to account for additive and multiplicative scatter effects. This preprocessing workflow was selected because it improved spectral comparability and enhanced the resolution of overlapping bands in the analysed fingerprint region.

To ensure the objectivity and optimality of the selected preprocessing strategy, a systematic comparison of nine different preprocessing combinations—including raw spectra, baseline correction, single and double derivatives, and SNV normalisation—was performed. The classification performance of these pipelines was evaluated using the Correct Classification Rate (CCR) of the PLS-DA models (detailed in Supplementary Table S1).

2.4.2. Classification and Multivariate Analysis

For the purpose of classifying samples into their respective classes, Principal Component Analysis (PCA) and Partial Least Squares Discriminant Analysis (PLS-DA) were used. Prior to modelling, categorical class membership variables were converted into a binary matrix using one-hot encoding. PCA was applied as an unsupervised exploratory method

to visualise the natural distribution of spectral fingerprints, whereas PLS-DA was used as a supervised classification approach.

To prevent overfitting and to determine the optimal number of latent variables for the PLS-DA model, 5-fold cross-validation was performed. Model performance was evaluated using a confusion matrix, from which the overall correct classification rate (CCR) and per-class classification rates were calculated.

Variable Importance in Projection (VIP) scores were used as a post hoc interpretative tool to identify spectral variables contributing most strongly to class separation. The reported PLS-DA classification performance should therefore be interpreted as being derived from the full preprocessed spectral dataset rather than as being based solely on variables exceeding the $VIP > 1$ threshold.

2.4.3. Software and Libraries Used

All calculations, data processing, and visualisations were performed in the Python programming language (version 3.12) (Python Software Foundation, Wilmington, DE, USA). The NumPy library was utilised for mathematical operations and multidimensional matrix manipulations, while data structuring was carried out using the pandas library. The SG2 calculation was implemented using the `scipy.signal` module from the SciPy library. Chemometric modelling (PCA and PLS-DA) and cross-validation were performed using the `cross_decomposition` and `model_selection` modules from the scikit-learn machine learning library. Graphical outputs, including the visualisation of clusters and 95% confidence ellipses, were generated using the Matplotlib library.

3. Results and Discussion

3.1. Raman Spectral Profiles of Insect Sample Extracts

Raman spectra of the analysed insect extracts prepared in PBS buffer exhibited broad and partially overlapping bands, reflecting the complex biochemical composition of PBS-extractable insect-derived material (Figure 1). Raman spectroscopy is sensitive to organic molecules, particularly proteins, lipids, and carbohydrate-containing structures, which represent major biochemical components of insect-derived material [22,24,30]. In contrast, PBS buffer exhibited only limited Raman signals in the fingerprint region above 550 cm^{-1} .

The spectral range below approximately 550 cm^{-1} was excluded from interpretation and modelling because this region showed a strong low-wavenumber background and poor reproducibility in both buffer and sample spectra, which precluded reliable band assignment.

The most relevant spectral differences among samples were observed in the regions of approximately 700–1050, 1200–1350, 1440–1460, 1600–1670, and 1700–1800 cm^{-1} . These intervals can be tentatively associated with protein-, lipid-, and carbohydrate-related structures [22,24,30]. Because the extracts were centrifuged and filtered through a $0.45\text{ }\mu\text{m}$ membrane prior to Raman analysis, intact structural chitin was likely retained predominantly in the solid residue. Therefore, bands in the 700–1050 cm^{-1} region should not be interpreted as direct evidence of structural chitin alone. Instead, they are more appropriately described as polysaccharide- and chitin-associated spectral contributions, potentially including soluble carbohydrate-containing compounds, protein-bound carbohydrates, glycoprotein-like structures, or residual fine cuticular components that may have passed through the extraction and filtration workflow. Cuticle-associated proteins and elastic structural proteins such as resilin may also contribute characteristic Raman bands to insect-derived spectra [31,32].

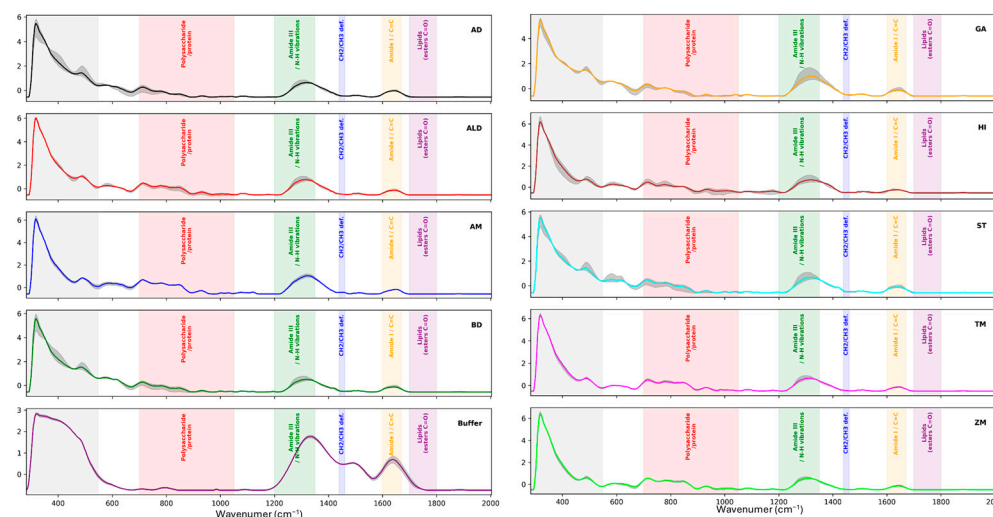


Figure 1. Mean Raman spectra of the analysed insect extracts and PBS buffer (recorded in the spectral range 200–2000 cm^{-1}), with grey shading indicating $\pm$ standard deviation. Highlighted spectral regions indicate the main intervals used for spectral interpretation: 700–1050 cm^{-1} , polysaccharide-/protein-associated region; 1200–1350 cm^{-1} , amide III/C–N and N–H vibrations; 1440–1460 cm^{-1} , CH_2/CH_3 deformation; 1600–1670 cm^{-1} , amide I/C=C vibrations; and 1700–1800 cm^{-1} , lipid ester C=O region. Abbreviations: AD—*Acheta domesticus*; ALD—*Alphitobius diaperinus* larvae; AM—*Apis mellifera* drones; BD—*Blaptica dubia*; GA—*Gryllus assimilis*; HI—*Hermetia illucens*; ST—*Shelfordella tartara*; TM—*Tenebrio molitor*; ZM—*Zophobas morio*.

Within these broader regions, protein-related contributions may include the phenylalanine ring breathing mode near 1004 cm^{-1} , amide III vibrations in the 1200–1350 cm^{-1} region, and amide I-related vibrations in the 1600–1670 cm^{-1} region [22]. Bands around 1440–1460 cm^{-1} are mainly associated with CH_2 and CH_3 deformation vibrations originating from both lipids and proteins [30]. The 1700–1800 cm^{-1} region is generally associated with ester C=O stretching modes of triacylglycerols and fatty acid esters near 1740 cm^{-1} , a spectral interval particularly informative for lipid-associated differences [30,33].

The present study was designed as a spectroscopic fingerprinting and classification study rather than as a quantitative compositional analysis of proteins, lipids, or carbohydrate-/chitin-associated components. In complex biological extracts, Raman band intensities are influenced not only by compound concentration but also by extraction efficiency, matrix effects, overlapping bands, baseline variation, and preprocessing. Therefore, relative peak intensities were not used as direct quantitative measures of biomolecular content. Quantitative interpretation would require independent reference compositional data and dedicated calibration models [20,21].

Visual comparison of the spectra indicated that larval samples, particularly *Tenebrio molitor* larvae (TM), *Zophobas morio* larvae (ZM), *Alphitobius diaperinus* larvae (ALD), and *Hermetia illucens* larvae (HI), showed relatively more pronounced spectral features in the 700–960 cm^{-1} region compared with several adult insects. These differences may reflect combined contributions from carbohydrate-containing structures, protein-associated vibrations, and possibly soluble or fine particulate cuticular-derived components. However, because the analysed samples represent different species and different developmental stages rather than controlled developmental stages within the same species, the observed spectral differences cannot be attributed to developmental stage alone. They should be interpreted as combined effects of species identity, developmental stage, and PBS-extractable biochemical composition.

In contrast, adult insect samples including the cricket species *Acheta domesticus* (AD) and *Gryllus assimilis* (GA), and the cockroach species *Blaptica dubia* (BD) and *Shelfordella*

tartara (ST) showed relatively similar spectral profiles, particularly in the 1200–1350 and 1600–1670 cm^{-1} regions. These regions encompass amide III bands associated with protein secondary structure, amide I contributions, and C=C stretching modes of unsaturated lipid chains, suggesting partially comparable biochemical compositions among these adult species [22,30]. The spectral similarity between BD and ST was especially pronounced across the entire measured range, which may reflect their classification within the same insect order Blattodea and comparable cuticular lipid and protein composition, as the biochemical profiles of cockroach species have been shown to differ in a species-specific manner [34]. This visual observation was subsequently confirmed by the classification analysis, as discussed below.

Overall, the spectra indicate that Raman spectroscopy captured compositional differences between insect extracts and the buffer, as well as partial differences among insect groups. Nevertheless, the overlap of several bands across species confirmed that visual spectral interpretation alone is not sufficient for reliable discrimination, and therefore multivariate analysis was required [35,36].

3.2. Exploratory and Supervised Multivariate Analysis

Principal component analysis (PCA) was first applied as an exploratory method to evaluate the natural distribution of Raman spectral fingerprints without using prior class information (Figure 2) [36,37].

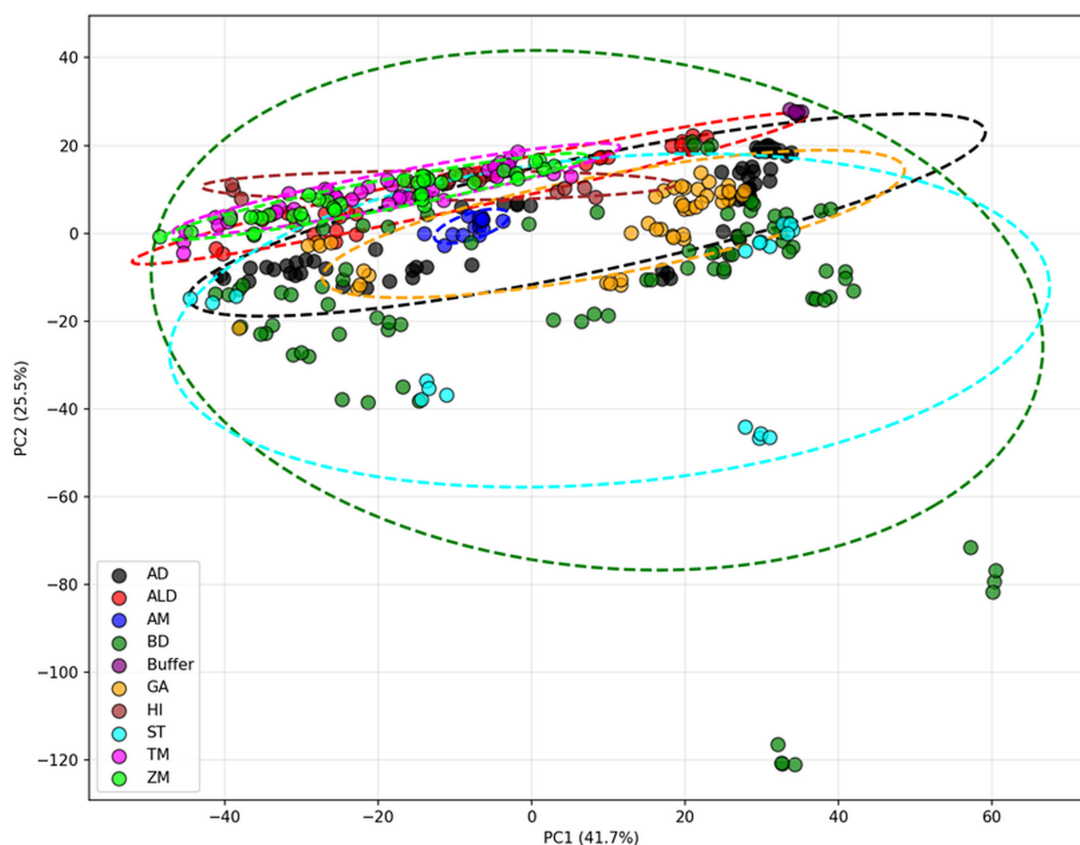


Figure 2. PCA score plot of Raman spectra of insect extracts and PBS buffer. Each point represents one sample; dashed ellipses indicate 95% confidence regions for each taxon. PC1 and PC2 account for 41.7% and 25.5% of the total spectral variance, respectively. Abbreviations: AD—*Acheta domesticus*; ALD—*Alphitobius diaperinus* larvae; AM—*Apis mellifera* drones; BD—*Blattica dubia*; GA—*Gryllus assimilis*; HI—*Hermetia illucens*; ST—*Shelfordella tartara*; TM—*Tenebrio molitor*; ZM—*Zophobas morio*.

PCA reduces the dimensionality of large spectral datasets, increasing interpretability while minimizing information loss, by creating new uncorrelated variables that successively maximize variance. When applied to the entire spectrum, PC1 and PC2 explained 97.3% and 1.0% of the variance, respectively (Supplementary Figure S1). This clearly demonstrates that Rayleigh scattering and fluorescence dominate this region. Excluding this spectral range is therefore crucial to ensure that subsequent classifications are based strictly on chemical composition. The PCA score plot showed broad dispersion of the analyzed samples, with PC1 and PC2 explaining 41.7% and 25.5% of the total variance, respectively. Although several grouping tendencies were visible, considerable overlap among insect species was observed, with the confidence ellipses of most classes intersecting substantially in the PC1–PC2 space. Notably, *Shelfordella tartara* (ST) displayed a particularly wide confidence ellipse due to the presence of spectral outliers, suggesting high intra-class variability for this species. Similarly, *Blaptica dubia* (BD) exhibited several extreme scores along PC1, reflecting pronounced spectral heterogeneity likely associated with variable cuticular lipid composition [34].

These observations confirmed that the main sources of spectral variability captured by PCA were not sufficient for complete species-level discrimination using unsupervised analysis alone [20]. The inherent limitation of PCA in this context stems from the fact that the principal components are defined solely by variance maximization, without accounting for between-class differences, which may result in relevant discriminatory information being distributed across higher-order components not visible in the two-dimensional score plot [37,38]. These findings motivated the subsequent application of a supervised classification approach.

Although PCA provided an initial overview of spectral variation, complete separation of all insect species was not achieved in the unsupervised space. This indicates that the main discriminatory information was not fully captured by the first principal components and supports the use of supervised classification by PLS-DA for further evaluation.

To further evaluate whether additional classification-relevant information was present in higher-order principal components, additional PCA score projections were examined. In the final PCA model, PC1 and PC2 explained 41.7% and 25.5% of the total variance, respectively, accounting for 67.2% of the total spectral variability. PC3, PC4, and PC5 explained 4.2%, 3.2%, and 2.8% of the total variance, respectively. Although the additional projections provided further insight into the distribution of the samples, including PC1 vs. PC3, PC1 vs. PC4, and PC3 vs. PC4, they did not result in complete unsupervised separation of all insect species. This confirms that PCA was useful for exploratory visualisation but insufficient for robust class discrimination, supporting the subsequent use of supervised classification by PLS-DA. The additional PCA score projections are provided in Supplementary Figure S2.

To enhance discrimination among predefined classes, PLS-DA was subsequently applied to the same spectral dataset. The resulting PLS-DA score plot is shown in Figure 3. Similar supervised chemometric workflows have been widely used in spectroscopic classification studies [20,39]. Unlike PCA, PLS-DA maximizes the covariance between the spectral data and the class membership matrix, rotating the latent variables to achieve maximum group separation [39,40]. The PLS-DA score plot showed clearer separation trends compared to PCA, with LV1 and LV2 explaining 39.3% and 31.9% of the spectral variability, respectively, accounting for 71.2% of the total variance in the LV1–LV2 subspace. The PBS buffer samples were separated from most insect extracts along LV1, confirming that the dominant spectral information originated primarily from PBS-extractable insect-derived organic compounds rather than from the extraction medium [24,30].

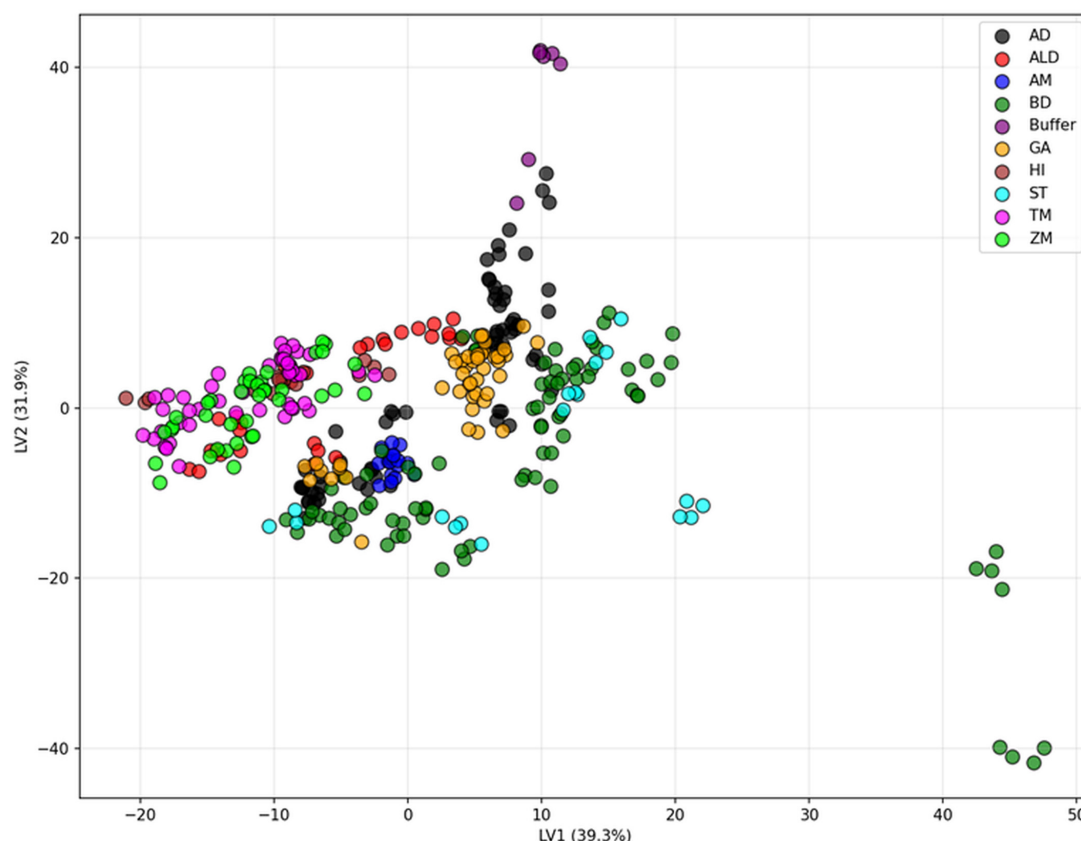


Figure 3. PLS-DA score plot of derivative-transformed Raman spectra of insect extracts and PBS buffer. Each point represents one sample; LV1 and LV2 account for 39.3% and 31.9% of the total spectral variance, respectively. Abbreviations: AD—*Acheta domesticus*; ALD—*Alphitobius diaperinus* larvae; AM—*Apis mellifera* drones; BD—*Blattella germanica*; GA—*Gryllus assimilis*; HI—*Hermetia illucens*; ST—*Shelfordella tartara*; TM—*Tenebrio molitor*; ZM—*Zophobas morio*.

Partial separation tendencies were also observed between species belonging to different orders and developmental stages, which may reflect differences in biochemical composition associated with phylogenetic relatedness and ontogenetic stage. Dynamic analyses of major components across developmental stages have shown that the proportions of protein, fat, and chitin differ substantially between larval, pupal, and adult stages, as demonstrated for example in yellow mealworm [41], which likely contributes to the spectral variability observable in the score plot. However, despite these trends, considerable overlap remained among the majority of insect species in the LV1–LV2 space, indicating that two latent variables alone were insufficient to achieve complete class separation [20,38].

Additional PLS-DA score projections were examined to visualise class distribution across higher-order latent variables. These plots confirmed clearer separation for some classes, particularly *Apis mellifera* and the PBS buffer class, whereas substantial overlap persisted among several insect species, especially closely related taxa. The additional PLS-DA projections are provided in Supplementary Figure S3.

3.3. Cross Validation

Prior to final model validation, the influence of various spectral preprocessing strategies on classification accuracy was systematically evaluated (Supplementary Table S1). The baseline PLS-DA model using raw, untreated spectra yielded an overall CCR of 57.3%, whereas SNV normalisation alone did not improve classification performance (CCR of 56.4%). The combination of a second-order Savitzky–Golay derivative followed by SNV normalisation provided the most favourable performance among the tested preprocessing

strategies. Although automatic latent-variable selection yielded a higher CCR of 71.2% with 10 latent variables, the conservative fixed five-component model was retained for the main classification analysis to limit model complexity and reduce the risk of overfitting. Therefore, all main classification results reported in the manuscript, including the confusion matrix and the overall CCR of 61.6%, refer to this conservative final model.

Model validation was performed using a 5-fold cross-validation scheme (corresponding to a 20% data split per fold) to robustly determine the classification performance parameters. The classification performance was evaluated using CCR and the confusion matrix (Table 2). The overall CCR reached 61.6%, corresponding to 225 correctly classified samples out of 365, indicating moderate classification performance. This result is broadly comparable to values reported for Raman spectroscopy-based classification of other complex biological matrices [42,43] and confirms that complete species-level discrimination was not achieved for all analysed insects.

Table 2. Confusion matrix showing classification results of the PLS-DA model for nine insect species and PBS buffer. Correctly classified samples are shown on the diagonal. CCR values represent the proportion of correctly classified samples within each actual class.

Actual Class (Taxon)	Abbr.	Predicted Class (Taxon)										n	CCR (%)
		Buffer	AD	ALD	AM	BD	GA	HI	ST	TM	ZM		
Buffer (PBS 9.4)	Buffer	4	2	0	0	1	0	0	0	0	0	7	57.1%
<i>Acheta domesticus</i>	AD	0	45	0	0	8	15	0	0	0	0	68	66.2%
<i>Alphitobius diaperinus</i>	ALD	0	0	8	0	3	0	0	0	10	9	30	26.7%
<i>Apis mellifera</i>	AM	0	0	0	15	0	0	0	0	0	0	15	100.0%
<i>Blaptica dubia</i>	BD	0	14	0	0	56	1	0	12	0	0	83	67.5%
<i>Gryllus assimilis</i>	GA	0	16	0	0	4	25	0	0	0	0	45	55.6%
<i>Hermetia illucens</i>	HI	0	0	0	0	3	0	12	0	0	0	15	80.0%
<i>Shelfordella tartara</i>	ST	0	0	0	0	19	0	0	0	0	0	19	0.0%
<i>Tenebrio molitor</i>	TM	0	1	0	0	3	0	0	0	36	5	45	80.0%
<i>Zophobas morio</i>	ZM	0	0	1	0	2	0	0	0	11	24	38	63.2%
Total (n)		4	78	9	15	99	41	12	12	57	38	365	Total: 61.6%

The highest classification performance was observed for *Apis mellifera* drones (AM), which were classified with a CCR of 100.0%. This result is consistent with the compact and partially separated AM cluster visible in the PLS-DA score plot, suggesting that the Raman fingerprint of this taxon contains sufficiently distinct biochemical features to allow unambiguous classification [20,39]. This exceptional performance may be related to the specific biochemical composition of drone body material, which together produces a spectral profile sufficiently distinct from other species [22]. Relatively good classification was also obtained for *Hermetia illucens* (HI) and *Tenebrio molitor* (TM), both showing a CCR of 80.0%. The higher performance of these larval species may reflect distinct PBS-extractable biochemical signatures, including lipid- and protein-associated spectral features as well as carbohydrate-/cuticle-associated contributions [30,41].

In contrast, low classification performance was observed for *Alphitobius diaperinus* larvae (ALD), with a CCR of 26.7%, and especially for *Shelfordella tartara* (ST), for which no samples were correctly classified. The confusion matrix showed that ST samples were exclusively misclassified as *Blaptica dubia* (BD), indicating near-identical spectral profiles between these two cockroach species.

The complete misclassification of ST as BD can be interpreted as a consequence of several combined factors. Both taxa belong to the order Blattodea and were analysed as nymphal cockroach material, which may result in highly similar PBS-extractable bio-

chemical fingerprints. In particular, overlapping lipid- and protein-associated Raman bands, together with carbohydrate-/cuticle-associated contributions, may have reduced the availability of species-specific spectral features for the PLS-DA model [22,24,30,34].

The overlapping spectral profiles observed for ST and BD illustrate a key challenge in spectroscopic species discrimination. Previous studies have shown that Raman spectroscopy combined with machine learning can discriminate closely related arthropod species, such as *Anopheles gambiae* and *Anopheles arabiensis* mosquitoes [42]. However, the present ST/BD result indicates that successful discrimination strongly depends on the presence of sufficiently distinct spectral features in the analysed matrix. When closely related taxa exhibit highly similar PBS-extractable biochemical fingerprints, the discriminatory information available to the model may be insufficient.

In addition, the number of physical ST samples was relatively low compared with BD, which may have limited the ability of the model to capture within-class variability for this taxon. The complete failure to discriminate ST from BD also manifested in the PLS-DA score plot, where ST and BD clusters overlap substantially, leaving no separable latent variable space for the model to exploit [39]. Therefore, the ST/BD confusion should be interpreted as a limitation of the current dataset and screening model rather than as definitive evidence that Raman spectroscopy cannot distinguish these taxa under improved sampling and validation conditions.

A similar but less severe pattern of partial misclassification was observed between the cricket species *Acheta domesticus* (AD) and *Gryllus assimilis* (GA). The broadly similar spectral shapes of AD and GA, differing mainly in relative band intensities rather than in the presence of distinct taxon-specific marker bands, suggest that their Raman fingerprints share comparable biochemical features, particularly in protein- and lipid-associated spectral regions [22,30]. This similarity is consistent with the fact that both species belong to the order Orthoptera and were analysed as adult insect material.

Although these spectral similarities can be interpreted in relation to shared taxonomic affiliation and overlapping protein- and lipid-associated Raman bands, the present study did not include independent targeted measurements of cuticular lipid composition, protein profiles, or chitin content. Therefore, the biochemical explanation of misclassification among taxonomically related species remains tentative. Future studies should combine Raman spectroscopy with reference techniques such as GC-MS lipid profiling, proteomics, or validated chitin quantification to directly link spectral discrimination to compositional differences.

3.4. Important Spectral Regions Contributing to Classification

To identify the spectral variables contributing most strongly to the discrimination among samples, VIP scores from the PLS-DA model were evaluated (Figure 4) [44]. Several spectral regions with VIP values above the commonly used threshold of 1 were identified, indicating that classification was not driven by a single isolated band but by a combination of multiple spectral features [20]. The most relevant regions were located between approximately 600–1300 cm^{-1} , 1400–1500 cm^{-1} , and 1700–1800 cm^{-1} , with the highest individual VIP score observed near 1750 cm^{-1} . The VIP score plot was aligned with the global mean Raman spectrum to facilitate direct interpretation of the spectral regions contributing most strongly to class separation.

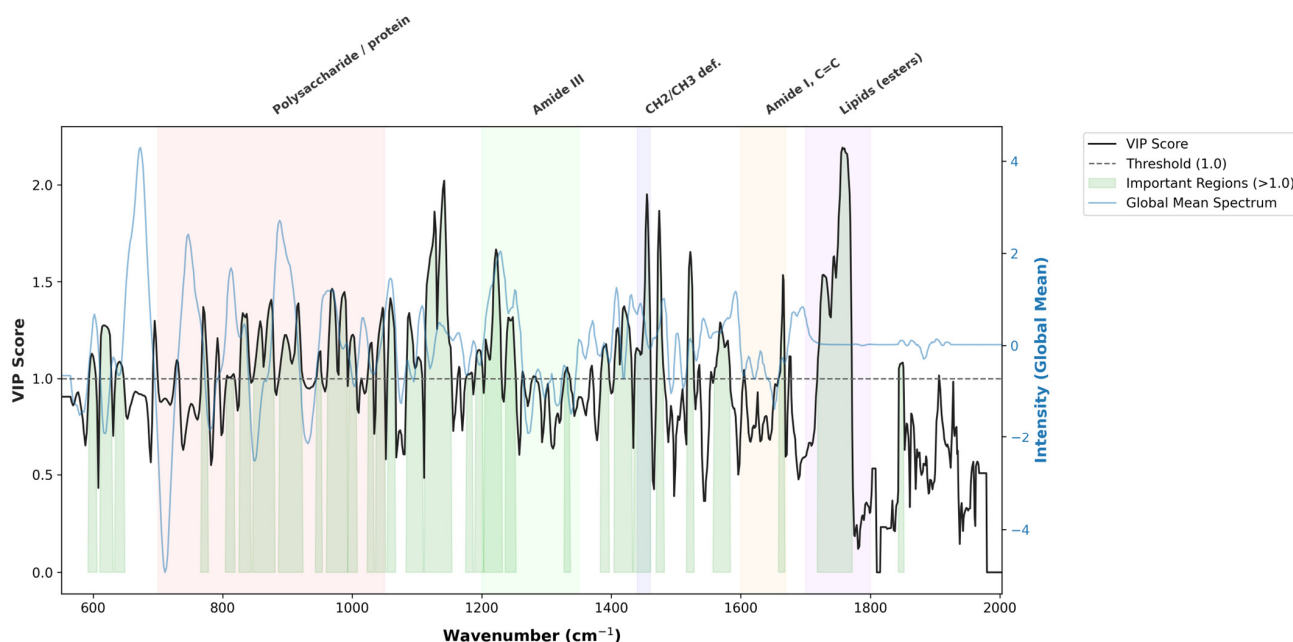


Figure 4. Variable Importance in Projection (VIP) scores obtained from the PLS-DA model aligned with the global mean Raman spectrum. The red dashed line indicates the VIP threshold of 1. Highlighted spectral regions correspond to the main intervals discussed in the text, including polysaccharide-/protein-associated contributions, amide III, CH_2/CH_3 deformation, amide I/C=C vibrations, and lipid ester C=O stretching. VIP regions were used for post hoc interpretation and should not be interpreted as single-compound biomarkers.

To avoid ambiguity, VIP scores were used primarily for post hoc interpretation of the PLS-DA model rather than as direct proof of individual biomarkers. Variables with $\text{VIP} > 1$ indicate spectral regions contributing strongly to class separation; however, these regions contain overlapping biochemical contributions and should not be treated as single-compound markers. Nevertheless, the prominence of lipid-associated spectral regions within these bands may reflect interspecific variability in insect cuticular hydrocarbons and related lipid compounds [45].

The selection of PBS at pH 9.4 as the extraction medium was an important methodological factor affecting the acquired Raman fingerprints. Under mildly alkaline conditions, ionisation of acidic amino acid residues may promote the solubilisation of protein-associated compounds, which is consistent with previous reports on alkaline extraction of proteins from insect biomass [46]. Because the extracts were analysed immediately after preparation, prolonged storage-related protein degradation was minimised. Nevertheless, exposure to pH 9.4 may induce partial conformational changes or denaturation of some protein structures, which may affect their Raman signatures [47], even if extensive alkaline hydrolysis of proteins or saponification of ester lipids is unlikely under the short extraction conditions used here. Therefore, the resulting spectra should be interpreted as fingerprints of PBS-extractable components obtained under the defined extraction conditions rather than as pristine whole-biomass or native-protein fingerprints. This limitation should be considered when interpreting taxon-specific spectral differences.

Several limitations of the present study should be acknowledged. First, each physical sample was extracted once, and the technical replicates therefore primarily reflected spectral acquisition repeatability rather than extraction-level repeatability. Independent extraction replicates should be incorporated in future validation studies to quantify variability introduced during sample extraction and to further improve the robustness of the proposed screening approach [38,43]. Second, the moderate overall CCR indicates that the current

dataset and classification model are not sufficient for robust species-level authentication across all analysed insects. Misclassifications may reflect true biochemical similarity among closely related taxa, particularly in lipid- and protein-associated spectral regions, but they may also be influenced by class imbalance, limited numbers of physical samples in some groups, and residual heterogeneity of insect-derived material [20,30,34,35,45]. Although the use of filtered liquid extracts partly reduces particle-level heterogeneity compared with direct powder measurements, future studies should include a larger and more balanced reference library, independent extraction replicates, additional measurement positions, systematic comparison of preprocessing strategies, and external validation in complex food matrices [20,38,43]. These steps are necessary to further develop the method towards reliable practical screening applications.

4. Conclusions

This study demonstrates that Raman spectroscopy of PBS-extractable insect components, combined with chemometric analysis, has potential as a preliminary screening approach for differentiating selected insect-derived materials. The method showed promising performance for some taxa, particularly *Apis mellifera*, *Hermetia illucens*, and *Tenebrio molitor*, while the discriminatory information was most pronounced in lipid-associated spectral regions. However, the moderate overall classification rate of 61.6%, together with the complete misclassification of *Shelfordella tartara* as *Blaptica dubia*, confirms that the approach is not yet suitable as a stand-alone method for regulatory species authentication.

The results should therefore be interpreted as a proof-of-concept for Raman-based fingerprinting of PBS-extractable insect components rather than as validation of a routine authentication method. Further development should focus on expanding and balancing the reference library, increasing the number of physical samples and independent extraction replicates, improving model validation, and testing the approach in complex food matrices. Raman spectroscopy may ultimately serve as one component of a multi-method authentication strategy for insect-derived materials.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pr14142337/s1>, Table S1: Comparison of PLS-DA classification performance using different spectral preprocessing methods; Figure S1: PCA score plot of the full measured Raman spectral range; Figure S2: Additional PCA score projections; Figure S3: Additional PLS-DA score projections; Supplementary Data S1: Raw Raman spectral data used to generate Figure 1.

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Abbreviations

AD	<i>Acheta domesticus</i>
ALD	<i>Alphitobius diaperinus</i> larvae
AM	<i>Apis mellifera</i> drones
ATR-FTIR	Attenuated Total Reflectance Fourier-Transform Infrared spectroscopy
BD	<i>Blaptica dubia</i>
CCR	Correct Classification Rate
ELLA	Enzyme-Linked Lectin Assay
EU	European Union
GA	<i>Gryllus assimilis</i>
HI	<i>Hermetia illucens</i>
MIR	Mid-Infrared spectroscopy
NIR	Near-Infrared spectroscopy
PBS	Phosphate-Buffered Saline
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PLS-DA	Partial Least Squares Discriminant Analysis
SG2	Savitzky–Golay 2nd derivative filter
SNV	Standard Normal Variate
ST	<i>Shelfordella tartara</i>
TM	<i>Tenebrio molitor</i>
VIP	Variable Importance in Projection
ZM	<i>Zophobas morio</i>

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