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RESEARCH ARTICLE

Insect meal as feed: discrimination of particles issued from authorised and unauthorised species using Near Infrared Microscopy (NIRM)

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Abstract

Since 2017, insect meals have been authorised for animal feed in the European Union, but only eight insect species can be used to produce these meals. This legislation brings with it new analytical challenges, such as the need to identify the insect species processed in the meal. This paper investigated the ability of Near Infrared Microscopy (NIRM) to discriminate meal particles from authorised insect species and differentiate them from unauthorised species. The spectral data was analysed using chemometrics, enabling the assessment of classification specificity and sensitivity for each species to be discriminated. Using Partial Least Squares Discriminant Analysis (PLS-DA), insect species could be efficiently discriminated with specificity and sensitivity values generally above 0.90. However, the discrimination between particular species appeared more difficult. Most of the observed confusion is probably due to the chemical composition of the insects, which can be very similar between closely related species. These results were encouraging, but also indicated that the use of the NIRM technique alone in case of fraud or natural contamination should be complemented by other techniques such as RT-PCR or mass spectrometry.

Keywords

authentication – chemometrics – holometabolous species – paurometabolous species – vibrational spectroscopy

1 Introduction

Over the past few years, many studies have focused on the growing of nutritional, ecological and economic interest of insect farming (Makkar *et al.*, 2014; Verbeke *et al.*, 2015; Arru *et al.*, 2019). The emergence of insect-derived products both in human and animal nutrition revolutionized our vision of the food of the future, making it more sustainable and environmentally friendly (FAO, 2019). Insects are known to be very rich in protein and fat (Rumpold and Schlüter, 2013; Kim *et al.*, 2020; Mokaya *et al.*, 2022), but also have adequate amounts of essential amino acids (Sánchez-Muros *et al.*, 2014; De Marco *et al.*, 2015; Malla *et al.*, 2022). These specific nutritional properties offer an alternative to conventional protein sources, such as soybean meal and fishmeal, in animal feeding.

According to European legislations, insect meal can be used since 2017 in aquaculture (European Commission, 2017), and since 2021 for non-ruminants (European Commission, 2021a,b). Insect meals must be produced from one of the eight species allowed by the legislation, namely *Acheta domesticus* (Orthoptera, Gryllidae), *Alphitobius diaperinus* (Coleoptera, Tenebrion-

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idae), *Bombyx mori* (Lepidoptera, Bombycidae), *Gryllodes sigillatus* (Orthoptera, Gryllidae), *Gryllus assimilis* (Orthoptera, Gryllidae), *Hermetia illucens* (Diptera, Stratiomyidae), *Musca domestica* (Diptera, Muscidae) or *Tenebrio molitor* (Coleoptera, Tenebrionidae) (European Commission, 2021b). This conditional approval of insects in animal feed brings new analytical challenges in their control.

Firstly, the use of insect meal is and will still be banned for ruminant feed according to EU authorities. Therefore, it is crucial to be able to analytically detect the presence of any insects in these feeds. In the current legislation, the detection of processed animal proteins (PAPs) and insect particles is based on two official methods: light microscopy (LM) and Real-Time Polymerase Chain Reaction (RT-PCR) (European Commission, 2003, 2013, 2022). Regarding LM a new protocol for the isolation and detection of insects in aquaculture feeds contaminated with four different insect species was tested and proved to be very effective, also in comparison to the current protocol (Veys and Baeten, 2018). Recent advances have also demonstrated the efficacy and applicability of RT-PCR for the targeted detection of insect species authorised by the legislation (Debode *et al*., 2017; Garino *et al*., 2021; Marien *et al*., 2018, 2022).

Secondly, it is necessary to identify the insect species used in the manufacture of the product in order to eliminate the presence of insect species not listed in European legislation. Although not yet integrated in the current legislation in force, an investigation into this aspect seems necessary to detect possible fraud or natural contamination, for example by mites (e.g. *Ephestia kuehniella*) or beetles (e.g. *Alphitobius laevigatus*). As for natural contamination of other matrices, it is not uncommon for grain storage silos or compound feeds to be infested with pests such as weevils (Curculionidae). In the case of LM, entomological knowledge is required to identify the insect(s). Furthermore, taxonomic sorting using LM is only considered reliable at order level but not at lower taxonomical levels (Veys and Baeten, 2018). In the case of RT-PCR, a targeted analysis method, detecting an insect species that is not specifically targeted may pose difficulties (Anselmo *et al*., 2023b). To date, only Belghit *et al*. (2019) have focused on the discrimination of different insect species used to produce meals for animal feed. This discrimination was achieved by tandem mass spectrometry and therefore required sample pre-treatment, specialised chemicals and expertise.

Near Infrared Microscopy (NIRM) is the combination of near infrared spectroscopy and microscopy. This technique has demonstrated its ability to detect and discriminate PAPs of terrestrial vertebrates from fish particles as well as poultry particles from mammalian particles (Piraux and Dardenne, 2000; Baeten *et al*., 2004, 2005; Baeten and Dardenne, 2005; Delarozadelgado *et al*., 2007; Tena *et al*., 2014). Besides, it has the advantage of not requiring any pre-treatment for the sample and not altering it (Fernández Pierna *et al*., 2013). Therefore, it would likely have some potential to discriminate PAPs from different insect species, providing characterisation at least at the genus level and without sample preparation constraints. If it proves viable, this method could complement the official methods to provide information on the species origin of insect meals. In this study, we thus investigated the ability of NIRM to discriminate between pure insect meals from nine distinct species, authorised and unauthorised.

2 Materials and methods

Insect meal samples

The samples collected covered all authorised insect species except *Musca domestica*, i.e. *A. diaperinus*, *A. domesticus*, *B. mori*, *G. assimilis*, *G. sigillatus*, *H. illucens* and *T. molitor*. To this list of seven species, two unauthorised insects, *Z. morio* (Coleoptera, Tenebrionidae) and *L. migratoria* (Orthoptera, Acrididae), were added (Table 1). *Z. morio* was selected because of its close taxonomic relationship with *T. molitor* and *A. diaperinus*, which are authorised. As for *L. migratoria*, this species was included as it is only authorised for human consumption, contrary to other species of the same order. All species samples came from European producers and were obtained through collaboration between the International Platform of Insects for Food and Feed (IPIFF), the Walloon Agricultural Research Centre (CRA-W) and the producers. They were stored in a refrigerated container in the global feed sample bank maintained at CRA-W in the framework of the European Union Reference Laboratory for Animal Proteins (EURL-AP) activities (https://www.eurl.craw.eu/).

Different super mixes were produced. The term "super mixes" refers to a mixture of meals from different suppliers to obtain a single reference sample that is representative of all the insect meal samples available to the CRA-W (Table 1). Consequently, these super mixes provide an average sample for each insect species studied. These super mixes were mainly used to simplify the NIRM analysis procedure by focusing on particle differentiation rather than differentiation that could be due

TABLE 1 Summary of the insect meal super mixes used to perform the different NIRM analysis. The assignment to the different groups (H and P) was made based on the metabolism of the species (holometabolous or paurometabolous)

Order	Family	Species	Group	Number of meal batches used to obtain the super mix	Developmental stage	Authorised (Y/N)
Coleoptera	Tenebrionidae	*A. diaperinus*	H	6	Larvae	Y
	Tenebrionidae	*T. molitor*	H	7	Larvae	Y
	Tenebrionidae	*Z. morio*	H	2	Larvae	N
Orthoptera	Gryllidae	*A. domesticus*	P	4	Imago	Y
	Gryllidae	*G. assimilis*	P	3	Imago	Y
	Gryllidae	*G. sigillatus*	P	5	Imago	Y
	Acrididae	*L. migratoria*	P	1	Imago	N
Diptera	Stratiomyidae	*H. illucens*	H	10	Larvae	Y
Lepidoptera	Bombycidae	*B. mori*	H	2	Chrysalis	Y

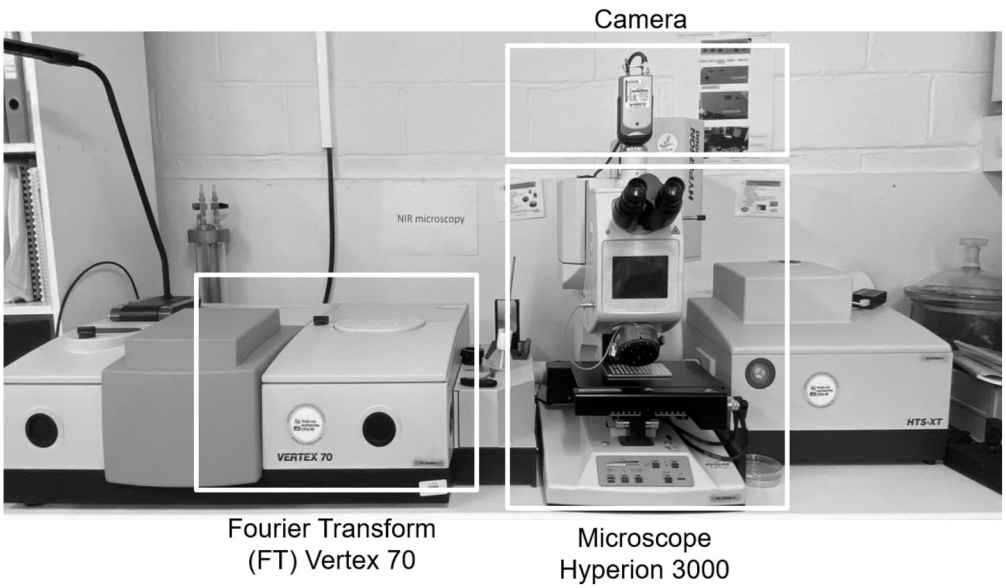

FIGURE 1 Photograph of the Near Infrared Microscope device used to perform the analysis.

to the different suppliers or the origin of the different insect meals used.

The super mixes obtained were not subjected to any pre-treatment, i.e. neither ground nor sieved, the aim being to use the NIRM method as a preliminary sample screening study.

Near Infrared Microscope analysis

NIR spectra were collected on a near infrared microscope consisting of a Hyperion 3000 microscope connected to a Fourier Transform (FT) near infrared spectrometer (Bruker Belgium SA, Kontich, Belgium). The instrument was connected to a camera (Figure 1) and equipped with the OPUS 7.5 software (Bruker Belgium SA, Kontich, Belgium) for spectra extraction and analysis. A multi-well aluminium plate (Bruker Belgium SA, Kontich, Belgium) has been covered with a thin layer of each sample and analysed with a 20 × 20 mapping with a 150 μm pitch, resulting in 400 spectra per sample. All spectra were collected in the range from 9,000 cm^{-1} to 4,000 cm^{-1} (1,111 nm to 2,500 nm), with a resolution of 16 cm^{-1} and a total of 8 co-added scans per spectrum. Each resulting spectrum is the ratio of the raw spectrum, i.e. the total information received by the detector (combining the instrument and the atmosphere), and the spectrum of the background, i.e. the gold well of the plate (Figure 2A).

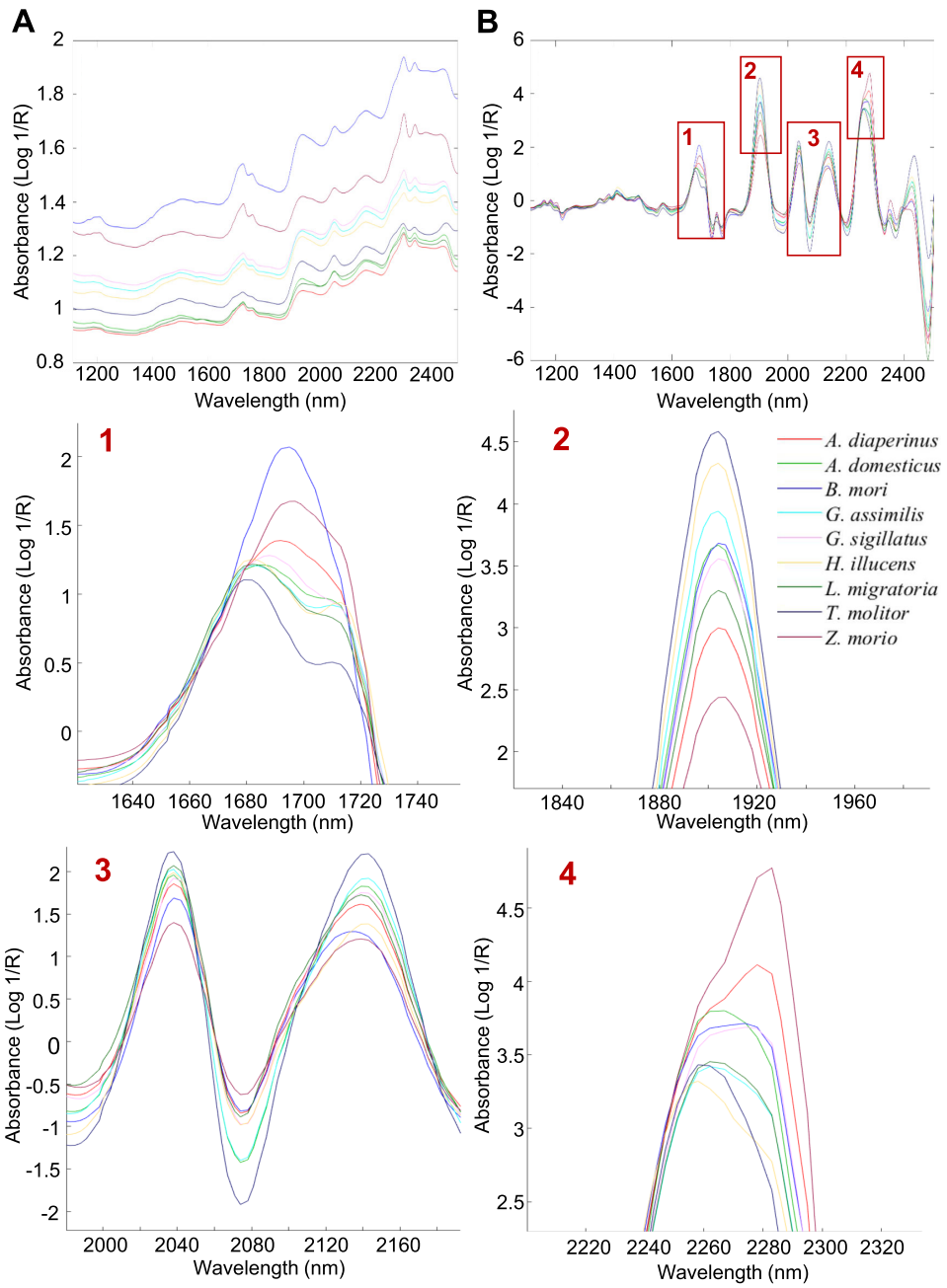

FIGURE 2 Raw mean spectra (A) and pre-processed (B) mean spectra for different insect meal super mixes and zoom on specific regions of interest: (1, 4) oil bands, (2) moisture band and (3) protein bands. In these regions, differences in intensity were observed between the different insect meals (1, 2, 3, 4), as well as variability in the number of bands observed (1, 4). Interpretation of the bands based on Fernández Pierna *et al.* (2012).

Based on the size of the mapping carried out, as well as the pitch and variability in particle size, it was estimated that between 200 and 400 particles were finally analysed for each sample.

Data set strategy

In a first step, the super mixes of the nine species were considered together and the feasibility to discriminate one species from another by NIRM was assessed. In a second step, the super mixes were divided into two distinct groups that were analysed separately. This separation was based on the type of life cycle, a group with the holometabolous (H) species and another one with the paurometabolous (P) species. Assessing the feasibility to discriminate species separately within these groups seemed relevant as the spectral data might behave differently according to the following rationale. In fact, holometabolous species present less morphological differentiation and therefore a more homogeneous structural and spectral composition, since they are in the

larval stage. Paurometabolous species have more diversified fragments (legs, wings, eyes, etc.), as they are individuals with adult morphology, which can lead to very high spectral diversity, which affects the visualization of the data for holometabolous species. Information on these two groups is shown in Table 1. Group H includes species from the Tenebrionidae family, while Group P is composed entirely of species from the Orthoptera order. Each group includes one unauthorised species, *Z. morio* for Group H and *L. migratoria* for Group P.

Data preprocessing

Before starting the analyses, all collected spectra were pre-processed to remove the noise, enabling the composition of the samples to be visualised more accurately (Engel *et al.*, 2013). The first pre-treatment consisted in applying the Savitzky-Golay method (S-G) with a first derivative of order 2 and a window of 15 smoothing points to reduce baseline and background effects (Engel *et al.*, 2013). Secondly, the standard normal variate (SNV) method was used to remove the variability in the reflectance spectra related to scattering effects (Engel *et al.*, 2013) (Figure 2B).

Chemometric analysis

All chemometric analyses were carried out using Solo 9.2.1 (2023) and the PLS toolbox plug-in included (Eigenvector Research, Inc., Manson, WA, USA).

For each group, 400 spectra were measured per insect meal, 100 random spectra of each insect meal were used to create a calibration set and the remaining 300 were used as a validation set. All spectra were classified by insect species.

To assess the ability of the NIRM technique to distinguish between different insect species, a supervised Partial Least Squares Discriminant Analyses (PLS-DA) (Barker and Rayens, 2003) was performed using k-fold (k = 10) venetian blinds cross-validation (CV), the default cross-validation in the PLS toolbox plug-in. The PLS-DA method is derived from partial least squares regression analysis (PLS-R). For a single target variable, PLS-R projects the spectra onto a limited number of factors called "latent variables" (LVs) which are linear combinations of the original variables (the spectral variables). The latent variables are calculated successively by maximising the covariance with the target variable (reference values). Then, these reference values are regressed on the LVs. The predictive model consisted in the coefficient of regression on the original variables and derived from both the projection and the regression on the LV. PLS-DA is an extension of PLS-R in which the target variable is no longer quantitative, but is a vector consisting of 0 and 1, representing the sample's membership of a specific class. In practice, as the aim was to discriminate between multiple classes, several single-class models were calibrated, each representing one insect species against all other species considered. During CV, the same number of LVs was used for all single-class models and the selected number of LVs was the one that minimized the classification error for the whole – multi-class – approach. This multi-class approach was applied separately for the whole dataset (9 single-class models), the group H (5 single-class models) and the group P (4 single-class models) in order to determine whether reducing the number of single-class model improved classification results.

The VIP scores representing the importance of the spectral variables into each insect meal, were also studied in order to support the interpretation of the results.

Discrimination results were assessed using the sensitivity and the specificity. For each insect species, the sensitivity refers to the ability of the model to correctly identify samples of this species. It measures the ratio of true positive (TP) predictions (well classified samples of this insect species) to actual positive instances (the total number of samples of this species), including false negative (FN) predictions (Westerhuis *et al.*, 2008) (Equation 1).

$$\text{sensitivity} = \text{TP}/(\text{TP} + \text{FN}) \quad (1)$$

Specificity refers to the ability of the model to correctly identify samples that are not of this insect species. It measures the ratio of true negative (TN) predictions (well classified samples that are not of this insect species) to actual negative instances (the total number of samples that are not of this species), including false positive (FP) predictions (Westerhuis *et al.*, 2008) (Equation 2).

$$\text{specificity} = \text{TN}/(\text{FP} + \text{TN}) \quad (2)$$

Sensitivity and specificity values vary between 0 and 1, with 1 being the optimum value for a prediction model.

For each multi-class PLS-DA analysis, a confusion matrix was provided. It compares the actual species with the one predicted by the model.

3 Results

The PLS-DA model for all insect meal super mixes included 12 latent variables. The classification results are given in Table 2. Regarding the calibration set, the classification of the different insect species is practically free of errors. Indeed, only a few classification errors are observed for 3 species: *A. domesticus*, *G. assimilis* and *G. sigillatus*. This indicates that the spectral data contain systematic differences between species and that the PLS-DA modelling approach is flexible enough to accommodate these differences. For the validation set, larger classification errors are observed for all species, especially with 113 mispredicted spectra for the species *G. assimilis* and 105 mispredicted spectra for *T. molitor*. These observations are supported by sensitivity values of around 0.6 for *G. assimilis* and *T. molitor* species. This indicates that not all the variation captured by the model during calibration is relevant, and that the relationships learned cannot be fully generalised to new unseen samples of these species.

The PLS-DA model for Group H (*A. diaperinus*, *B. mori*, *H. illucens*, *T. molitor* and *Z. morio*) included 10 latent variables. The classification results are given in Table 3. The reduction of the dataset seems to have improved the classification of the different insect species included in Group H. First, the classification in the calibration set is error-free. For the validation set, prediction errors are observed, but their number is less important compared to those obtained for the full dataset. Again, most of the prediction errors are observed for the *T. molitor* species. The improvement of the model is also supported by the sensitivity and specificity values, which are generally higher than those obtained for the full dataset.

For Group P (*A. domesticus*, *G. assimilis*, *G. sigillatus* and *L. migratoria*), the PLS-DA model included 13 latent variables. The classification results are given in Table 4. Again, reducing the number of species improved the results, compared to the full dataset, for the species included in Group P. Indeed, the sensitivity and specificity values were globally higher than those obtained with the full dataset, in both calibration and validation. Predictions in the calibration dataset were free of error including those for *A. domesticus*, *G. assimilis* and *G. sigillatus*. Prediction errors were still observed in the validation set, but sensitivity and specificity values were globally higher, in particular for *G. assimilis*, whose sensitivity value had increased from 0.620 to 0.766. However, *G. sigillatus* exhibited a slightly decrease of the sensitivity and the specificity values.

4 Discussion and conclusion

Overall, the results demonstrated that NIRM can differentiate between various insect species. First, the discrimination of different samples by NIRM is influenced by their chemical composition. Several studies as summarised by Sánchez-Muros (2014) showed that the chemical composition of insect meals varies from one species to another but also within a species according to the substrate used during rearing (Pinotti and Ottoboni, 2021). This may explain why, for the entire dataset, prediction errors largely vary depending on the species. Typically, the performance of classification is not as good for *G. assimilis* and *T. molitor* as for the other species. In the case of *G. assimilis*, the majority of the misclassifications occur with the species *G. sigillatus*, while for *T. molitor*, most misclassifications involve *G. assimilis*. For *G. assimilis*, the confusion is likely due to its taxonomic proximity to *G. sigillatus*, as both species belong to the Gryllidae family and may therefore be expected as close in terms of chemical composition. For instance, in the VIP scores of *T. molitor*, *G. assimilis* and *G. sigillatus* in the complete dataset (Figure 3A), among the bands that seem to have the greatest impact on the model, are those around 1,700 nm, 1,900 nm and 2,300 nm. These spectral bands have been associated to moisture, proteins and lipids (Fernández Pierna *et al.*, 2012). Regarding lipids, the literature mentions a content of 21.8 g/100 g for *G. assimilis* (Soares Araújo *et al.*, 2019) and 19.5 g/100 g for *G. sigillatus* (Józefiak *et al.*, 2019). These values are relatively close to the typical differences observed between different insect species and this would explain the observed misclassifications.

Regarding the confusion between *T. molitor* and *G. assimilis*, misclassifications could be explained by similarities in protein, lipid or moisture content. As mentioned above, based on the VIP scores of these species in the entire dataset (Figure 3A), it appears that the bands corresponding to lipids, proteins and moisture have the greatest impact on discrimination. In fact, the fat content of *T. molitor* is referenced at 25.3 g/100 g (Józefiak *et al.*, 2019) and is quite close to that obtained for *G. assimilis* (Soares Araújo *et al.*, 2019). This could be part of the explanation but more in-depth chemical analyses, like mass spectrometry, need to be carried out on the samples to confirm this point. Subsequently, when the dataset is divided into two groups that are analysed separately, the prediction errors are reduced. Regarding *T. molitor*, the classification is also much better, as confusion with *G. assimilis*, which belongs to the other group, is no longer possible. An overall improvement

TABLE 2 Confusion matrix of calibration and validation sets and performance parameters obtained by PLS-DA for all insect meals analysed by NIRM. Unauthorised species are in bold

Predicted class	Actual class								
	A. diaperinus	*A. domesticus*	*B. mori*	*G. assimilis*	*G. sigillatus*	*H. illucens*	***L. migratoria***	*T. molitor*	***Z. morio***
Calibration									
A. diaperinus	100	0	0	0	0	0	0	0	0
A. domesticus	0	91	0	1	0	0	0	0	0
B. mori	0	0	100	0	0	0	0	0	0
G. assimilis	0	6	0	92	2	0	0	0	0
G. sigillatus	0	3	0	7	98	0	0	0	0
H. illucens	0	0	0	0	0	100	0	0	0
L. migratoria	0	0	0	0	0	0	100	0	0
T. molitor	0	0	0	0	0	0	0	100	0
Z. morio	0	0	0	0	0	0	0	0	100
Sensitivity	1	0.910	1	0.920	0.980	1	1	1	1
Specificity	1	0.998	1	0.990	0.987	1	1	1	1
Validation									
A. diaperinus	267	0	0	0	1	0	0	6	0
A. domesticus	4	283	0	10	4	0	1	7	0
B. mori	0	0	299	2	0	0	0	0	10
G. assimilis	18	17	0	186	36	0	1	84	0
G. sigillatus	0	0	0	100	253	0	0	0	0
H. illucens	0	0	0	1	2	300	0	1	1
L. migratoria	4	0	0	1	2	0	298	7	4
T. molitor	0	0	1	0	0	0	0	195	0
Z. morio	7	0	0	0	2	0	0	0	285
Sensitivity	0.890	0.943	0.996	0.620	0.843	1	0.993	0.650	0.950
Specificity	0.997	0.989	0.995	0.935	0.958	0.997	0.992	0.999	0.996

TABLE 3 Confusion matrix of calibration and validation sets and performance parameters obtained by PLS-DA for Group H composed of *A. diaperinus*, *B. mori*, *H. illucens*, *T. molitor* and *Z. morio* larvae meals analysed by NIRM. Unauthorised species are in bold

Predicted class	Actual class				
	A. diaperinus	*B. mori*	*H. illucens*	*T. molitor*	***Z. morio***
Calibration					
A. diaperinus	100	0	0	0	0
B. mori	0	100	0	0	0
H. illucens	0	0	100	0	0
T. molitor	0	0	0	100	0
Z. morio	0	0	0	0	100
Sensitivity	1	1	1	1	1
Specificity	1	1	1	1	1
Validation					
B. mori	0	300	0	2	4
H. illucens	0	0	300	2	0
T. molitor	0	0	0	281	0
Z. morio	7	0	0	0	294
Sensitivity	0.976	1	1	0.936	0.980
Specificity	0.985	0.995	0.998	1	0.994

TABLE 4 Confusion matrix of calibration and validation sets and performance parameters obtained by PLS-DA for group P composed of *A. domesticus*, *G. assimilis*, *G. sigillatus* and *L. migratoria* meals analysed by NIRM. Unauthorised species are in bold

Predicted class	Actual class			
	A. domesticus	*G. assimilis*	*G. sigillatus*	***L. migratoria***
Calibration				
A. domesticus	100	0	0	0
G. assimilis	0	100	0	0
G. sigillatus	0	0	100	0
L. migratoria	0	0	0	100
Sensitivity	1	1	1	1
Specificity	1	1	1	1
Validation				
A. domesticus	289	4	19	0
G. assimilis	11	230	30	1
G. sigillatus	0	66	251	1
L. migratoria	0	0	0	298
Sensitivity	0.963	0.766	0.836	0.993
Specificity	0.974	0.953	0.925	1

of the classification is also observed for Group P. With these two groups, as with the full dataset, it appears that the bands for moisture, proteins and lipids are the most influential in discriminating the different species (Figure 3B,C). This means that, in both cases the discrimination is based on the chemical composition but the accuracy increases when the number of classes is reduced, as the possibility of confusion is reduced. This can also be explained through the fact that PLS-DA analysis requires the same number of LVs for all single-class models. As a result, the optimal global LVs is not necessarily the optimal LVs for each insect species. This constraint is therefore more detrimental if the number of classes to be distinguished is higher.

The results also demonstrate that NIRM analysis can discriminate between species belonging to the same

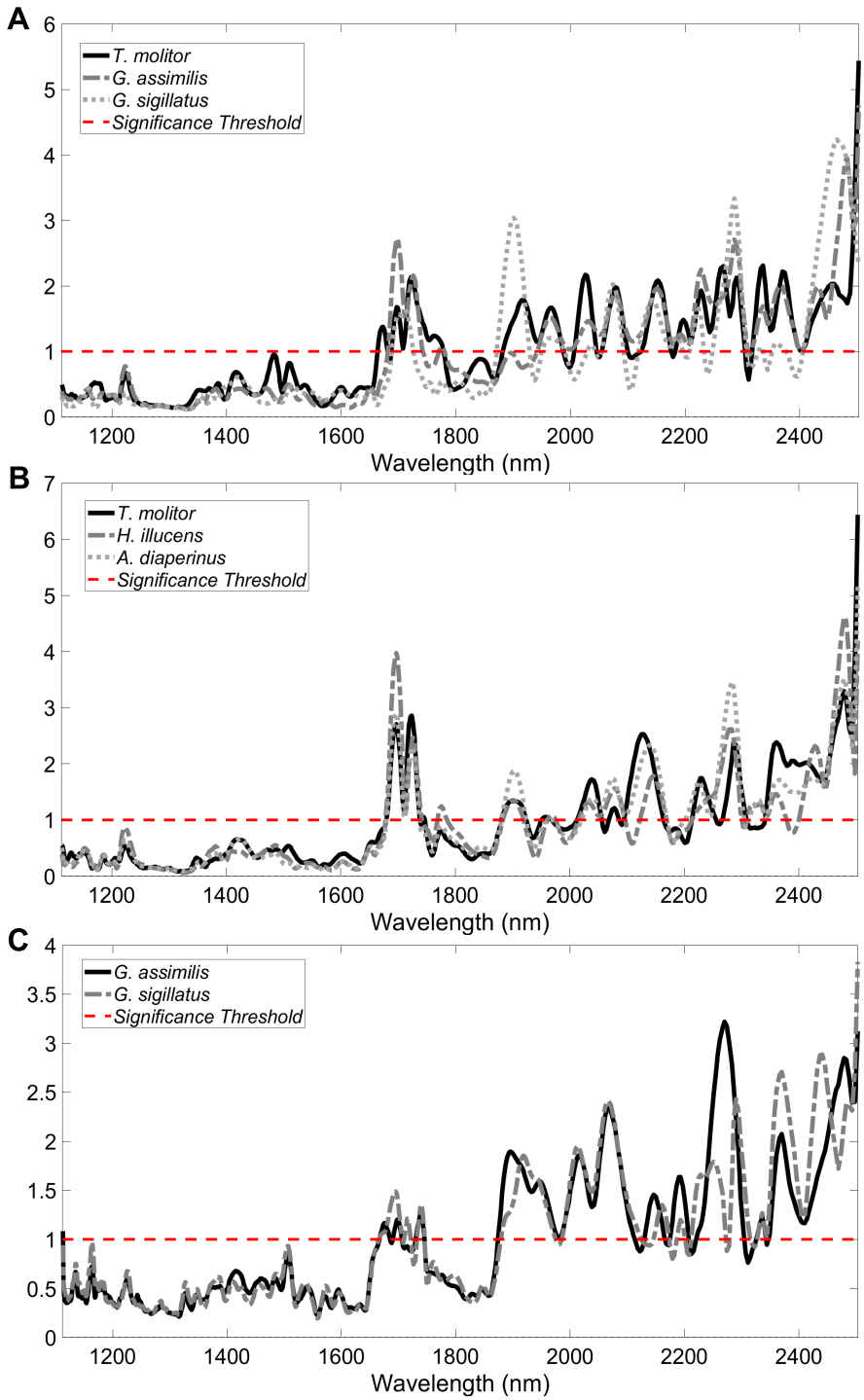

FIGURE 3 Importance of the different spectral variables (VIP scores) in PLS-DA models for the insect species for which the majority of the misclassifications were observed, for the classification scenarios with (A) all insect species, (B) group H and (C) group P.

family. This is the case, for example, for the family Tenebrionidae, which, in this study, is represented by *A. diaperinus*, *T. molitor* and *Z. morio*, a species, not authorised in the EU. Very few misclassifications are observed between them. The variation between them in terms of nutritional value and protein, lipid and fatty acid profiles in particular (Adámková *et al.*, 2016) is a likely explanation.

So far, our focus was mainly on the lipid content but, as highlighted by the VIP scores, the moisture content also has an influence. Very few studies deal with moisture content and, unfortunately, these do not cover all the insect species studied here. However, Khatun *et al.*

(2021) provided interesting data on the moisture content of insects by showing that, depending on the treatment applied to the cricket meal sample, the moisture content can vary significantly, while the lipid content does not. Taking this into account, it would mean that the manufacturing process of the insect meals could also influence the discrimination of the different insect species studied. Regarding the different meals used to create the super mixes, information on the drying process is not known. In this study, it would have been ideal to dehydrate the samples and then measure the residual moisture before analysing them by NIRM, as this could be influenced by the storage conditions of the meal. However, an analysis of the moisture content of the samples without dehydration would already provide an indication.

Another important parameter in insects is the presence of chitin, a polysaccharide of glucosamine an N-acetylglucosamine, which mainly constitutes their cuticle. Depending on the stage of development, but also on the moulting process, the chitin content varies (Hong *et al.*, 2020; Eggink and Dalsgaard, 2023) and can therefore potentially influence the results obtained from NIRM analyses. Chitin has a structure similar to that of cellulose. Thus, Finke (2007) specify that using the acid detergent fiber (ADF) value would allow for an estimation of the chitin content in insects. The article by Finke (2015) reports ADF-based values of 17.8 g/kg for *A. domesticus*, 22.3 g/kg for *T. molitor*, and 23.4 g/kg for *Z. morio*. These values could potentially explain the correct differentiation between *A. domesticus* and *T. molitor* or *Z. morio*. However, they may not necessarily explain the differentiation between *T. molitor* and *Z. morio* obtained through PLS-DA analysis.

More recently, Hahn *et al.* (2018) proposed using the ADF-ADL value, correcting the ADF value using acid detergent lignin (ADL), to obtain a better estimation of insect chitin content. In the same article, the author presents chitin content estimations for *A. diaperinus*, *G. assimilis*, *H. illucens*, and *T. molitor*. It can be observed that there seems to be no difference in the ADF-ADL content between *H. illucens* and *T. molitor*, but there appears to be a difference between these two species and the other two presented, *A. diaperinus* and *G. assimilis*. These results could explain the correct distinction between *A. diaperinus* and *H. illucens* or *T. molitor*, but do not seem to justify both the confusions obtained between *G. assimilis* and *T. molitor* and the results obtained for differentiation between *H. illucens* and *G. assimilis* or *T. molitor*.

Taking into account these various studies and results, it is clear that the variation in chitin content does not seem to be able to explain all the results obtained during our study through PLS-DA analyses. As a result, it would be useful to evaluate the level of chitin present in the super mixes, for example, through NIRS analysis (Chen *et al.*, 2008), in order to relate it to the obtained results.

Finally, in the case of *B. mori* and *H. illucens*, whatever the dataset, their classification in the calibration set or the validation set is almost error-free. Firstly, these two species are taxonomically quite distant from the other species studied, but also from each other (Diptera versus Lepidoptera), so their chemical compositions are probably sufficiently different to allow easy discrimination. The article of Sánchez-Muros *et al.* (2014) also mentioned that the chemical composition of insects varies according to the developmental stages (egg, larva, pupa, nymph, adult). It should be pointed out that the *B. mori* super mix sample is the only sample created using chrysalis meal, but, in this respect, as samples are in meal form, it is difficult to conclude on possible spectral differences based on this. In this case, it would be useful to have access to meals manufactured at other stages of development to compare the results obtained with NIRM. Additionally, mass spectrometry analyses on whole individuals, at different stages, could complement the obtained results and thus enable conclusions regarding potential spectral differences exist between the various morphological structures of the insects.

The reduction of the dataset for group H and P in the case of NIRM analysis thus seemed to improve the performance of the different models and to better differentiate insect species up to their genus. However, this point should still be nuanced; prediction errors can still occur, even if their number were small, and it is therefore not possible to state with certainty that a species is perfectly classified during a NIRM analysis. Although this seems to be one of the limitations of the NIRM method, it is important to bear in mind that in a routine analysis of a sample of insect meal or a mixture of feed and insect meal, it is unlikely that there will be a large number of different species that could interfere with the analysis. In this context, the samples could contain no more than 3 or 4 different species.

In conclusion, the NIRM method remains a screening method that gives an idea of the insect species present in the meal, but does not conclusively confirm the presence of a particular species. In case of fraud, natural contamination or mislabelling, the NIRM method would not be able to reach a conclusion if the chemical composition is, for example, too similar to that of the

contaminated species. However, in the presence of contamination, the probability of having a chemical composition profile as homogeneous as that of the samples used in this study is very low. In fact, during contamination, insects at different stages of development will be found and could influence detection by NIRM. Furthermore, it is also important to bear in mind that in this type of situation, contamination usually occurs in low percentages and, to date, it has not been demonstrated that the NIRM technique has a LOD that can compete with LM or RT-PCR. This technique should be combined with methods that allow further classification, such as genomic or proteomic techniques. Nevertheless, these results remain encouraging and are ultimately in line with what has been already demonstrated with the NIRM technique. Indeed, past studies have shown that this technique is able to distinguish poultry particles from mammalian particles in the field of PAPs and their identification within the feed ban framework (Baeten *et al.*, 2004). Ultimately, this new study on pure insect meals aims to push the boundaries of species discrimination using NIRM even further. It also opens the way to other perspectives, such as using NIRM to detect and identify insect species used to adulterate feed (Anselmo *et al.*, 2023a). However, there is still to be done in relation to the use of this technique to detect low concentrations of insects and to truly compete with official methods such as LM and RT-PCR.

In order to improve the NIRM analyses, it could be considered to analyse separately the different meals used to form the super mixes in order to obtain a more contrasted data set. With regard to the use of PLS-DA, it could be suggested using another classification method such as the Super Vector Machine (SVM) (Fernández Pierna *et al.*, 2004), but there is no guarantee that this would improve on the results already obtained. Moreover, to check whether the chemical composition of insect meal varies according to the treatment undergone during the production process, it would be useful to carry out chemical composition analyses, like mass spectrometry or even NIR techniques (Cruz-Tirado *et al.*, 2023), on the super mixes but also on each insect meals used to create them. This could indeed help in the interpretation of the results obtained by NIRM.

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