Rapid discrimination and classification of edible insect powders using ATR-FTIR spectroscopy combined with multivariate analysis

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Running header: Insect powder's authentication by infrared spectroscopy and chemometrics

Abstract

Insects are being proposed as an alternative way to ensure world's food and feed security. Methods to determine edible insect powder's origin and specie will be needed for quality control purposes. Infrared spectroscopy has been extensively used in rapid chemical fingerprinting of food products. The present research explores a new approach to discriminate and classify commercial edible insect powders using attenuated total reflectance mid-infrared spectroscopy combined with multivariate analysis. Infrared spectra of seven commercial edible insect powders from different species (*Tenebrio molitor*, *Alphitobius diaperinus*, *Gryllodes sigillatus*, *Acheta domesticus* and *Locusta migratoria*) and origins (Netherlands and New Zealand) were collected to build up soft independent modelling of class analogy (SIMCA) models. SIMCA models clearly discriminated insects by their specie and origin linking their differences to lipids and chitin. SIMCA models performance was tested using five spectra of each class not used to build up the training set. ~~100% correct predictions were obtained for most of the samples analysed .~~ predictions were obtained for all the samples analysed with the exception of one sample of *Alphitobius diaperinus*. Infrared spectroscopy coupled to multivariate analysis provided a powerful method for the assurance of insect powder's authenticity.

Keywords: rapid method, food authentication, chemometrics, edible insects

Introduction

World population growth combined with an increasing demand for animal-derived products in both developed and developing countries requires finding other and more sustainable protein sources. Insects are being considered as an alternative way to ensure food and feed security (Belluco *et al.*, 2017; Belluco *et al.*, 2013; van Huis *et al.*, 2013; van Huis and Oonincx, 2017). Entomophagy, namely the habit of eating insects, arachnids and arthropods, has been practiced by humans for centuries in several countries of Africa, Asia, Australia and Latin America, with more than 2,000 insects considered edible (Belluco *et al.*, 2017, Belluco *et al.*, 2013; Premalatha *et al.*, 2011; Rumpold and Schlüter, 2013; Sun-Waterhouse *et al.*, 2016; van Huis *et al.*, 2013; van Huis and Oonincx, 2017). Edible insects are good sources of proteins, polyunsaturated fatty acids, vitamins and minerals such as iron, calcium and zinc. Despite the fact that insects can also produce greenhouse emissions and ammonia, most commercially reared edible insect species have lower environmental impact than conventional livestock (Belluco *et al.*, 2017; Sánchez-Muros *et al.*, 2014; Sun-Waterhouse *et al.*, 2016; van Huis *et al.*, 2013; van Huis and Oonincx, 2017). In terms of productivity, insects have a higher growth rate and fecundity as

well as higher feed conversion efficiencies since they are poikilothermic (i.e. they do not invest energy to maintain a constant body temperature). Additionally, insects can be reared on organic side streams and therefore they are able to transform waste into high value food and feed resources (Makkar *et al.*, 2014; Premalatha *et al.*, 2011; Rumpold and Schlüter, 2013; Sánchez-Muros *et al.*, 2014; van Huis *et al.*, 2013; van Huis and Oonincx, 2017).

Even though the use of insects seems encouraging, its incorporation to the market could be slowed down by the long approval process stated by the current legislation and the poor acceptance of entomophagy in Western countries (Belluco *et al.*, 2017). Concerning the low acceptance of entomophagy, innovative ways to incorporate insects into the human diet have been proposed. Edible insects can be processed into more palatable forms by drying and grinding them, thus obtaining a powder that can be used as an ingredient to increase the protein content of several food products (van Huis *et al.*, 2013).

Accordingly, methods are required in order to discriminate and classify edible insect powder's origin and specie to prevent fraud and adulterations. Ulrich *et al.* (2017) have already discriminated several whole insects (*Tenebrio molitor, Alphitobius diaperinus, Acheta domesticus* and *Locusta migratoria*) commercially available for human consumption through protein profiling using mass spectrometry (MS). Moreover, Köppel *et al.* (2019) developed a multiplex real-time PCR method for the detection of insect DNA and determination of contents of *Tenebrio molitor*, *Locusta migratoria* and *Acheta domesticus* in several food matrixes. Even though aforementioned techniques have been gaining attention from both scientific and industrial communities because of their accuracy and reliability, both of them present several drawbacks. In one hand, DNA-based molecular methods need to be cost-effective, as well as they require highly trained operators and set-up optimization, which is not easily achieved (Ali *et al.*, 2014; Levin *et al.*, 2018). On the other hand, MS implies a high initial cost due to the purchase of the equipment and a fractionation of the sample must be done before collecting the spectra in some applications (Sébédio and Malpuech-Brugère, 2016; Singhal *et al.*, 2015).

Infrared spectroscopy combined with multivariate analysis has also been applied for authentication of fruit juices, edible oils and dairy products, among others (Rodriguez-Saona and Allendorf, 2012). This technique is fast, cheap, non-destructive, robust,simple to use and a minimum training for the operator is needed. However, infrared spectroscopy is highly affected by changes in sample's preparation (Wenning *et al.*, 2014), which is easily overcome when the procedure is properly standardized. As far as we know, no previous research has investigated the potential of using attenuated total reflectance Fourier transform mid-infrared spectroscopy combined with supervised pattern recognition techniques to discriminate and classify commercial edible insect powders by species and origins. The objective of the present work was to obtain mid-infrared spectroscopy profiles from commercial edible insect powders and to develop multivariate classification models to rapidly discriminate and classify edible insect powder's specie and origin.

Materials and methods

Edible insect samples

Tenebrio molitor (mealworm), *Alphitobius diaperinus* (Buffalo worm), *Gryllodes sigillatus* (banded cricket), *Acheta domesticus* (house cricket) and *Locusta migratoria* (grasshopper) powders were purchased from Kreca Ento-Food BV (Ermelo, Netherlands), DeliBugs

(Lelystad, Netherlands) and Eat Crawlers (Auckland, New Zealand) (more details are shown in Table 1).

Attenuated total reflectance Fourier transform mid-infrared spectroscopy

Insect powders were mixed thoroughly and 4 mg were taken randomly (per each spectrum collected) and placed onto the sample stage of a portable spectrometer Cary 630 (Agilent Technologies Spain SL, Madrid, Spain), equipped with a single bounce ATR diamond crystal accessory and a deuterated triglycine sulfate (DTGS) detector. A pressure clamp was used to ensure optimal contact between samples and the diamond crystal. A background scan was taken before every sample scan to prevent the environment from interfering the measurements. Spectra were obtained from 4000 to 800 cm^{-1} with 8 cm^{-1} of resolution. Data acquisition was controlled using MicroLab PC software (Agilent Technologies SL, Madrid, Spain). Two different batches of each edible insect powder were analysed during three different days obtaining five spectra per day and batch (30 spectra in total per edible insect powder).

Multivariate analysis

Multivariate analysis and data preprocessing were performed using a chemometric software (Pirouette version 4.0. Infometrix Inc., Washington, US). Second derivative transformation (13-point window second order polynomial-fit Savitzky-Golay function) and multiplicative scatter correction (MSC) were performed on mean-centred data. A PCA-based pattern recognition method, Soft Independent Modelling of Class Analogy (SIMCA), was performed to obtain classification models in order to discriminate the chemical differences between insect powders and for predicting unknown samples (Wold and Sjöström, 1977). Sample residuals and Mahalanobis distances were used for outlier diagnostics (Shah and Gemperline, 1990). Three SIMCA models were built up in order to assess differences between edible insect powders, a 7-class SIMCA model with all samples tested (whole model), a 3-class SIMCA model with Mealworm, Buffalo worm A and Buffalo worm B (worm model) and a 3-class SIMCA model with Banded cricket, House cricket A and House cricket B (cricket model). SIMCA models were interpreted according to class projections, interclass distances and discriminating power. Predicting ability of SIMCA models was tested using five spectra of each class not used to build up the training set (25 spectra per class) (Vandeginste *et al.*, 1998).

Results and discussion

Mid-IR raw spectra

Mid-infrared spectroscopy can provide information on the composition of complex chemical mixtures by studying the IR bands arising from their functional groups, whose assignment is known in most of the cases (Rodriguez-Saona and Allendorf, 2012). Raw IR spectra of several edible insect powders are shown in Figure 1. Different spectral regions linked to several components could be identified from the insect powder's spectra. In all samples, one broad band was observed at 3000-3500 cm^{-1} caused by the H-bonded O-H stretching of chitin, other polysaccharides and residual water. The spectral region at 2800-3000 cm^{-1} represented the C-H stretching of methyl groups of lipids and chitin (Ibitoye *et al.*, 2018; Marchessault *et al.*, 2003; Paulino *et al.*, 2006). The IR band located at 1740 cm^{-1} was assigned to C=O stretching of esters of lipids. IR bands from amide I, II and III regions linked to proteins or chitin were observed as well at 1700-1600, 1600-1500 and 1300-1200 cm^{-1}, respectively. Infrared spectra also showed an IR band around 1200-1000 cm^{-1} mainly attributed to carbohydrates (Socrates, 2001; Stuart, 2012; Talari *et al.*, 2017).

Discrimination and classification of commercial edible insect powders by ATR-FT-MIR combined with SIMCA

Class projections plots, namely PCAs of the entire training set, were generated through SIMCA to visualize class separation among samples and spectra reproducibility (Figure 2). The ellipses represent the regions in which samples belonging to a certain class fall into with a 95% of confidence (Kvalheim and Karstang, 1992). Each data point in the plot represents one insect powder's spectrum. Tight clustering and good separation was obtained among powders of different insect species for all three SIMCA models. Spectra collected from insect powders made from same insect species but produced by different manufacturers (i.e. House cricket A and House cricket B, see Table 1) displayed clearly distinguishable clusters as well (Figure 2 b). On the other hand, Buffalo worm powders (i.e. Buffalo A and Buffalo B), which were elaborated by the same manufacturer, showed overlapping clusters and poor differentiation (Figure 2 c).

Another way to evaluate class separation (i.e. distance between two classes) is the interclass distance (ICD) value. In SIMCA, PCA models are built up for all the classes in the training set. In Table 2 number of factors and outliers per each class for the whole model are shown. After building the PCA class models, the residuals are computed by fitting the objects of every class of the training set to the PCA model of each class. The overall standard deviations of the residuals (i.e. Euclidean distances) are used for calculating the ICD, which is a ratio of interclass to intraclass distance. ICD values close to 0 indicate that the two classes compared are almost identical and values larger than 1 show differentiation (Wold and Sjöström, 1977). As a rule of thumb, ICDs above 3.0 are considered significant to discriminate two clusters of samples as distinct classes (Dunn and Wold, 1995). ICDs among insect powders for the whole model are displayed in Table 3, and are comparable to the clustering patterns obtained in class projections plots (Figure 2 a). ICD values above 3.0 were achieved when comparing powders made of different insect species, but lower values (ICD < 3.0) were obtained when comparing products elaborated with the same insect species (Buffalo worm A-Buffalo worm B and House cricket A-House cricket B clusters). In spite of that, the ICD for house cricket clusters was higher than the one obtained for Buffalo worm clusters. The nutritional and bioactive profiles of edible insects are affected by factors such as habitat, feed, sex, stage and preparation/processing methods applied prior to consumption, among others (Rumpold and Schlüter, 2013; Sun-Waterhouse et al., 2016). Since house cricket powders were produced by different manufacturers (see Table 1), our data confirmed that product origin plays an important role in insect powder differentiation. On the other hand, the ICD between House cricket B-Banded cricket clusters was 2.5. Considering that both species belong to the same family (Gryllidae), low ICD values could be expected (Krinsky, 2019). However, the same outcome was not observed in House cricket A-Banded cricket powders (ICD = 3.5), which were produced by different manufacturers, confirming again the assumption of differentiation by origin. From the results presented in both class projections plots and ICD values, it is possible to conclude that product origin (i.e. manufacturer) was a key factor for the differentiation in powders made from same insect species, even though no significant differences were found on them (i.e. ICD < 3.0).

Wavenumbers in the spectral range analysed were plotted against their capability to classify and discriminate insect powders tested (Wold and Sjöström, 1977). Discrimination power plots of all models are shown in Figure 3, displaying the IR bands responsible for the discrimination among classes. For the whole model (Figure 3 a), the IR bands at 2945, 2919 and 2851-2825 cm^{-1} could be attributed to CH_3 and CH_2 asymmetric stretching and CH_2 symmetric stretching

of lipids and chitin, respectively (Ibitoye *et al.*, 2018; Marchessault *et al.*, 2003; Paulino *et al.*, 2006; Socrates, 2001; Stuart, 2012). The IR bands at 1744-1722 cm^{-1} might be linked to C=O stretching of lipids. Aforementioned IR bands were also present in the regions previously commented in the insect powder's raw spectra (Figure 1). Nonetheless, SIMCA analysis revealed which of them were mainly responsible of the discrimination of the samples analysed, hardly detected from just observing their raw spectra. The data obtained suggests that the chemical differences among the insect powders analysed related to their chitin and fat fractions when all products were included in a single model (i.e. whole model).

Similar IR bands were obtained for cricket (Figure 3 b) and worm models (Figure 3 c), as well as new ones related to the insect protein and carbohydrate fractions, which were also present in the raw spectra (Figure 1). The IR band at 3114 cm^{-1} could be associated to O-H stretching of carbohydrates (chitin) or N-H stretching of amide A of proteins or chitin. Furthermore, several IR bands in the spectral region of 1700-1500 cm^{-1} were detected, and could be attributed to amide I and II regions of proteins or chitin (Ibitoye *et al.*, 2018; Marchessault *et al.*, 2003; Paulino *et al.*, 2006; Socrates, 2001; Stuart, 2012; Talari *et al.*, 2017). Once again, the chemical differences between cricket powders were mainly related to lipid and chitin components. Worm powders gave the same output as well.

Prediction of unknown samples

SIMCA models performance (whole, worm and cricket models) was tested using five spectra of each class not used to build up the training set. Every data point (spectrum) falling inside the 95% confidence interval boundary of a certain class (i.e. class' PCA model) would be assigned as a member of that class, otherwise would be rejected. Class prediction in SIMCA provides three possibilities, whether the observation belongs to one, more than one (next best prediction) or none of the predefined classes (He *et al.*, 2007). 100% of correct predictions into the appropriate class were obtained for most of the insect powders. Nonetheless, it is important to mention that for Buffalo worm clusters (A and B) some of the next best predictions were assigned to the other Buffalo worm cluster (see Table 4). These results, which are in line with both ICD values and class projections plots obtained through SIMCA, confirmed that there are not significant differences between Buffalo worm A and Buffalo worm B. Lastly, even though an ICD lower than 3.0 was obtained when comparing House cricket A and House cricket B clusters, 100% of the spectra tested in the validation set were correctly assigned to each class without obtaining next best predictions. These results confirmed that our SIMCA models could discriminate and properly classify the commercial insect powders tested by specie and origin.

Conclusions

The current study has given the framework for a cheap, rapid and easy technique in edible insect powder's discrimination (i.e. origin and specie) by using ATR-FTIR combined with SIMCA. Our findings are the first step towards a reliable and easy-to-implement way of preventing fraud and adulterations in the emerging insect sector. A further important implication is the possibility of extrapolating this approach to any insect-derived product. For this reason, future work should concentrate on the creation of models that include a wide range of products and, in collaboration with insect farmers, taking into consideration factors such as feed, sex and stage when discriminating them. Additionally, to further our research we plan to determine the feasibility of this technique for detecting insect powders in several food matrixes.

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Figure 1. Attenuated total reflectance Fourier transform mid-infrared spectroscopy raw spectra of seven edible insect powders.

Figure 2. Soft independent modelling of class analogy (SIMCA) class projections plots of transformed (second derivative, 25 points window) attenuated total reflectance Fourier transform mid-infrared spectroscopy (ATR-FT-MIR) spectra (4000-800 cm^{-1} region) of (A) 7-class SIMCA model with all samples tested (whole model), (B) 3-class SIMCA model with Mealworm, Buffalo worm A and Buffalo worm B (worm model) and (C) 3-class SIMCA model with Banded cricket, House cricket A and House cricket B (cricket model).

Figure 3. Soft independent modelling of class analogy (SIMCA) discriminating power plots of transformed (second derivative, 25 points window) attenuated total reflectance Fourier transform mid-infrared spectroscopy (ATR-FT-MIR) spectra (4000-800 cm^{-1} region) of (A) 7-class SIMCA model with all samples tested (whole model), (B) 3-class SIMCA model with Mealworm, Buffalo worm A and Buffalo worm B (worm model) and (C) 3-class SIMCA model with Banded cricket, House cricket A and House cricket B (cricket model).

Figure 1

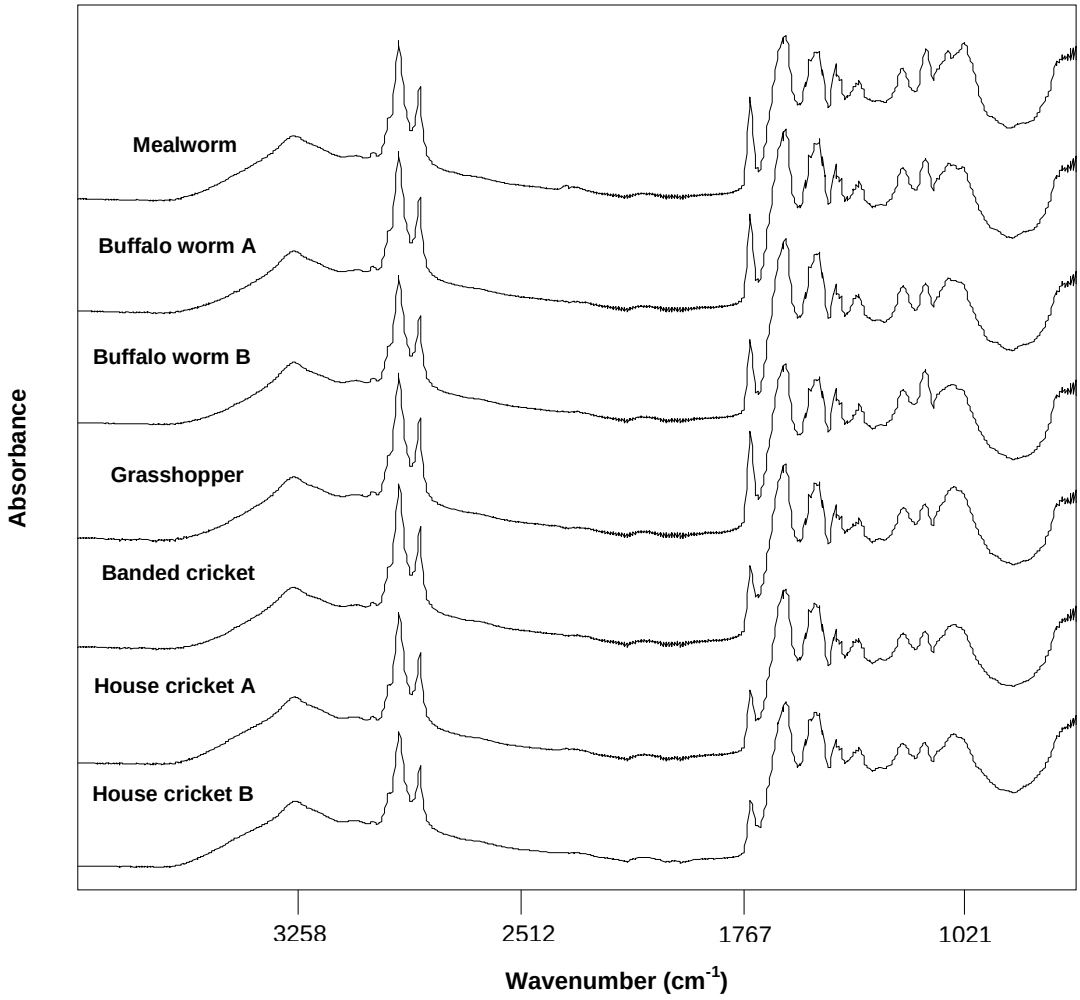
Mealworm
Buffalo worm A
Buffalo worm B
Grasshopper
Banded cricket
House cricket A
House cricket B
Absorbance
3258
2512
1767
1021
Wavenumber (cm-1)

Figure 2

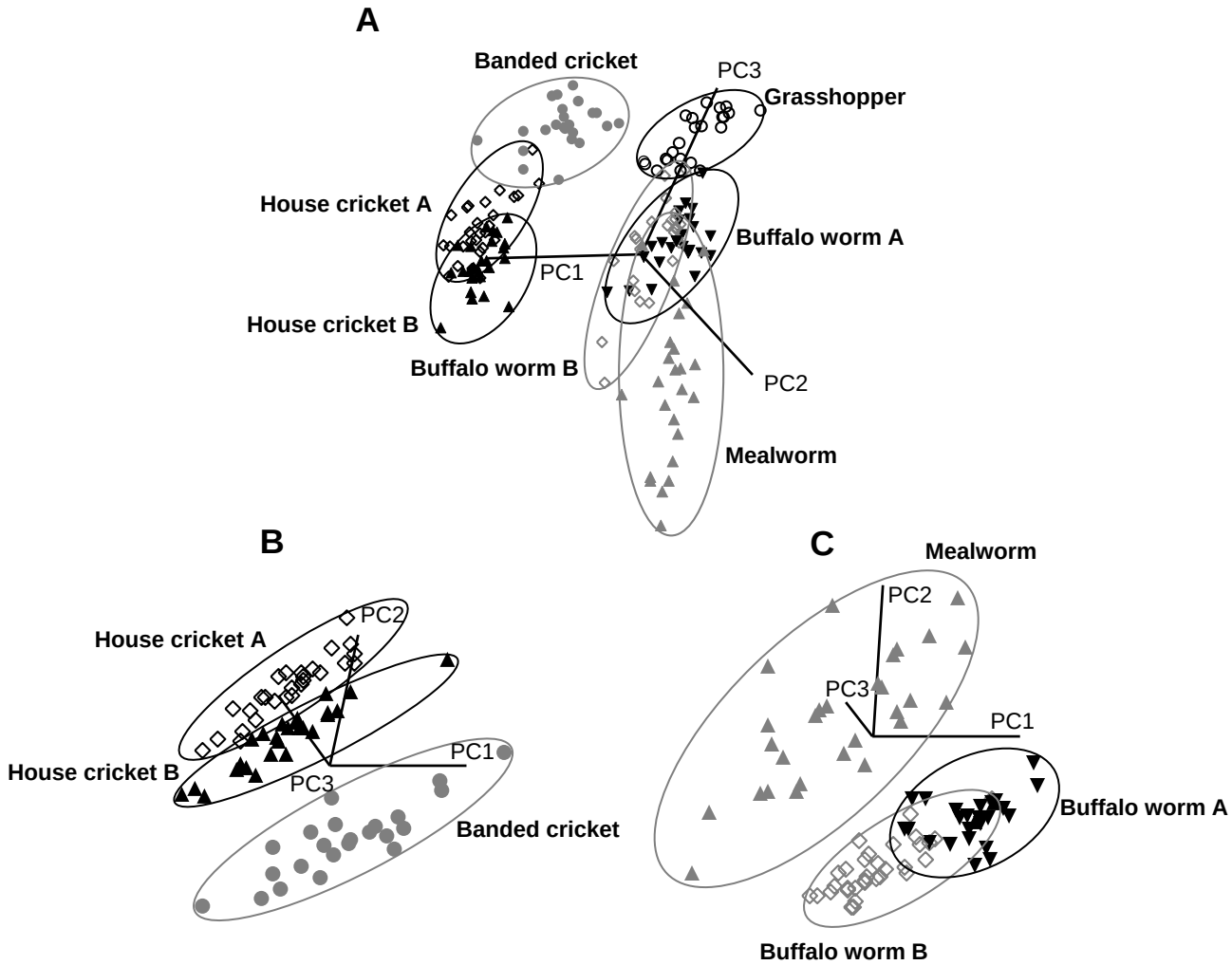
A
Banded cricket
PC3
Grasshopper
House cricket A
Buffalo worm A
PC1
House cricket B
Buffalo worm B
PC2
Mealworm
B
House cricket A
PC2
PC1
House cricket B
PC3
Banded cricket
C
Mealworm
PC2
PC3
PC1
Buffalo worm A
Buffalo worm B

Figure 3

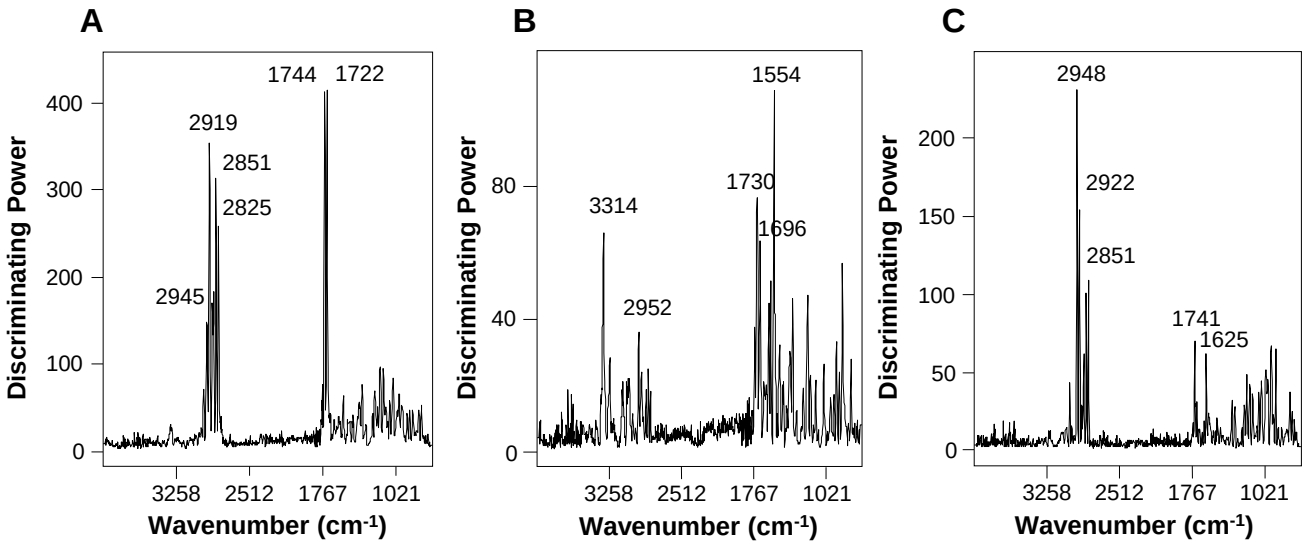
A
B
C
Discriminating Power
Wavenumber (cm-1)
3258
2512
1767
1021
1744
1722
2919
2851
2825
2945
1554
1730
1696
3314
2952
2948
2922
2851
1741
1625

Table 1. Edible commercial insect powders tested in this research.

Insect powder	Supplier	Manufacturer	Amount per packaged[c] (g)	Sample tag
Mealworm	Kreca Ento-Food	1[a]	100	Mealworm
Buffalo	DeliBugs	1	50	Buffalo worm A
	Kreca Ento-Food	1	100	Buffalo worm B
Grasshopper	Kreca Ento-Food	1	100	Grasshopper
Banded cricket	Kreca Ento-Food	1	100	Banded cricket
House cricket	Eat Crawlers	2[b]	50	House cricket A
	Kreca Ento-Food	1	100	House cricket B

[a] Netherlands (origin).

[b] New Zealand (origin).

[c] Two different batches analysed per product tested.

Table 2. Cumulative variance obtained for each factor and number of outliers considered per each class (disjoint PCA model) of 7-class soft independent modeling of class analogy whole model.

Class	Factor 1 (%)	Factor 2 (%)	Factor 3 (%)	Factor 4 (%)	Factor 5[a] (%)	Number outliers[b]
Mealworm	78.7	91.9	94.8	96.9	97.6	3
Buffalo worm A	76.5	84.9	90.3	94.1	95.5	0
Buffalo worm B	47.7	73.8	87.9	91.5	94.5	2
Grasshopper	83.1	94.7	96.9	97.8	98.4	2
Banded cricket	61.4	83.5	90.0	92.6	94.5	8
House cricket A	44.4	75.0	83.2	89.1	91.8	3
House cricket B	62.7	82.4	87.9	91.3	93.8	1

[a] The number of factors selected per model was to obtain at least 90% of variance. This criteria was established according to the number of optimal factors obtained through Pirouette's SIMCA algorithm (seven for the whole model), which performs an F-test with 95% confidence on the reduced eigenvalues (i.e. latent factors' variances; Malinowski, 1989).

[b] Sample residuals and Mahalanobis distances were used for outlier determination.

Table 3

Table 3. Interclass distances of transformed (second derivative, ~~25~~ 13 points window) attenuated total reflectance Fourier transform mid-infrared spectroscopy (ATR-FT-MIR) spectra (4000-800 cm^{-1} region) of all edible insect powder model (7-class soft independent modeling of class analogy whole model).

	Meal-worm	Buffalo worm A	Buffalo worm B	Grass-hopper	Banded cricket	House cricket A	House cricket B
Mealworm	0.0						
Buffalo worm A	3.3	0.0					
Buffalo worm B	3.1	0.9	0.0				
Grasshopper	4.5	3.3	3.6	0.0			
Banded cricket	6.3	5.0	4.9	4.3	0.0		
House cricket A	8.2	7.2	6.7	7.1	3.5	0.0	
House cricket B	7.8	7.1	7.1	6.4	2.5	2.2	0.0

Table 4. Insect powder model predictions~~validation~~ of all edible insect powder model (7-class soft independent modeling of class analogy whole model) by internal validation using 5 spectra per sample.

Prediction	Insect powders						
	Meal-worm[1]	Buffalo worm A[2]	Buffalo worm B[3]	Grass-hopper[4]	Banded cricket[5]	House cricket A[6]	House cricket B[7]
Best[a]	100%	100%	80%	100%	100%	100%	100%
Next best[b]	-	60%[3] 20%[1]	75%[2]	-	-	-	-

[a] Percentages refer to spectra that were correctly identified by SIMCA model.

[b] Percentages were obtained from the number of correctly identified samples. Next best prediction is indicated by superscript numbers (1-7).