Fruzsina Bettina TÓTH[1], Mátyás LUKÁCS[1], Zoltán GILLAY[1], István KERTÉSZ[11]

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Possibilities for discriminating between Tenebrio molitor *larvae fed with plastic and conventional substrates*

Keywords: *Tenebrio molitor,* plastic waste, mealworm, circular economy, Near Infrared Spectroscopy

1. Abstract

The rapid increase in the global population is driving up the demand for high-quality biological proteins and contributing to rising plastic production and plastic waste. Research indicates that certain larvae may break down plastic, which is resistant to biodegradation, due to bacteria and enzymes in their gut. This study aims to identify a measurement method suitable for industrial use to detect plastic consumption by larvae, which were fed three distinct diets: a mixture of flour and carrot (control), white Styrofoam (wPs), and grey Styrofoam (gPS) enriched with activated carbon. In addition to Styrofoam (polystyrene (PS)), the second two groups were given a mix of flour and carrots. The measurements were conducted using near-infrared spectroscopy, and the data were analysed using Matlab2019a software. A variety of classification algorithms were employed to separate the three groups following pretreatment of the spectra. The linear support vector machine (SVM) model demonstrated an 88% accuracy rate in separating the three groups. Larvae fed on polystyrene-free feed were accurately identified, while larvae fed on two types of polystyrene were less well differentiated. The classification methods were also tested for two groups: larvae fed with polystyrene-containing and polystyrene-free feed. The results indicate that the linear SVM was 92% efficient. At the same time, the quadratic SVM and cubic SVM demonstrated 100% efficiency. The results show that the appropriate model can be employed to differentiate between larvae that consume polystyrene and those that consume plastic-free diets. The underlying mechanism responsible for this separation, whether it be undegraded microplastics in the larval gut or other substances accumulated from polystyrene, requires further investigation.

[1] Department of Food Measurement and Process Control, Hungarian University of Agriculture and Life Sciences Institute of Food Science and Technology, Budapest, Hungary

Fruzsina Bettina TÓTH toth.fruzsina.bettina@phd.uni-mate.hu https://orcid.org/0009-0002-9840-9551
Mátyás LUKÁCS https://orcid.org/0009-0004-9613-910X
Zoltán GILLAY https://orcid.org/0000-0002-2801-0847
István KERTÉSZ https://orcid.org/0000-0001-8336-865X

2. Introduction

The global population is increasing rapidly. It is projected to reach 9 billion by 2050 and 10 billion by 2100 (Internet 1). This rapid growth will increase demand for high biological value animal proteins, which the agricultural and food industries will find challenging to meet. (Kemenczei et al., 2016). The speed of livestock production and processing, along with the amount of land, feed production, and water used for livestock production, can also present a challenge (Miglietta et al., 2015). One potential solution is the utilisation of alternative proteins, including those derived from plants, aquaculture and insect proteins (National Academies of Sciences, Engineering, and Medicine, 2022). The processing of insect proteins presents several advantageous characteristics, in addition to their exceptionally high nutritional value. They are potential food and feed ingredients due to their high protein content, essential amino acid content, and ideal fatty acid composition (Costa et al., 2020). Their production is also environmentally beneficial, as they have significantly lower greenhouse gas and ammonia emissions than other agricultural meat processing methods and require less water and land (van Huis et al., 2021).

In addition to their excellent nutritional properties, it is important to consider the various food hygiene and food safety factors and risks associated with edible insects in the food and feed industry. “The food consumed must, therefore, be expected to contain the appropriate proportions of proteins, carbohydrates, fats, macro- and microelements, vitamins, and other active substances necessary for life. However, it must not contain pathogenic microbes, other biological agents harmful to health, chemical substances (in excess of the limits set forth by law), and physical contaminants” (Laczay, 2012). In the case of insects, chemical hazards may include the presence of toxins and heavy metals, which accumulate in their bodies and enter the food chain. Insects may come into contact with these substances in their natural habitat or through ingesting contaminated feed. A further significant issue in food hygiene is the potential presence of microbiological hazards. Regarding edible insects, microbiological hazards include, in addition to the individual microbiota of the insects, bacteria and viruses that attack the insects and which currently do not pose a risk to humans. However, paying attention to this area is important to avoid possible adverse effects. The FAO’s Edible Insects: Future Prospects for Food and Feed Security (Forestry Papers 171, 2013) addresses the potential risks of zoonotic infections. Given the significant taxonomic differences between insects and humans, the risk of zoonotic infections is not expected to be significant. However, there is a potential risk of contamination by viruses, bacteria, and parasites that may threaten humans and animals due to inadequate husbandry and handling and cleaning practices.

As a secondary consequence of population growth and economic change, income levels have risen, leading to an increase in plastic production and waste by 2019. Consequently, the quantity of plastic waste produced has increased by more than 100% over the same period (OECD,2022). Plastics are employed in many applications across numerous industrial sectors, including construction, textiles, transportation, electronics, and packaging for various consumer products and foodstuffs. The most significant issue is the use of single-use plastics for packaging and other applications, given their limited lifespan of less than one year and subsequent disposal as waste (Rhodes, 2018). The natural breakdown of plastic waste is a challenging process. Various environmental, physical, and physicochemical processes result in fragmentation and disintegration into micro- and nanomaterial particles. These particles are now ubiquitous in all parts of the world, including on land, freshwater basins, seas and oceans, marine sediments, and even air. These substances present a significant environmental and wildlife hazard and pose a considerable risk to human health, contributing to major public health concerns. Micro- and nanomaterials can gain access to the body via ingestion, inhalation or dermal exposure, with the potential to cause a range of significant health issues (Ali et al., 2024; Pilapitiya & Ratnayake, 2024; Hirt & Body-Malapel, 2020). Styrofoam, or foamed polystyrene (PS), is one of the most commonly used types of plastic, produced by the polymerisation of styrene monomers. Styrofoam is made by heating polystyrene granules (Kik et al., 2020). Styrofoam is also known as expanded polystyrene (EPS) and its properties include being thermoplastic, durable, permanent, and resistant (Febriansya et al., 2024).

It is important to identify solutions to the food safety and waste management issues that arise from a growing population. The European Commission’s Circular Economy Action Plan may offer a suitable solution to these problems. The Action Plan will focus on utilising farming techniques that minimise the consumption of raw materials to a level that the Earth can sustain without further deterioration to ensure the planet’s future (Internet wang2). Research by Madau et al. (2020) indicates that processing edible insects as food and feed represents a natural and essential component in the evolution of a circular economy. This is because there is growing evidence that some species of invertebrates can consume and break down food waste, other by-products, and various plastics. The larvae of the *Tenebrio molitor* have been the subject of extensive research due to their notable ability to degrade polystyrene. In their 2022 study, Wang and Tang investigated the effects of different polystyrenes on the intestinal rumen of *Tenebrio molitor*. They evaluated the vital signals in the gut and faecal fluid using RNA-Seq technology. The findings suggest that consuming polystyrene may result in delayed larval development. The findings of an additional study indicate that larvae fed with polystyrene foam exhibited comparable survival rates to those nourished with conventional (wheat bran) feed

during the initial month. The study employed gel permeation chromatography, nuclear magnetic resonance spectroscopy, and thermogravimetric Fourier transform infrared spectroscopy to analyse faecal samples from larvae fed Styrofoam. The results corroborate the hypothesis that polystyrene molecules in the intestinal tract are cleaved and depolymerised (Yang et al., 2015). In a study by Nakatani et al. (2024), expanded polystyrene (EPS) was provided as a dietary supplement to *Tenebrio molitor* larvae. The resulting decrease in the molecular weight of the polystyrene was examined using FT-IR and py-GC/MS spectrometry. The results demonstrated a 33% reduction in the molecular weight of digested polystyrene following a one-week feeding period. Furthermore, the findings indicated that the survival rate of larvae reared on EPS was higher than that of starved larvae, suggesting that EPS may serve as a nutrient source for the larvae. However, there was no observable weight gain in larvae reared on EPS feed. In a 2022 study (Machona et al., 2022), larvae were fed on three distinct types of feed for seven days. The feed settings were the following: a control group (fed wheat flour and carrot), a polystyrene group, and a third group that received expanded polystyrene as a substrate. After seven days, the survival rate and weight variation were investigated, and distinct bacterial strains were isolated and identified from the intestinal tract of the polystyrene-fed larvae. The survival rate of the feeding experiments was 90% for the larvae fed with the control diet and the beet-polystyrene mixture. In comparison, a survival rate of 85% was observed for larvae fed with polystyrene alone. Following isolation, the bacteria were subjected to morphological and DNA sequence analyses for identification. Gram-negative bacteria were found in the intestinal tract of the mealworms based on three isolates, and the sequencing results showed that the bacteria responsible for the degradation in the isolates were the same as those found in the study: *Klebsiella oxytoca* ATCC 13182, *Klebsiella oxytoca* NBRC 102593 and *Klebsiella oxytoca* JCM 1665. In 2022, an in vitro cytotoxicity test was conducted on starch derived from *Tenebrio molitor* larvae fed polystyrene. The objective was to assess the effect of this substance on the viability of an oestrogen-dependent MCF-7 cell line. Additionally, in vivo experiments were performed on male Sprague-Dawley rats. In the study, rats were fed daily for 5 weeks with a powder made from larvae previously fed with polystyrene as a forage. Following this, a series of toxicological tests were conducted including clinical signs, body and organ weights, food consumption, haematology, serum chemistry, haematoxylin and eosin staining of the liver and kidney. The results demonstrated that the powder did not elicit any specific adverse effects. Consequently, the study concluded that it was acceptable to utilise the larval powder in high-protein animal feed (Choi et al., 2022).

1.1 Regulation

In the European Union, the conditions for the marketing of novel foods are governed by Regulation (EU) 2015/2283 of the European Parliament and of the Council. “Novel Food is defined as food that had not been consumed to a significant degree by humans in the EU before 15 May 1997, when the first Regulation on novel food came into force.” (Internet 3). In accordance with EU regulation 2015/2283 of the European Parliament and of the Council, the placement of novel foods on the market within the European Union is contingent upon their inclusion in the list of novel foods. The aforementioned list is the EU regulation 2015/2283, which was subsequently amended on 1 June 2021 (amendment to the European Commission Implementing Regulation 2017/2470: European Commission Implementing Regulation 2021/882) to permit placing four insect species within the EU on the market. The European Commission Implementing Regulation 2017/2470 specifies the insect species that are permitted for use, the forms in which insect-based ingredients may be incorporated, and the maximum permitted levels of such ingredients in various food types.

The legislative framework governing the marketing of insects as animal feed is more complex. The conditions for the utilisation of insects as animal feed are established in a number of legislative instruments. The eight species of insects that may be used as animal feed are listed in the amendment of Regulation (EU) No 142/2011/EU 2017/893. Following the amendment of Regulation (EU) No 999/2001 by Regulation (EU) No 2017/893, protein extracted from insects may be used for feeding aquatic animals as of 24 May 2017. Subsequently, in 2021, Regulation (EC) No 999/2001, as amended by Regulation (EC) No 2021/1372, will allow the feeding of pig and poultry with feed containing insect protein. The regulation stipulates that feed containing processed protein derived from farmed insects may contain less than 50% crude protein.

Furthermore, Commission Regulation (EU) 2017/893, which amends Annexes I and IV to Regulation (EC) No 999/2001 of the European Parliament and of the Council, as well as Annexes X, XIV and XV to Commission Regulation (EU) No 142/2011, states the following with regard to the provisions on processed animal protein: The term “farmed animals,” as defined in Article 3(6) of Regulation (EC) No 1069/2009, is extended to include insects reared for the production of processed protein derived from insects. The feed prohibitions set forth in Article 7 of Annex IV to Regulation (EC) No 999/2001, along with the feed regulations outlined in Regulation (EC) No 1069/2009, are applicable to the feeding of insects.

In the face of these regulations, it is expected that some farming ventures use prohibited materials as cheap feed for insects, such as certain plastics, which pose a potential health risk further down the feed and food chain. Also, such materials imported from third countries require investigation for safety purposes upon

entering the EU. Therefore, our study aimed to test an industrially applicable and possibly scalable approach for monitoring, with the use of Near Infrared Spectroscopy to detect whether insects have ingested plastic.

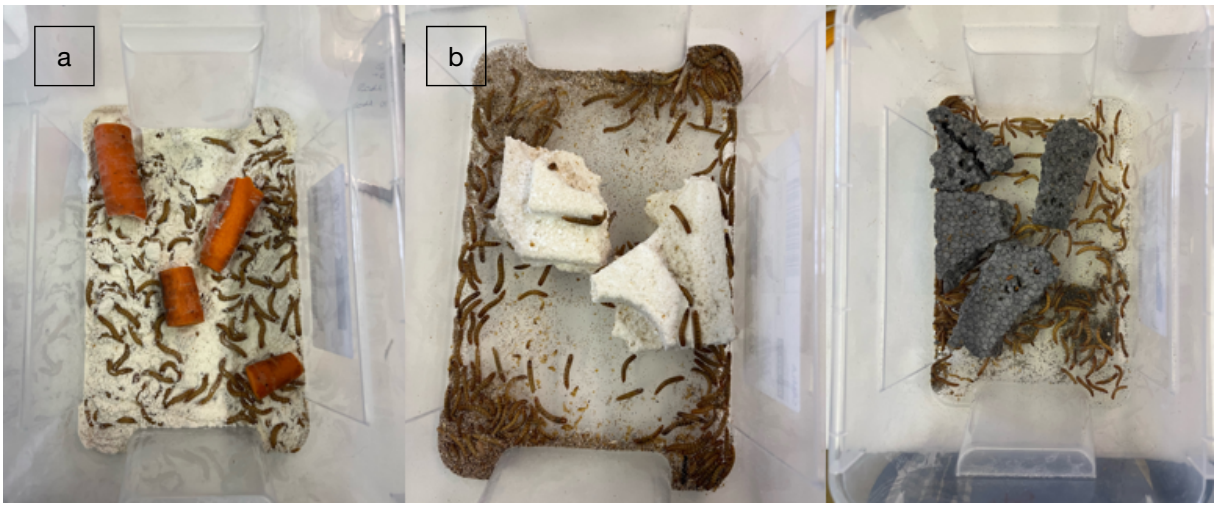
Materials and Methods

Tenebrio molitor

The larvae of Tenebrio molitor are one of four edible insect species that are permitted for marketing in the European Union for human consumption and animal feed (Internet 4). The *Tenebrio molitor* belongs to the order *Coleoptera*, family *Tenebrioidae*, class *Insecta*. The larvae of the mealworm are cold-blooded and environmental factors markedly influence their development. They have optimal conditions in dark, damp environments. The diet of the mealbug is omnivorous, with a preference for cereals and vegetables (Dalton et al., 2019).

The larvae used in the experiments were sourced from various pet and feed animal shops in Budapest. Since October 2023, we have been developing our omme with insects from the same source.

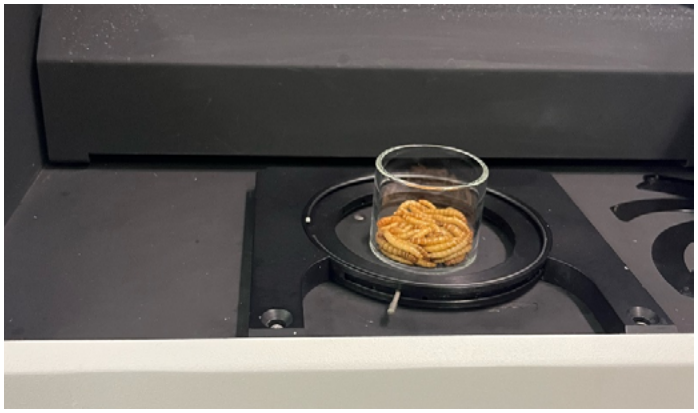
The larvae were given three different diets for varying lengths of time. The diets included a conventional mixture of carrot and flour (control) (**Figure 1/a**), white Styrofoam(wPS) used for packaging (**Figure 1/b**), and grey Styrofoam (gPS) enriched with activated charcoal (**Figure 1/c**), leftover from a previous project in our department. Larvae fed on polystyrene (PS) were given flour and carrots in addition to the PS.



1. Figure: The different feed types.
a: mixture of carrot and flour; b: diet contained white Styrofoam; c: diet contained grey Styrofoam

Near Infrared Spectroscopy (NIRS) Measurements

The NIRS measurements were conducted using a Metrohm NIRS XDS RapidContent Analyzer within the wavelength range of 400-2500 nm, with a resolution of 0.5 nm. The spectra were recorded from live larvae with three repetitions per sample (**Figure 2**).



2. Figure: Tenebrio molitor *larvae during the NIRS measurement*

The larvae are part of a multi-measurement experiment, therefore we used live larvae for the NIR scans. Since the goal of the study is to detect the fact of feeding with polystyrene, no chemical analyses were conducted, however some assumptions regarding the possible components and metabolic changes are explained in the Results section. Also, as Tsochatzis et al. (2021) conclude in their study, the gut microbiome can be vastly

different among distinct populations, as studies have found dissimilar bacteria in Tenebrio molitor larvae. This finding and the fact that the whole larvae is subject to further processing makes separate gut microbiome or gastrointestinal tract analysis superfluous.

Our design for the experimental setup was based on the work of Nina Kröncke and Rainer Benning (2022), in which live larvae were measured for moisture and protein content using near-infrared reflectance spectroscopy. The working principle and measuring method of the instrument ensures that the motility of the insects does not affect the readings. This is confirmed by the virtually identical spectra of the triplicates per sample measurement (average correlation coefficient is 0.9993).

Data Pretreatment and Analysis

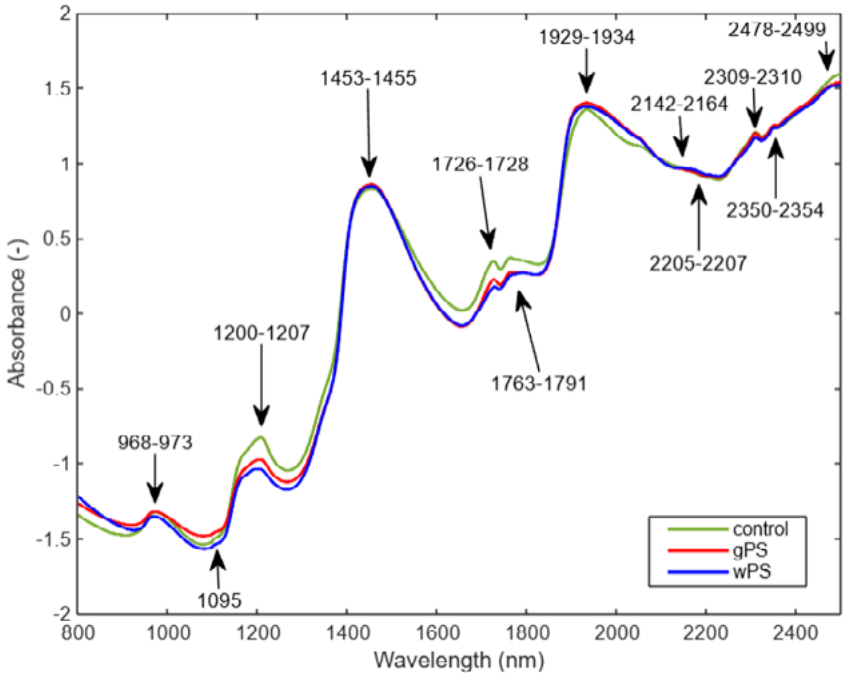
The raw spectral data were processed using MATLAB 2019a, between 800-2500 nm wavelengths. The preprocessing stage included smoothing with a second-order Savitzky-Golay filter and normalisation using Standard Normal Variate (SNV) correction. The input variables were the coefficients of the pretreated spectra, while the estimated classes were the different feed types. Various classification methods were employed for estimation, and the method yielding the best results was selected. The settings included three-fold cross-validation and principal component analysis (PCA) with 99% explained variance for efficient model building. The classification methods used were Discriminant Analysis (DA), K-nearest neighbours (KNN), Decision Trees, and Support Vector Machines (SVM).

3. Result and discussion

The correspondence between wavelengths and molecular bonds was assessed using the above-mentioned literature (Kröncke & Benning, 2022), the Practical Guide and Spectral Atlas for Interpretive Near-Infrared Spectroscopy, 2nd edition (Workman & Weyer, 2012) and the Handbook of Near Infrared Analysis 4th edition (Ciurczak et al., 2021). The wavelength values below indicate the chemical background of the peaks based on the above three references.

To be able to analyse the spectra of different feed types, the spectra were averaged by groups, followed by a preprocessing protocol described in 2.3. This process already showed visible dissimilarities between the groups, as shown in **Figure 3.**

3. Figure: Average of processed spectral data and main peaks of average spectras, by feed type (control - mixture of



carrot and flour; gPS - diet contained grey Styrofoam, wPS - diet contained white Styrofoam)

Several of the peaks are observed in all three averaged spectra, and are attributed to water, lipid, protein and chitin content, normally present in mealworms. The peaks around 970 nm most likely indicate the presence of water (Peñuelas et al., 1993). However, certain peaks, that are present in the PS-fed groups align with the absorbance spectrum and its specific peaks of pure crystalline polystyrene detailed in the works of Workman and Weyer (2012) and Ciurczak et al. (2021). These peaks occur at 1766 nm, 1791 nm, 1933 nm, 2144 nm, and 2354 nm, while certain plasticizers used in the manufacturing process contain aromatic rings, resulting in a peak at 2487 nm, but based on the table in the literature used, the peak may also refer to a C-H stretching &

C-C stretching combination in cellulose. According to other literature (Ozaki et al., 2021), peaks around 1767 nm can be attributed to the first overtone of C-H stretching in the CH2 groups of hydrocarbons and aliphatic compounds. In their work, Nina Kröncke and co-workers (2023) attributed peaks appearing between 1390 and 1450 nm to combined CH stretching and first overtone OH stretching, peaks appearing between 1925 and 2030 nm to combined and first overtones of OH and NH stretching, mainly related to water and protein content, and peaks at approximately 1146 nm and 1196 nm to second overtone C-H stretching. Peaks appearing between 1699 and 1797 nm may generally be related to fat and fatty acid content. On the other hand, based on the work of Workman and Weyer (2012) and Ciurczak et al. (2021), the peaks around 1935 nm can be attributed to O-H stretching and the HOH combination bonds of polysaccharides. The peaks around 2310 nm are probably C-H bending in lipids, while around 2350 and 2500 nm, probably the C-H stretching & C-C & C-O-C stretching combination of polysaccharides. Other manufacturing byproducts and chemical residues might contribute to the differences in the observed spectra, but these are not identified within the scope of the present study, and, therefore, are subject to speculation, and ultimately are not described in the present paper in detail.

The peaks in the 1010-1095 nm and 1140-1155 nm range are attributed to the second overtone of O-H and C-H, respectively, in accordance with another source on NIR spectroscopic measurement methods for determination of protein, total carbohydrate, and crude fat content in foxtail millet (Chen et al., 2013).

It is important to note that consumption of PS by the larvae may have an effect on the metabolism and therefore the over- or underproduction of certain compounds naturally present in them, and, as a result, can have an effect on the performance of classification (Tsochatzis et al. 2021). For instance the peaks around 1200 nm are generally attributed to lipids (Kröncke & Benning, 2022), but are more pronounced for the control group, and therefore we can assume that higher relative fat content can be associated with the consumption of PS. Larval fat/oil content can also be influenced by substrate quantity and quality, and environmental factors such as temperature and relative humidity (Bordiean et al., 2021; Syahrulawal et al., 2023).

Again, these are assumptions but they demonstrate the possible complexity of a correlative measurement technique such as NIRS.

4. Figure: Absolute values of spectral loadings of the first two principal components (PC1, PC2)

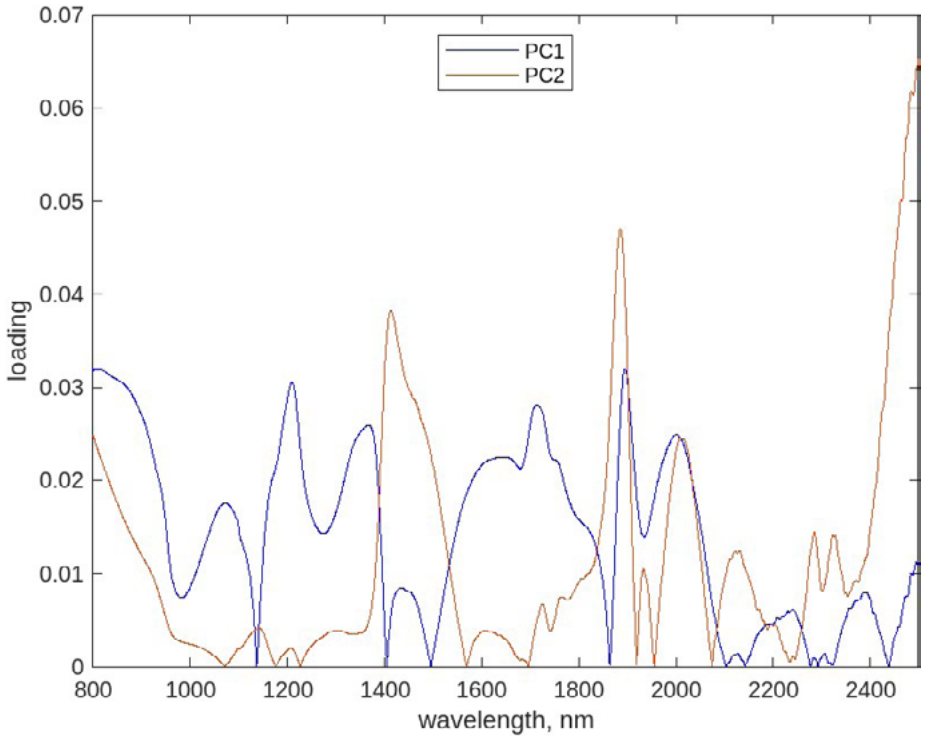
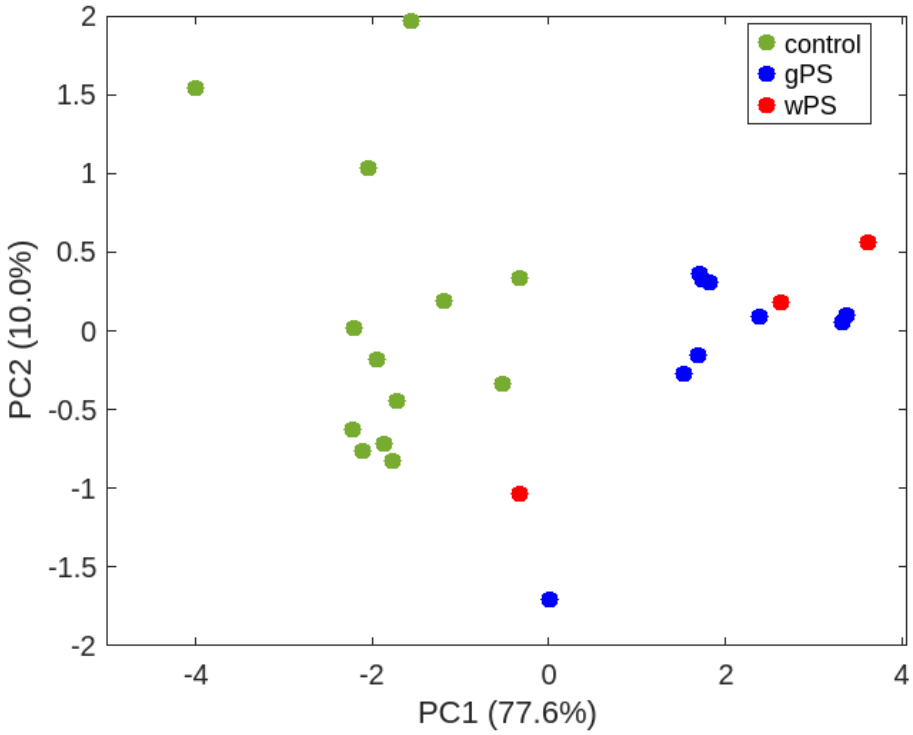


Figure 4 illustrates the absolute values of spectral loadings for the first two principal components. This plot explains which wavelengths contribute most to the variance within the datasets, and therefore, if a separation between the groups emerges, it indicates which wavelengths and hence chemical bonds cause the difference in the feed groups. The 1209 nm peak in the first principal component (PC1) is probably the second overtone of methylene C-H stretching, which can overlap with the peak of lipids at 1200 nm (Workman & Weyer, 2012). The peak at 1366 nm may be a C-H from aromatic hydrocarbons. The peak at 1633 nm may indicate C-H stretching in vinyl groups or C-H stretching in aromatic hydrocarbons caused by pollutant residues from the manufacturing process (Ciurczak et al., 2021). A peak at 1647 nm is the first overtone of C-H stretching of the double bond in the 1645-1675 nm range. A peak at 1894 nm may indicate vibrations of O-H hydrogen bonding

(Workman & Weyer, 2012). The peak observed in principal component 2 (PC2) at 1412 nm may be associated with aromatic bending in the C-H plane or methylene C-H, combined with branched aliphatic compounds as assumed contaminants. At 2008 nm, it may be an N-H or C-N combination band from primary amides (Workman & Weyer, 2012). The peaks were determined entirely based on the three literature sources cited (Workman & Weyer, 2012; Ciurczak et al., 2021; Kröncke & Benning, 2022) and are, therefore, assumptions and not conclusive. Further studies are required to determine the exact composition.



5. Figure: Scatterplot derived from the initial two principal components (PC1, PC2), control - mixture of carrot and flour; gPS - diet contained grey Styrofoam, wPS - diet contained white Styrofoam

Figure 5 represents the scatterplot derived from the initial two principal components (PC1 and PC2), showing a clear separation between the control group and the PS-fed groups. Principal component analysis is typically employed to reduce the dimensionality of a data set by summarising the data set with a smaller number of variables (Bartholomew, 2010), which enables us to efficiently run classification algorithms using only seven latent variables with practically the same outcome as with all 3400 original variables. In this instance, the initial principal component accounts for 77.6% of the variance in the data set, while the second principal component describes 10.0% of the variance, and the first three principal components collectively account for 94.9% of the data variance. The loading values corresponding to the wavelengths for the first two PCs are shown in **Figure 4**, in comparing this figure with the pre-processed averaged spectra the correspondence between the observed differences and the loading peaks are apparent. Figure 5 demonstrates that larvae nourished with a diet devoid of polystyrene can be discernibly differentiated from those fed a diet containing Styrofoam. This suggests the existence of a discernible discrepancy in the spectral data.

As previously mentioned (Chapter 2.3), the estimation was conducted by selecting the optimal model from the evaluated models. The Support Vector Machine (SVM) gave the best result, which exhibited an accuracy rate of 88% for three distinct feed types, indicating the model's overall performance. No misclassification of larvae was observed in the polystyrene-free food samples. However, the model results indicated some degree of uncertainty regarding the two polystyrene types. Further, run models discriminated between larvae fed with the three feed types with 76-84% accuracy.

Matlab 2019a software was used to run the models, with the two different Styrofoam feeds treated as a single feed. This resulted in the three feed types being reduced to two, comprising a conventional polystyrene-free and polystyrene-containing feed. Once more, the input variables were the pretreated spectral coefficients, while the estimated variables were the two feed types. In this instance, the linear discriminant analysis yielded 96% accuracy, with the linear support vector machine (SVM) model discriminating the samples with a marginally inferior accuracy of 92% compared to the previous case. However, the quadratic SVM and the cubic SVM produced 100% accuracy.

4. Conclusions

The rapid growth of the global population is resulting in a surge in demand for high biological value proteins, which is, in turn, leading to a corresponding increase in the production of plastic and plastic waste (Internet 1, OECD 2022). Research suggests that edible insects, like *Tenebrio molitor* larvae, could play a role in the European Commission's Circular Management Plan (Madau et al., 2020). Their intestinal tract hosts bacteria and enzymes capable of degrading hard-to-break-down plastics (Yang et al., 2015; Choi et al., 2022; Machona et al., 2022; Wang & Tang 2022; Nakatani et al., 2024).

This study aimed to find an effective method of confirming larval plastic consumption. Three diets were tested: a mixture of flour and carrot, white polystyrene packaging, and grey polystyrene with activated carbon.

The linear support vector machine (SVM) gave the best results, discriminating the groups with 88% accuracy and showing no misclassification of larvae fed the polystyrene-free substrate. Without distinguishing between the two types of polystyrene, two diet categories were created: with and without polystyrene. In this case, the linear SVM showed a separation efficiency of 92%, while the quadratic and cubic SVMs reached 100%.

The results demonstrate that, with the appropriate methodology, it is possible to differentiate between larvae that have been fed polystyrene and those that have been fed a diet that does not contain polystyrene with NIR spectroscopy. The spectra were analysed based on three existing literature sources (Workman&Weyer, 2012; Ciurczak et al., 2021; Kröncke & Benning, 2022). Therefore these results are not definitive. Further research is necessary to ascertain the complete picture of the precise composition in order to determine whether the presence or accumulation of undegraded microplastics in the larvae is responsible for the observed spectral differences.

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