



Biodegradation of expanded polystyrene by mealworm larvae under different feeding strategies evaluated by metabolic profiling using GC-TOF-MS

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ABSTRACT

The present study investigated the biodegradation of polystyrene (PS) plastic by mealworm (*Tenebrio molitor*) on different diets followed by untargeted screening of larvae gut intestine tissue and frass (manure and feed residuals) to investigate the existence of polymer-generated organic residues. Three different diets, consisting of PS, rolled barley and water were tested. PS degradation rates ranged from 16% to 23% within 15 days, with no statistical differences in survival rates. The larvae fed with ad libitum barley:PS (20:1 w/w) and water had the highest growth rate, while higher PS consumption was observed for barley:PS of 4:1 w/w. A GC-TOF-MS analysis revealed no contaminating substances in the gut intestine tissue, nor styrene or PS oligomers, whilst several bioactive compounds and traces of alkanes, mostly with small carbon chains, were present. Metabolomics analysis on the collected frass, either on the lipophilic (CHCl₃) or the polar fraction (MeOH-H₂O) was performed. Styrene and PS oligomers (dimers, trimers) were identified, though in a relatively low total amount, up to a total of 346.0 ng/mg 2,4 di-tert butylphenol was identified in both frass and tissue, coming from the PS polymer (Non-intentionally added substances; NIAS). Finally, in the polar fraction of frass, bioactive molecules (fatty acids, amides) were identified, together with several hydrocarbons, mostly with longer carbon chains. The formation of these substances indicated enzymatic and biochemical activity in the larvae-gut intestine. It was shown that degrading and contaminating organic compounds occur at low levels, in both gut intestine and frass, during biodegradation of PS.

1. Introduction

Plastic packaging is associated with raised concerns about the potential harm to the environment and human health. Polystyrene (PS) is a thermoplastic polymer considered the third most important for consumers and industry. It is used extensively to produce food contact materials (FCMs) due to its low cost, durability and mechanical strength (International Life Sciences Institute, 2002). Though possible to recycle mechanically, PS is not presently of great market interest to be used in its recycled form (Ho et al., 2018; International Life Sciences Institute, 2002).

Biodegradation is defined by the International Standards Organization (ISO) as the “degradation caused by biological activity, leading to a significant change in the chemical structure of a material” (International Standards Organization (ISO), 2018). During recent years there has been an increasing amount of interest and research conducted on the biodegradation of plastic-based materials promoted by the activity of bacteria and fungi. These organisms are found to be capable of degrading plastics, and together with insects, can convert natural polymers into smaller fragments that facilitate enzymatic reactions (Brandon et al., 2019; Oliveira et al., 2019). Recently, an increasing number of studies have been reported focusing on the bio-recycling of PS

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by insects, such as the common mealworm (*Tenebrio molitor*) (Brandon et al., 2018, 2019, 2021; Matyja et al., 2020; Nukmal et al., 2018; Peng et al., 2019; Tsochatzis and Alberto Lopes, 2020; Yang et al., 2015a, 2015b, 2018a, 2018b, 2021; Zielińska et al., 2021), superworms (*Zophobas morio*) (Yang et al., 2020) and waxworms (*Galleria mellonella*) (Lou et al., 2020). Bio-recycling by insects was reported also for polyethylene (PE) (Bombelli et al., 2017; Kundugal et al., 2019; Lou et al., 2020) and polyvinylchloride (PVC) (Peng et al., 2020).

The European Commission with the “Action Plan for Circular Economy, focusing on Sustainable and Green recycling” adopted in 2015, stressed the importance of plastic recycling and bio recycling. Therein, a particular focus is being given in plastic production and use, towards a goal of ensuring that all plastic packaging is recyclable by 2030 (European Commission, 2019; Ojha et al., 2020). The effect of bio-degradation of PS is currently being investigated by Stroka et al. at the Joint Research Centre (JRC) of the European Commission.

The scope of this research was to perform an untargeted metabolomics screening evaluation of the frass (manure and feed residuals) and larvae gut intestine tissue generated from mealworms after biodegradation of PS, using different feeding strategies. It aimed at identifying and quantifying the most relevant substances (degradation products, PS oligomers or other bioactive compounds) in frass resulting from the bioprocess itself or due to metabolic effects. This evaluation allows us to observe if compounds of significant added value are produced for later exploitation. The work assessed the PS bio-recycling capacity and metabolic effects in these particular insects, providing new insights in this biological process. Furthermore, no published work exists, highlighting the metabolic alteration in mealworms while degrading PS by applying different feeding strategies.

2. Materials and methods

2.1. Chemicals

MSTFA (2,2,2-Trifluoro-N-methyl-N-(trimethylsilyl)-acetamide) containing 1%TMCS (trimethylchloro silane) (Thermo Scientific, USA). Stearic acid-d₂₇ was used as internal standard were obtained from Agilent Technologies (Agilent, USA). Methoxime hydrochloride (98%), chloroform (CHCl₃), pyridine and methanol (MeOH) LC-MS grade and 2-propanol (IsOH) LC-MS grade were supplied by Sigma Aldrich (Steinheim, Germany) and Ultrapure water (18.2 MΩ), for the preparation of stock solutions (Millipore, Bedford, USA).

Styrene (>99%), acetophenone (>99%) and 1,1-diphenyl-ethylene (97%) were purchased from Sigma Aldrich (Steinheim, Germany), while the other PS oligomers, namely 2,4,6-triphenyl-1-hexene, 1,3,5-triphenylcyclohexane, 2,4-diphenyl-1-butene, 1,1-diphenyl-ethylene, *trans*-1,2-diphenylcyclobutane, 1a-phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthalene (1a-tetralin) and 1e-phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydro-naphthalene (1e-tetralin) were supplied by FUJI-FILM Wako Chemicals Europe GmbH (Milano, Italy). Polytetrafluoroethylene (PTFE) 17 mm, 0.2 μm membrane filters were used for filtration of all the samples.

2.2. Test materials and *Tenebrio molitor* larvae

An expanded PS (EPS) foam packaging (density 0.014 g/cm³) were bought at the local market store. PS material was assessed by attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy and differential scanning calorimetry (DSC). Recorded spectra and thermograms were evaluated and confirmed the PS nature of the samples (matching FTIR spectra and a glass transition of 100 °C). *T. molitor* larvae were reared at Aarhus University, Department of Animal Science. Prior to the experiment the diet for the larvae consisted of rolled barley and a mixture of vegetable as a source of water.

2.3. Biodegradation of PS

In addition to the control diet of rolled barley, three experimental setups were compared, as reported in Table 1. In order to assess larvae survival and degradation of PS, 75 larvae (weighting 78 ± 3 mg/larvae) were transferred to 50 cm² cylindrical plastic containers, given a density of 1.5 larvae/cm². All experiments were carried out in duplicate, and except for the control, received identical amounts of PS, cut into 2–3 cm cubes (Table 1). They were conducted in a climate controlled room maintained at 25 °C ± 1 °C and 55 ± 5% relative humidity. Mealworm survival rate was assessed by counting dead mealworms every 4 days during a total of 15 days, and dead larvae were then removed from the container. Frass were separated from barley, PS and larvae at the end of each experimental day, by using a 500 μm sieve, and stored at –18 °C until further analysis. In addition to a control of rolled barley, three experimental setups were compared (Table 1), whereas seventy five larvae (weighting 78 ± 3 mg/larvae) were transferred to 50 cm² cylindrical plastic containers, given a density of 1.5 larvae/cm².

Diet 1 was based on previously studies (Brandon et al., 2018) and was chosen to ensure comparable results. However, because of the higher portion of unavailable nutrition from the shell of the grain compared to wheat bran, an increased amount of feed was used to avoid starvation of larvae. Water is known to have a large influence on larvae growth (Urs and Hopkins, 1973) and to test the effect of water on PS degradation by larvae, water was added to Diet 2 together with an increased bran:PS (20:1). The latter ratio was implemented as stand-alone diet (no water) to Diet 3, in order to investigate whether larvae were more or less prone to eat PS when more feed was present, together with (or without) the presence of water.

2.4. Analytical methods

2.4.1. GC-based metabolomics

2.4.1.1. Extraction of frass. An amount of 100 mg of frass was placed in an Eppendorf tube and extracted with 500 μL of CHCl₃ for 12 h at 25 °C using a vibrational thermostatic mixer. The extract was then filtered with a 0.22 μm PTFE syringe filter and placed into a GC vial and injected. The same amount of frass (100 mg) was extracted following the same procedure but using 500 μL of a MeOH–H₂O 1:1 (v/v) mixture. After the extraction, the tubes were centrifuged at 15,000 g for 15 min at 4 °C, after which 200 μL aliquots were transferred to 1.5 mL Eppendorf tubes. These were then evaporated to dryness using a vacuum centrifuge at 30 °C. The residue was further derivatized using the method reported in paragraph 2.4.2.3.

2.4.1.2. Extraction of gut intestine tissue. Gut intestines were removed from the larvae and homogenized with an electrical homogenizer. An amount of 100 mg was extracted with 500 μL of CHCl₃, at 25 °C using an elliptical shaker. Subsequently, the sample was filtered using a 0.22 μm PTFE syringe filter and injected in the GC-TOF-MS system, in triplicates.

Table 1

Experiment condition and larvae feeding diets for the polystyrene (PS) biodegradation study.

Larvae and provided feed	Control	Diet 1	Diet 2	Diet 3
		Ad libitum barley:PS (4:1)	Ad libitum barley:PS (20:1) + H ₂ O	Ad libitum barley:PS (20:1)
Larvae [g]	5.68 ± 0.00	5.76 ± 0.10	6.01 ± 0.10	5.94 ± 0.10
Rolled barley [g]	20.00 ± 0.01	4.04 ± 0.04 ^a	20.20 ± 0.04	20.10 ± 0.02
PS [mg]	0	1000 ± 42	980 ± 14	1015 ± 21
water [ml]	0	0	1.5*	0

^a Same amount given at the beginning of the experiment and every fourth day.

A gravimetric method (oven 110 °C for 12 h) was used to derive a humidity of 57.8%. An aliquot of 5 µL of myristic acid-d27 stock solution in H₂O:MeOH:IsOH; 2:5:2 (v/v/v) was added to the biological extracts. The sample was evaporated to dryness using a vacuum centrifuge at 30 °C. The analysis was performed following the GC-MS metabolomics analytical method at split inlet mode (1:10 split).

2.4.2. Gas chromatography

2.4.2.1. Targeted analysis of monomers and oligomers. The quantification was based on a modified method of the fast GC method reported by Tsochatzis et al. using styrene and PS-oligomers standards, that were prepared in CHCl₃, and external calibration was applied, according to a previously reported method (Tsochatzis et al., 2020b). The organic CHCl₃ extracts were filtered with PTFE 0.22 µm filters and a volume 1 µL was injected in the GC-TOF-MS system in split-less mode into an Agilent Technologies 7890B gas chromatography system coupled to an Agilent Technologies 7200 Accurate-Mass Q-ToF mass spectrometer (Agilent Technologies, Waldbronn, Germany). Separation of the metabolites was performed on an HP-5MS capillary column coated with polyimide (30 m, 0.250 mm i.d., 0.25 µm film thickness; Agilent Technologies). The temperature of the ion source was 230 °C, the mass spectral analysis was performed in scan mode with a quadruple temperature of 150 °C and a fragmentation voltage of 70 eV, with the remainder settings being kept the same (pressure of 21.4 psi, a gas saver of 20 mL/min and 3 mL/min purge flow). A GC oven programs was applied, where the initial temperature was 60 °C and held for 2 min, followed by a temperature ramp of 10 °C/min up to a temperature of 320 °C, and then held for 5 min. The total analysis time was 34 min.

2.4.2.2. Derivatization. The derivatization protocol was based on the Fiehn GC/MS metabolomics using MSTFA/1% TMCS (Agilent Technologies, 2013) slightly modified. Prior to trimethylsilylation, ketones and aldehydes were protected by derivatization to methoxyamino groups (methoxyamination). An amount of 10 µL of a 40 mg mL⁻¹ solution of methoxyamine hydrochloride in pyridine was added to the frass extract and the mixture was gently shaken at 30 °C for 90 min. Following methoxyamination, N-Methyl-N- trimethylsilyl trifluoroacetamide with 1% Trimethylchlorosilane (MSTFA/1% TMCS) was added (90 µL) to the methoxyaminated samples, for silylation (Agilent Technologies, 2013). The mixture was incubated at 37 °C for 30 min. The derivatized samples were cooled to room temperature before transfer to GC vials and injected to the chromatographic system.

2.4.2.3. GC-ToF-MS metabolomics. For GC-based metabolomics, the samples (1 µL aliquot) were injected in split mode (1:10 split ratio) into an Agilent Technologies 7890B gas chromatography system coupled to an Agilent Technologies 7200 Accurate-Mass Q-ToF mass spectrometer (Agilent Technologies, Waldbronn, Germany) operating in ToF mode. Separation of the metabolites was performed on an HP-5MS capillary column coated with polyimide (30 m, 0.250 mm i.d., 0.258 µm film thickness; Agilent Technologies). The temperatures of the ion source was 230 °C, the mass spectral analysis was performed in scan mode with a quadruple temperature of 150 °C and a fragmentation voltage of 70 eV. Solvent delay was adjusted to 4.5 min. Scanning range was set from *m/z* 50.0 to *m/z* 600.0. The TOF-MS conditions were identical as presented in 2.4.2.1.

2.5. Data analysis and statistical analysis

All analytical data was processed with Masshunter 10.0 software (Agilent Technologies, USA), while Mass Profiler Professional (MPP) v.15.0 software (Agilent Technologies, USA) was used as the chemometrics platform for alignment, and exploitation of the received MS data. MPP is the only platform that provides integrated identification/

annotation of compounds and automated sample classification in combination with Masshunter. Pairwise analysis were performed by one-way ANOVA using RStudio (RStudio Team, 2020). On this basis, the identified components in frass fractions (organic; CHCl₃, and polar; MeOH:H₂O 1:1 v/v) were analyzed by principal component analysis (PCA) to assess the discrimination between the different feeding strategies, performed with SIMCA 16.0.2 software (Umetrics, Umea, Sweden).

3. Results and discussion

3.1. Biodegradation of PS

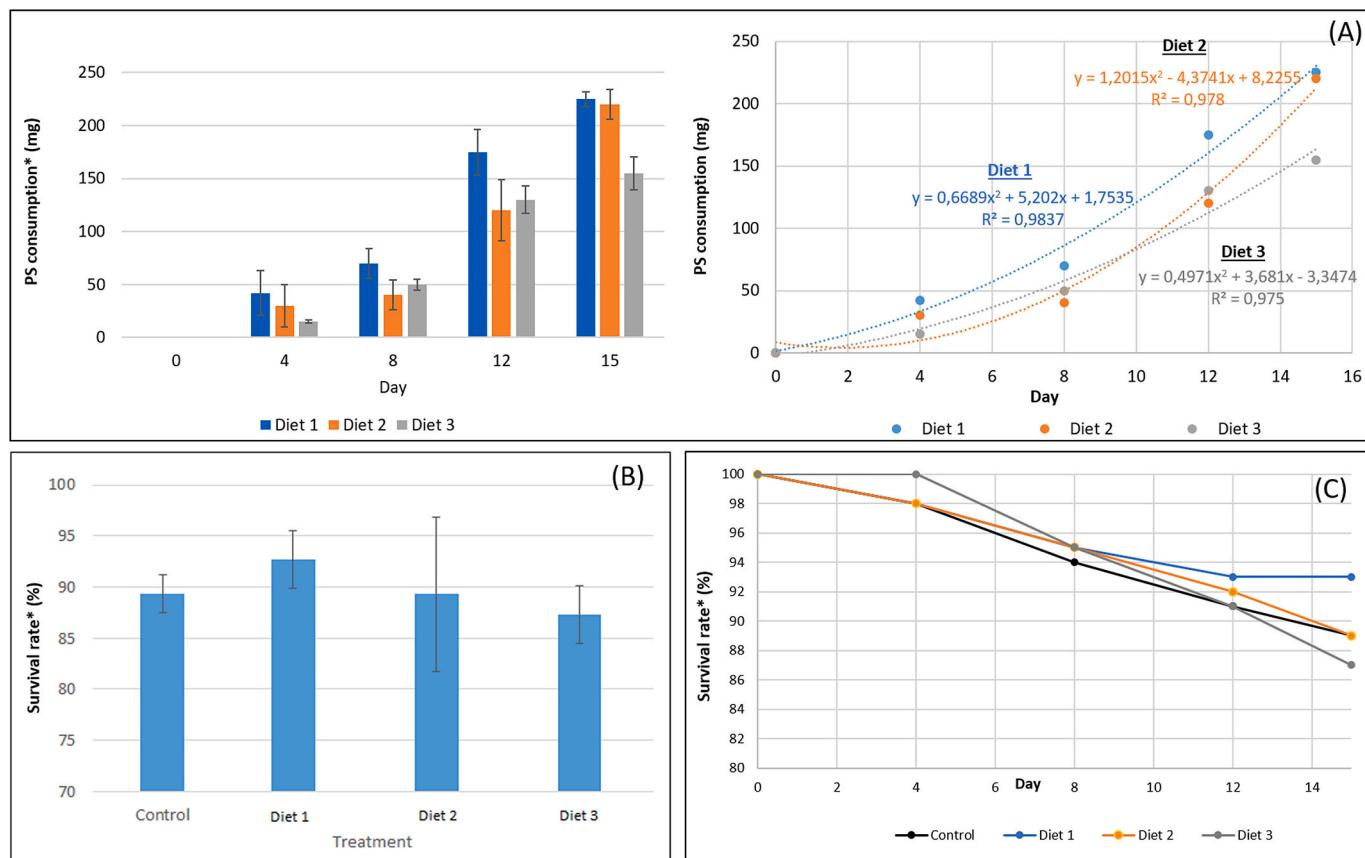
A mass balance on the PS-fed larvae was performed following a gravimetric approach previously reported (Brandon et al., 2018; Yang et al., 2015a). The weights of system inputs (plastic and/or diet containment), and the respective outputs (frass and accumulated larvae weight) were measured. The experimental containers with the mealworm larvae were kept at a constant humidity, and all weights were measured as wet weights, as previously reported (Brandon et al., 2018).

The mass differences originating by the degradation of the PS within the duration of the four experiments were recorded. The mass balance showed that from the initial amounts of PS a significant non-linear degradation rate (*p* < 0.05) is observed among the different sampling days (Fig. 1A and B), fitting to a polynomial fitting (Fig. 1B). An interesting outcome is that the insects in Diet 2 (high PS:bran, water included) required a significant time to start increasing PS consumption, whilst at the end of the growth period, the PS consumption reached identical levels with Diet 1 (low PS:bran). The addition of water within the diet, improved PS consumption, compared to the absence of water. The total degradation at the end of the experiment (15 days) varied according to the different feeding treatments, from 155 mg (PS/barley 20:1; Diet 3) to 225 mg (PS/barley ad libitum 4:1; Diet 1) corresponding to 15.5%–22.5%. Degradation of PS per gram of larvae increased throughout the experimental period from 0.4 mg PS/g larvae/day to 1.7 mg PS/g larvae/day, after 15 days. This may be due to: the observation period by the larvae before consumption of PS; when the larvae makes tunnels in the PS the surfaces increases which may increase the availability of PS; older and bigger larvae prefer PS over younger and smaller larvae. In all experiments, the PS consumption rate was considered sufficient, and in line with previously reported studies (Brandon et al., 2018, 2021; Tsochatzis and Alberto Lopes, 2020; Yang et al., 2021).

At the end of the experiment the survival ratio of the mealworms fed PS was 89.8% ± 4.5%; this ratio was not significantly different (*p* = 0.90) from the control (89.3% ± 1.9%). No significant difference was also observed between any of the PS treatments. The survival rates were similar to values reported by Brandon et al. (2018) and Yang et al. (2021), for the same period of experimentation and feed (PS).

Regarding average weight gain during the experiment, the larvae grew from an average weight of 78 ± 3 mg/larvae to 116 ± 7 mg/larvae. ANOVA revealed that the larvae provided with water (Diet 2) grew more than larvae in the three other treatments including the control (*p* < 0.01) (Fig. 2). The average weight gain of larvae provided with water in the 15 day experimental period was 46 ± 2.1 mg/larvae (Fig. 2A), compared to the 35 ± 1.3 mg/larvae (Fig. 2B) for the other treatments. Diet only showed an effect on growth which indicate that all diets tested can be used for experiments like the present. Diet 3 requires the least handling but if the experimental period is longer than the 15 days, water should be added to ensure the well-being of larvae.

The degradation of PS due to the effect of mealworms was extensively reported in the literature. Brandon et al. indicated that the molecular weights (M_n) of egested polymer residues decreased by 12.8 ± 3.1% in case of the PS-fed Mealworms (Brandon et al., 2018), while identical behaviour was identified by Yang et al. (2018a). Similarly, Yang et al. (2018a) reported a 14.7% decrease rate in the M_n of the PS residues extracted from the frass when compared to raw PS foam. The



* Error bars correspond to the range for the N=2 of each diet.

Fig. 1. Average consumption of PS (mg) (A) throughout the experiment; (B) final survival rates of *T. molitor* larvae within the different applied feeding experiments, and (C) survival rate during the experiment.

M_w decreased, at a rate of 9.2%, reducing from 256.4 to 220.7 kDa, representing a typical depolymerization pattern in case of mealworms larvae (Brandon et al., 2018; Yang et al., 2015a, 2015b, 2018a, 2018b). However, the fate of several regulated plastic additives (styrene monomer) and NIAS (such as styrene oligomers) is still unknown. This needs to be further investigated to highlight chemical degradation products and the potential accumulation into the insects' tissues, frass and to the environment. All the latter shall identify and highlight the importance of this bio-process, while the identified chemical products might reveal the existing risks.

3.2. Untargeted screening of organic content in insects tissue and frass

Apart from the degrading polymer, a screening of the organic content of both the frass and the tissue of larvae was performed. No post-degradation was applied (i.e. lyophilisation) in the case of larvae gut intestine tissue analysis, as reported in a previous study (Tsochatzis and Alberto Lopes, 2020).

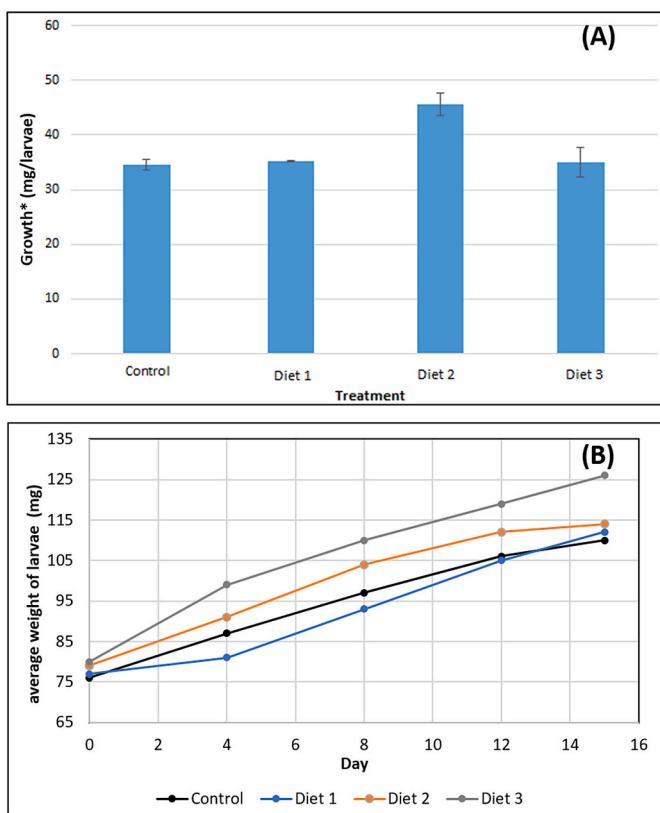
Unlike the frass extraction procedure, the intact gut intestine larvae was extracted, in order to see what was accumulated in terms of organic compounds coming from the polymer. Hence, the potential losses of certain volatiles (i.e. alkanes) or semi-volatile compounds (i.e. esters) was avoided. In both cases (frass and larvae gut intestine tissue) an extraction with CHCl_3 was performed to obtain the lipophilic fraction. The selection of a chlorinated solvent was based on previous studies, where its efficiency in extracting food contact materials (FCM) substances has been demonstrated (Tsochatzis et al., 2020a, 2020b). In this work CHCl_3 was chosen over dichloromethane to also facilitate the extraction of organic components, such as fatty acids and amides (Tsochatzis and Alberto Lopes, 2020).

3.2.1. Analysis of frass

Lou et al. analyzed the frass collected after the biodegradation of PS, where they identified a limited number of PS-oligomers and PS generated compounds (NIAS). Herein, the frass extracted with CHCl_3 also revealed the presence of several organic polymer-related compounds in relatively low amounts (Tsochatzis and Alberto Lopes, 2020). Therefore, the following compounds have been detected and quantified, using a validated analytical method (Tsochatzis et al., 2020b): acetophenone, styrene, 2,4,6-triphenyl-1-hexene, 1,3,5-triphenylcyclohexane, 2,4-diphenyl-1-butene, 1,1-diphenyl-ethylene, *trans*-1,2-diphenylcyclobutane, 1a-phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthalene (1a-tetralin) and 1e-phenyl-4e-(1-phenylethyl)-1,2,3,4-tetrahydronaphthalene (1e-tetralin) (Fig. 3).

Some of these compounds were not detected in a previous study, which included a lyophilisation step that may contributed to the loss of these volatile (Tsochatzis and Alberto Lopes, 2020). Furthermore, 2,4-DTBP was detected in the different commercial PS-material used. This NIAS compound originates from the degradation of Irgafos 168 (FCM 671; tris (2,4-di-*tert*-butylphenyl) phosphate), which is a typical antioxidant additive (European Commission, 2011), for which no-observed adverse-effect levels (NOAEL) has been observed in rats. The identification and quantification of 2,4-DTBP and Irgafos 168 is common when analysing FCM materials (Tsochatzis and Alberto Lopes, 2020; Tsochatzis et al., 2020a, 2020c). A GC chromatogram of the analysis of CHCl_3 extract from frass is provided in the supplementary material (Fig. S1).

Higher content of monomer and oligomers was observed when larvae were fed with PS, especially in case of the barley consumption (Diet 1), we had a higher amount of degrading compounds (Fig. 3). This can be explained by the higher consumption of PS (Fig. 1), while in case



* Error bars correspond to the range for the N= 2 of each diet.

Fig. 2. Average weight gain of the larvae.

of ad libitum barley:PS (20:1; +water; Diet 2) and ad libitum barley:PS (20:1; Diet 3) the degrading substances were significantly lower (Fig. 3), although the PS consumption rates were identical (Fig. 1). However, the fact that in case of Diet 2, after 15 days, the content of monomers/oligomers is lower, whilst the PS consumption is identical with Diet 1 (bran:PS = 4:1), highlights the importance of the water addition to the diet. The latter can be justified by the fact that in case of water absence, the higher amount of bran (ad libitum barley:PS = 20:1; Diet 3) did not increase the consumption of PS nor the mass fraction of the generated styrene and PS oligomers (Fig. 3). A tentative explanation could be that the presence of water can facilitate the growth of gut microbiota that increases PS degradation rates. The latter effect, concerning microbiota, can also justify the delay, in case of Diet 2, to reach higher PS consumption rates after eight days.

Principal component analysis (PCA) was performed (Fig. 4), where it can be seen that for the identified compounds, the samples are clustered in three distinct regions in the PCA score plot ($R^2X_1 = 57.5\%$; $R^2X_2 = 19.8\%$) according to the feeding strategies, indicating that the control diet is fully differentiated from the other diets. Furthermore, Diet 1 (barley:PS = 4:1) is also different from the other two (ad libitum barley:PS = 20:1; +water; Diet 3, and ad libitum barley:PS = 20:1; Diet 2).

Certain acids and amides occur at lower amounts in the frass of PS-fed larvae compared to the control, which maybe attributed to the enzymatic activity (Tsochatzis and Alberto Lopes, 2020). Table 2 indicates that formation of free fatty acids (FFA) in all diets, especially long-chain FFA. These compounds can be generated from enzymatic degradation of triacylglycerols and fatty acid metabolism, from insect's intestinal microbiota (Kong et al., 2019). The formation of long chain FFA indicates digestion and biodegradation of PS, as reported by Lou et al. (2020). The formation of amides, could be also correlated to the presence of enzymatic activity and biocatalytic amide bond formation from the respective FFA (Hara et al., 2018).

The obtained results from the PCA indicated the existence of certain groups of substances (fatty acids, amides) in relation to the presence of styrene monomers and oligomers in the frass (Fig. 3) where it was further explored the potential generated metabolites with the PS biodegradation, by applying univariate statistics. It was also observed the excretion of several long chain alkanes and cycloalkanes generated, which are also indicating enzymatic activity, and it has been reported for the microbiological synthesis of alkanes from acids (e.g. hexadecanoic acid) (Park, 2005). Based on genome data that exist, several alkane hydroxylases and monooxygenases, and ring-hydroxylating dioxygenases among other enzymes, can be involved in oxidation-reduction reactions. The cleavage of C-C bonds and oxidized-reduced aromatic ring compounds, generated from the decomposition of PS, seems plausible as recently reported recently by Hou and Majumber (Hou and Majumber, 2021).

Insects have the capability of storing energy reserves in the form of glycogen and triglycerides in the adipocytes, the main fat body cell, and the metabolism of lipids is crucial for their growth and reproduction, whilst is the source of energy, during non-feeding periods (Arrese and Soulages, 2010). The increase of the cholesterol in the frass can be correlated, to enzymatic activity on these stored lipids repositories of energy, due to metabolic stress, caused by the PS-feeding. Thus, enzymatic degradation and metabolism of fat, releases TAG and cholesterol, where TAG remains in the body (energy), while cholesterol excretes. Furthermore, the released TAG can be further enzymatically hydrolysed to FFA (= energy), and glycerol (see paragraph 3.2.2.), under stimulated metabolic conditions (Arrese and Soulages, 2010).

In addition, comparing the three different diets, the compounds that were significantly differentiating among them, were mainly fatty acids

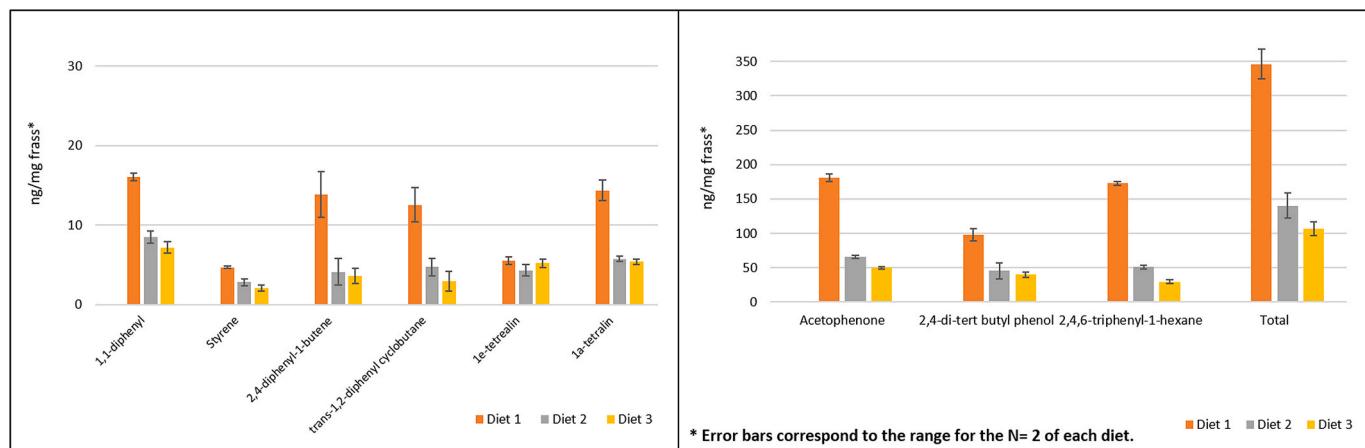


Fig. 3. Mass fractions (ng/mg) of monomer (styrene) and PS oligomers in the frass from PS biodegradation by *Tenebrio molitor* larvae, following different diets.

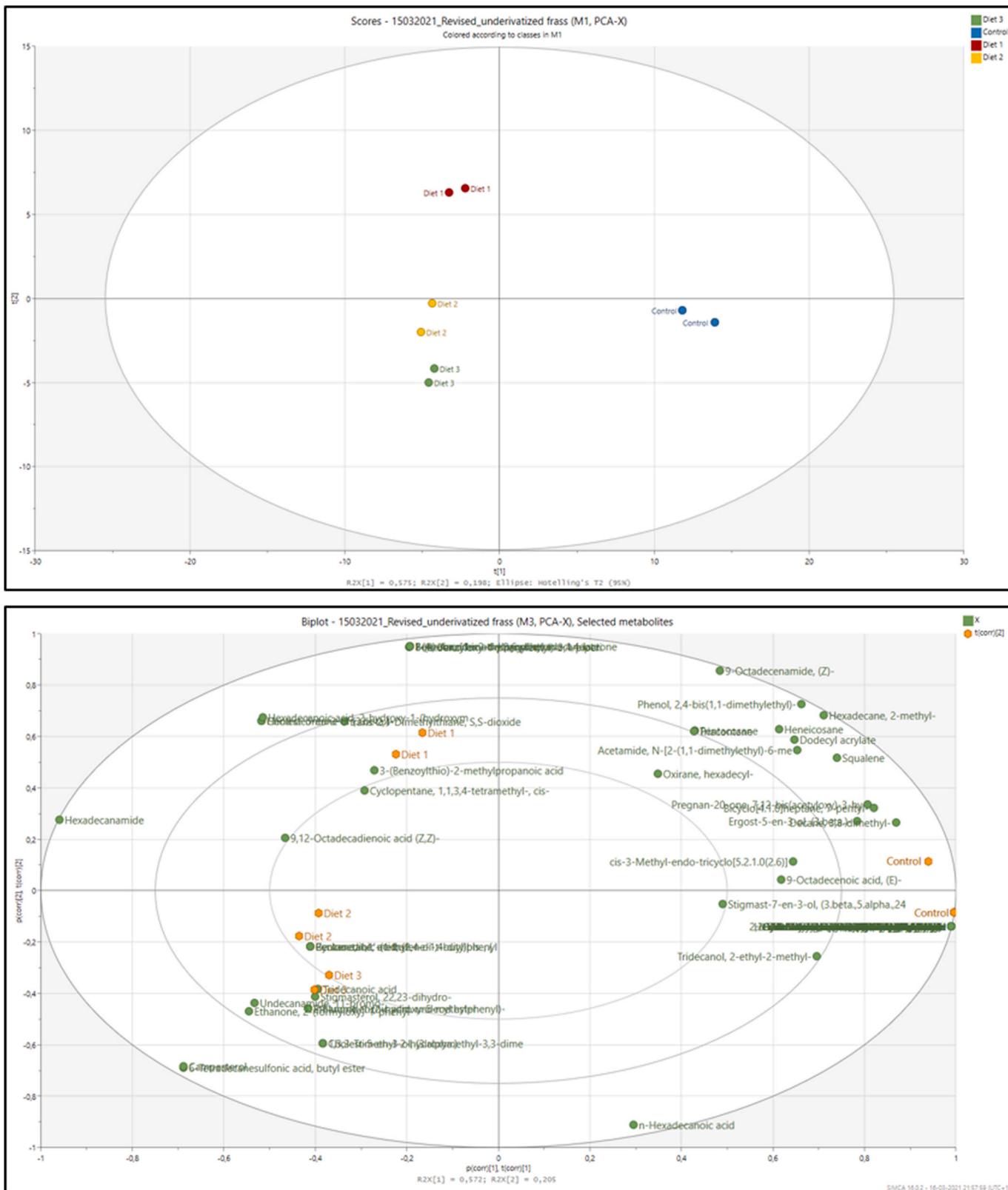


Fig. 4. PCA score plot (A) and biplot (B) of GC-TOF-MS analysis of underivatized chloroform extracts of control and of the applied feed strategies (paragraph 2.3).

(hexadecanoic, octadecanoic, tetradecanoic acid), amides (oleamide, tetradecanamide, hexadecanamide), cholesterol. All these metabolites are indicating the existing enzymatic activity, which is higher (Fig. 4, Fig. S2; PCA biplots) in case of Diet 1, while cholesterol is higher for increased bran:PS (Diets 2 and 3). Diet 1 facilitates the increase of

primary amides, while Diets 2 and 3 elaborated the excretion of fatty acids, probably due to biosynthesis. A comparison of the generated metabolites among the applied diets was performed, in order to identify and explore the potential effect of the PS ratio and the water during the metabolism process of mealworm with PS-containing diets. Fatty acids

Table 2Analytes identified on the CHCl₃ extract of frass (substances and metabolites).

Compound ^a	Diets ^c comparison		
	Diet 1 vs Control	Diet 2 vs Control	Diet 3 vs Control
n-Hexadecanoic acid ^b	down	down	down
Octadecanoic acid ^b	down	down	down
Tetradecanoic acid ^b	down	down	down
9-Octadecenamide, (Z)- ^b	down	down	down
Tetradecanamide ^b	down	down	down
Hexadecanamide ^b	up	up	up
26-Nor-5-cholest-3-beta-ol-25-one	down	down	down
Cholest-5-en-3-ol (3.beta.)-	up	up	up
Cholest-5-en-3-ol, (3.alpha.)- ^b	down	down	down
Cholesta-3,5-diene	down	down	down
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	up	up	up
Hexadecanoic acid, butyl ester	down	down	down
Hexanedioic acid, mono(2-ethylhexyl)ester ^b	down	down	down
n-Pentadecanoic acid, methyl ester ^b	down	down	down
Octadecanoic acid, methyl ester	down	down	down
Pentanedioic acid, (2,4-di-t-butylphenyl)mono-ester	down	down	down
2,3-Dimethyl-3-heptene, (Z)-	down	down	down
4-Heptanone, 2-methyl-	down	down	down
Benzene, (1-methylpropoxy)-	down	down	down
Benzene, 1,3-bis(1,1-dimethyl)- ^b	down	down	down
Benzene, 1,4-bis(phenylthio)-	down	down	down
Cyclohexane, (1,2-dimethylbutyl)-	down	down	down
Cyclopentane, (2-methylbutyl)-	down	down	down
Dodecyl acrylate	down	down	down
Hexadecane	down	down	down
Hexadecane, 2-methyl-	down	down	down
Octane, 2,6,6-trimethyl-	down	down	down
Pentacosane	down	down	down

^a Results significant at 95% CI ($p < 0.05$); fold change ≥ 1.5 .^b Statistical significant only among the 3 applied diets ($p < 0.05$).^c Diets are given in paragraph 2.3.

primary amides, are highly signalling molecules and they are related to the primary fatty acids metabolism (Ezzili et al., 2010), such as we identified in the CHCl₃ extract of the frass.

Furthermore, the observed differences in FA were almost reflecting in the different diets of larvae, thus suggesting selective FA absorption from diet and biosynthetic pathways promoted by the dietary source, as previously reported (Melis et al., 2019). In addition, interestingly we noticed a decrease in the cholesterol content (Cholest-5-en-3-ol) in all diets, where the higher amounts were observed in Diet 3 (Diet 1 > Diet 2 > Diet 3). Cholesterol is a sterol (a combination steroid and alcohol) and a lipid found in the cell membranes of all body tissues, and is involved in lipid metabolism in general (Wishart et al., 2018). As to conclude, main metabolic pathways involved in the PS biodegradation are involving the enzymatic degradation and metabolism of fat, as well as fatty acids metabolism.

3.2.2. Analysis of polar fraction of frass

PCA was performed (Fig. 5), where it can be seen that for the identified compounds, the samples are clustered in two distinct regions in the PCA score plot (R²X1 = 78.0%; R²X2 = 12.3%) according to the feeding strategies. This indicates that the frass content/profile of the control diet larvae differs from those of the other three diets, while their polar fraction do not differ significantly with the different feeding of the insects. Thus, all PS-fed treated insects did not show differentiation among their polar fraction (metabolites) (Fig. 5).

The majority of the metabolites observed to significantly down-regulate ($p < 0.05$) from the control to the PS-fed insects, while three of them upregulated (Table 3). Among them, ethylbis(trimethylsilyl) amine, a silyl derivative of NH₃, that may be excreted by the insects. Similarly to what was observed in Table 2, the oleic acid content

increased in the frass of the PS-fed mealworms, probably due to lipid metabolism (Arrese and Soulages, 2010) A GC chromatogram of the analysis of CHCl₃ extract from frass is provided in the supplementary material (Fig. S4).

The variation of glycerol may indicate that mealworms were under metabolic stress. Glycerol is synthesis precursor of triacylglycerols (TAG) and phospholipids, that an organism can use to store amounts of energy (Arrese and Soulages, 2010). However, enzymatic hydrolysis of TAG and phospholipids can occur, in case of reduced amount of available food or stress, like in case of the PS-fed larvae. Enzymatic reactions can be justified from the increase of excreted glycerol in frass (Table 3) and in combination with the formation of fatty acid content stored in gut intestine tissue (Table 4). The latter also supports the existence of enzymatic reactions within larvae' gut intestine. Moreover, it has been already reported, that TAG can be hydrolysed (by enzymes) in the intestine to free fatty acids (Pan and Hussain, 2012) and glycerol by adipose triglyceride lipase (AGTL) (Zechner et al., 2005).

In addition, in case of the polar fraction, PCA analysis without the control, in order to remove the main cause of variance of PC1, did not highlight any differences among the components of the variance (Fig. S3). Moreover, univariate analysis among the diets revealed a statistical significant decrease only for mannose (1-way ANOVA). Thus, among the three studied diets we observed only a decrease in mannose. Mannose is an important monosaccharide for protein glycosylation in mammals and is considered an non-efficient cellular energy source. Mannose is an abundant monosaccharide in N-linked glycosylation, which is a post-translational modification of proteins. Glycosylation is an important and highly regulated mechanism of secondary protein synthesis within cells (Sharma et al., 2014, 2018). The latter, can also be correlated with the observed enzymatic activity that resulted in higher PS degradation rates (Fig. 1). Finally, it cannot be underestimated that mannose can be converted into glucose, via glycan biosynthesis, during catabolism that can justify also the higher survival rates, which is highly associated with the increased enzymatic activity taking place, during PS biodegradation (Reily et al., 2019; Sharma et al., 2014). Regarding the polar fraction of the frass, we identified that glycolysis and fatty acid metabolism are the main metabolic pathways involved.

3.2.3. Analysis of larvae' tissue organic extract

The analysis of the biomass revealed no residues of any PS monomers or oligomers (Tsochatzis et al., 2020b), nor other regulated compounds and non-regulated (NIAS), coming from the polymer. However, several biologically active compounds have been identified, among several alkanes or bioactive components (acids, vitamins and amides), as presented in Table 4.

The identification of the compounds is in agreement with previously studies in biomass, although in this case samples were lyophilized (Tsochatzis and Alberto Lopes, 2020). Since the gut intestine samples were not lyophilized, a larger number of compounds were identified (Table 4). All identified compounds were bioactive substances (i.e. acids, amides, ester, squalene, and cholesterol) and no detectable accumulation of FCM substances was observed (i.e. monomers, oligomers). These volatile compounds may have been excreted by the insects, resulting in low mass fractions detected (Tsochatzis et al., 2020b). A typical GC chromatogram is provided in supplementary material (Fig. S5).

Similar observations were reported by Brandon et al. for mealworms fed with accumulating flame retardants, indicating a capability of larvae to excrete organic compounds from their body. A recent work from Brandon et al. reported that a typical flame retardant (hexabromocyclododecane, HBCD) consumed by the mealworms was excreted via the frass within 24 h, with very low amounts of the ingested HBCD being retained in the mealworm body tissue. Hence, it can be concluded that highly toxic and lipophilic compounds, may not be retained or accumulated in the mealworms after PS biodegradation (Brandon et al., 2019).

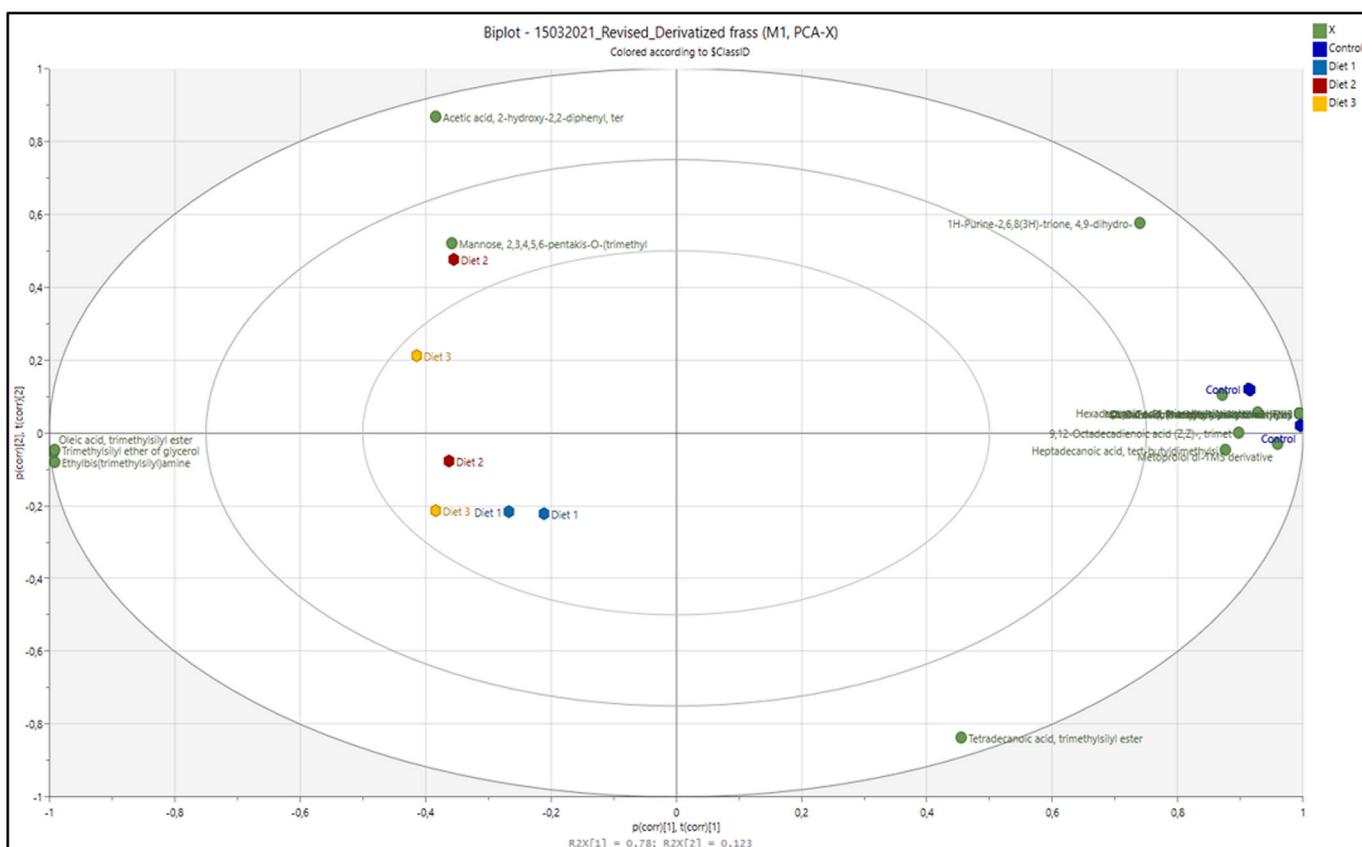
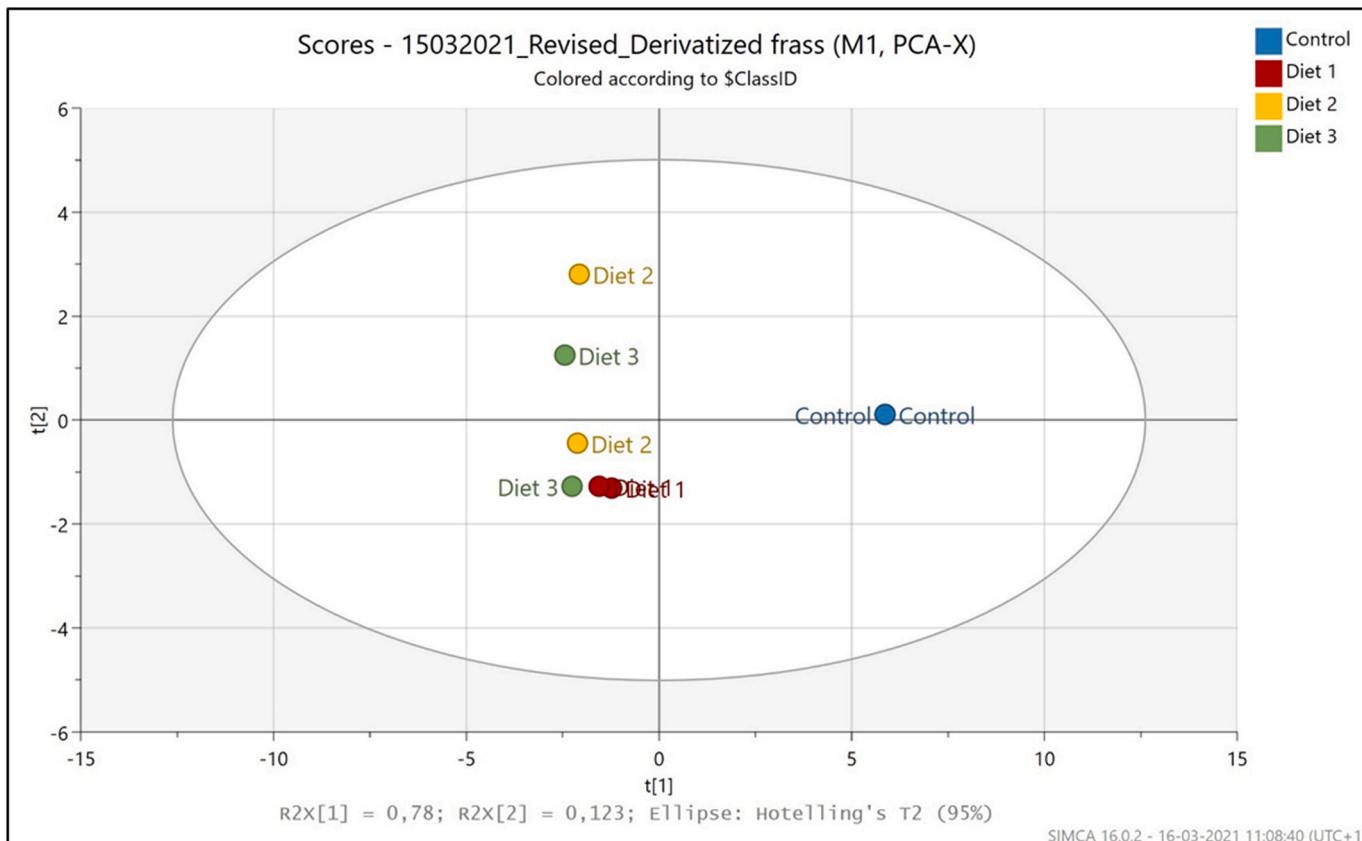


Fig. 5. PCA score plot (A) and biplot (B) of GC-TOF-MS analysis profiles of the MeOH:H₂O (1:1 v/v) extracts of frass (after derivatization) of control and of the applied feed strategies (paragraph 2.3).

Table 3

Metabolites identified in the analyzed MeOH:H₂O extracts after derivatization with MSTFA/1% TMCS.

Compound ^a	Diets ^c comparison		
	Diet 1 vs Control	Diet 2 vs Control	Diet 3 vs Control
Trimethylsilyl ether of glycerol	up down	up down	up down
Phenylethanolamine triTMS	up down	up down	up down
Oleic acid, trimethylsilyl ester	up down	up down	up down
Octadecanoic acid, tert-butyltrimethylsilyl ester	down	down	down
N,N,O-Tris(trimethylsilyl)-4-aminomethylcyclohexane carboxylic acid	down	down	down
Mannose, 2,3,4,5,6-pentakis-O-(trimethylsilyl)-, D- ^b	down	down	down
Hexadecanoic acid, trimethylsilyl ester	down	down	down
Hexadecanoic acid, 2,3-bis[(trimethylsilyl)oxyl]propyl ester	down	down	down
Heptadecanoic acid, tert-butyltrimethylsilyl ester	down	down	down
Ethylbis(trimethylsilyl)amine	up down	up down	up down
Acetic acid, (1-methyl-1H-indol-3-yl)ester	down	down	down
9,12-Octadecadienoic acid (Z,Z)-, trimethylsilyl ester	down	down	down
11-trans-Octadecenoic acid, trimethylsilyl ester	down	down	down

^a Results significant at 95% CI ($p < 0.05$); fold change ≥ 1.5 .

^b Statistical significant only among the 3 applied diets ($p < 0.05$).

^c Diets are given in paragraph 2.3 (Diet 1 = Control).

Table 4

Identified compounds in the CHCl₃ extract of gut intestine content of *Tenebrio molitor* larvae, following PS bio-degradation.

Analyte ^a	Molecular formula	Type
Pentanoic acid, methyl ester	C ₆ H ₁₂ O ₂	Acid
Octanamide, N-(2-hydroxyethyl)-	C ₁₀ H ₂₁ NO ₂	Ester
Decanamide, N-(2-hydroxyethyl)-	C ₁₂ H ₂₅ NO ₂	Amide
Butanamide, N-cyclohexylmethyl-1-propyl-	C ₁₄ H ₂₉ N	Amine
Methyl tetradecanoate	C ₁₅ H ₃₀ O ₂	Ester
Oxalic acid, 6-ethyloct-3-yl isobutyl ester	C ₁₆ H ₃₀ O ₄	Ester
9-Hexadecenoic acid, methyl ester, (Z)-	C ₁₇ H ₃₂ O ₂	Ester
<u>Hexadecanoic acid, methyl ester*</u>	C ₁₇ H ₃₄ O ₂	Ester
n-Hexadecanoic acid	C ₁₆ H ₃₂ O ₂	Ester
Hexadecanamide	C ₁₆ H ₃₃ NO	Amide
9-octadecenoic acid, (Z)-	C ₁₈ H ₃₄ O ₂	Acid
9-Octadecenamide, (Z)-	C ₁₈ H ₃₅ NO	Amide
13-octadecanal, (Z)-	C ₁₈ H ₃₄ O	Aldehyde
Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	Acid
Hexadecanoic acid, 2-hydroxy-1-(hydroxy-methyl)ethyl ester	C ₁₉ H ₃₈ O ₄	Ester
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	C ₁₉ H ₃₄ O ₂	Ester
<u>9-Octadecenoic acid, methyl ester, (E)-*</u>	C ₁₉ H ₃₆ O ₂	Ester
Octadecanoic acid, methyl ester	C ₁₉ H ₃₈ O ₂	Ester
Cholest-5-en-3-ol (3 beta.)	C ₂₇ H ₄₆ O	Cholesterol
<u>Cholecalciferol*</u>	C ₂₇ H ₄₄ O	Vitamin
Squalene	C ₃₀ H ₅₀	Triterpene

^a Only the organic compounds with an asterisk and underlined are presenting significant difference at a level of 95% (ANOVA, Tukey test).

4. Conclusions

This work presents an in-depth study of the generated, formed and potentially accumulated organic compounds, and polymer-based chemical substances, during biodegradation of PS with insects' larvae. A study of the biodegradation of PS with mealworms larvae was performed, applying different feeding diets, which did not have an effect on the degradation of PS and on the survival rate of PS-fed larvae. The untargeted screening of the larvae revealed no presences of plastic

polymer-generated compounds (monomers, oligomers, NIAS), indicating no bioaccumulation. Several of these compounds were identified and quantified in the frass, but at a very low content (mass fraction). In the case of frass, a GC-TOF-MS metabolomics study has been performed, in which the lipophilic and polar fraction were analyzed. It was identified that several bioactive compounds, such as long chain fatty acids, esters and amides, were generated, and probably related to the enzymatic activity taking place in their gut intestine, during bio-degradation of the plastic. The latter is also suggested by the fact that several long chain acids, besides hydrocarbons, were present due to the degradation of the PS. Finally, in all studied diets, together with the consumption of PS, the insects undergo through metabolic stress, due to the consumption of plastic, in comparison to their typical diet.

Credit author statement

Emmanouil Tsochatzis: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Project administration. **Ida Elisabeth Bergreen:** Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Writing – review & editing. **Jan Værum Nørgaard:** Writing – original draft, Writing – review & editing. **Georgios Theodoridis:** Writing – review & editing, Project administration. **Trine Kastrup Dalsgaard:** Writing – review & editing, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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