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Changes in the level of consolidation of the fatty acid profile of *Hermetia illucens* larvae grown on a substrate contaminated with heavy metals

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ABSTRACT

We conducted a comparative investigation to examine the alterations in the composition and content of the fatty acid complex in the larvae of the Black Lion fly (*Hermetia illucens*) as they were reared under different concentrations and combinations of heavy metals. The use of the method of mass spectrometric analysis of the obtained biomass showed that linoleic, lauric and oleic fatty acids predominated in the composition of the larvae. The use of the mathematical method of fractal analysis based on the data on the profile distribution of fatty acid components in the insect body according to the experimental variants showed that samples with metal concentrations of 20 mg of cadmium, 800 mg of cobalt and Mix (200 mg of copper, 20 mg cadmium, 200 mg cobalt, 20 mg aluminium and 50 mg lead) per kilogram of dry food. The variation in the values of the indices of the biosystemic consolidation of acids, based on the conversion to their molar masses, ranged from 0.41 to 0.82.

Keywords: *Hermetia illucens*, heavy metal, fatty acid, fractal, bioconsolidation index

INTRODUCTION

One of the topical issues in the development of society at the moment is the world's hunger problem. In 2050 the world population is expected to grow from 7 to 10 billion. However, out of 800 million people who are already malnourished, 650 million are in developing third-world countries. Overall, two billion people suffer from nutritional deficiencies. The problem of producing and using cheap feed additives is also felt in the livestock industry when grazing and keeping livestock. One of the possible solutions here is to introduce non-traditional sources of fodder protein into the diet of humans and animals, which can be larval forms of edible insects.

The most frequently produced species of forage insects is the Black soldier fly (*Hermetia illucens*), from the lionfish family (*Stratiomyia chamaeleon*) **[1]**, **[2]**. Individuals of this species in natural conditions are mainly distributed in tropical and subtropical countries. In Russia, the insect is called the "Black Lion", abroad - "Black Soldier" **[3]**. The first experiments on the cultivation of *Hermetia illucens* began in the 90s of the twentieth century in search of an effective way to utilize organic waste by converting it into biomass rich in proteins and fats. The biomass obtained from their larvae or pupae is a rich source of protein with essential amino acids (in particular, arginine, histidine, leucine and isoleucine, lysine, phenylalanine, tyrosine, valine and others), fatty acids (lauric, myristic, palmitic, stearic, oleic, linoleic acid, etc.), vitamins, macro- and microelements, and other biologically active substances **[4]**, **[5]**, **[6]**. For a long time, these ingredients were part of the food consumed by only most Southeast Asia. However, according to the FAO, in our time, dried insects are no longer an exotic food for Western European countries, including the Russian Federation. They are gradually becoming an ordinary source of high protein content for humans, without allergens and toxins **[6]**, **[7]**.

The use of *Hermetia illucens* larvae has so far gained great popularity in animal husbandry **[8]**, **[9]**. The larvae are also used in fish farming, especially aquaculture **[10]**, **[11]**, **[12]**. It is known that large areas of agricultural land with grassy vegetation are required for the production of animal protein. The feeding process here is very long and laborious. Therefore, dried insect biomass can serve as an excellent complementary feed supplement to existing animal food sources or as a promising alternative to replace their traditional food base completely. According to haematological and biochemical studies, this product did not cause serious deviations in the indicators of enzymatic activity in the blood and liver and violations of the main indicators of protein, lipid, and carbohydrate metabolism in animals **[5]**. Among the European industrial companies producing large-scale feed protein derived from Black Lion fly larvae, the following companies can be distinguished: Hermetia Baruth GmbH (Germany), Agri Protein Technologies (South Africa), Enterra Feed Corporation (Canada), Protix (Netherlands), Bühler Insect Technology Solutions (Switzerland) **[13]**. In the Russian Federation, this direction's representatives are the Biogenesis company. Because *Hermetia illucens* larvae are polyphagous, companies feed them on substrates derived from various waste sources.

It should be noted that in the European Union there are general principles of food and feed safety - the "General Food Law" and the "Hygiene Package", by which the use of manure and food waste for fattening is prohibited **[14]**. In Russia, however, the ecological assessment of plant and animal substrates used for feeding larvae may often differ from the generally recognized level (standard) of European quality. A particular problem here is the possible contamination of substrates with heavy metals. Laboratory experiments conducted with insect larvae reared using standardized chicken feed supplemented with various concentrations of cadmium (2-50 mg/kg), lead (5-125 mg/kg), and zinc (100-2000 mg/kg) showed that the bioaccumulation factor (the ratio of the amount of metal in the body compared to the amount of metal in the substrate) varied considerably. It was revealed that in insect prepupae, the value of the factor for cadmium was 2.32-2.94, for lead - 0.25-0.74. For zinc, the value of the factor decreased with an increase in its concentration in the substrate and ranged from 0.97 to 0.39 **[15]**. Based on the results of the work, it was also concluded that none of the three heavy metals significantly affected the life cycle determinants (pupal weight, development time, sex ratio) of larvae.

It is known that, compared with other insects, Black Lion larvae contain a large amount of fat, especially in the form of saturated fatty acids with an average carbon chain length **[5]**, **[16]**, **[17]**, **[18]**, **[25]**, **[26]**. However, more studies on the effect of their synthesis on the mechanism of regulation and adaptation of the insect to metal-induced stress is needed. This work aimed to study the composition and level of organization of fatty acids in the larvae of *Hermetia illucens*, grown under conditions of contamination of the nutrient substrate with heavy metals.

Scientific Hypothesis

Insects can be a new protein source in feed and food production upon risk assessment. But today, the presence of the availability of accurate data raises the suspicion of the danger of the risk associated with the presence of toxic elements in stable biomasses of insects. The aim of our work was to investigate the alleged detection of metals in the larvae of one of the most popular objects shortly for proposal as a source of protein - the Black soldier fly (*Hermetia illucens*). We are seeing an increase in the structure of acidic fat organization for permanent inclusion in the trophic food chain.

MATERIAL AND METHODOLOGY

Insect

The eggs of the black soldier fly were selected from the brood colony of insects kept in the insectarium of the laboratory for the structural processing of biomass of the All-Russian Research Institute of Food Additives (St. Petersburg, Russia).

Samples

Before the current experiment, newborn larvae were grown in a light chamber (insectarium), 60×50×50 cm in size. The front wall of the chamber was a glass window, and the rest were made of chipboard. The temperature in the working area for breeding flies was maintained at 30 ±2 °C, relative humidity was 65 ±5% Testo 174H (Testo SE & Co. KGaA, Germany) (Figure 1).

Figure 1 Insectarium: development of the authors.

Small ventilation holes were made on the cover of the insectarium. On the ceiling of the incubator, two fluorescent lamps with a power of 30 watts each were installed, connected to the network. The colour temperature of both lamps was 6500 K. The length of the day was 12 hours.

Feed

Initially, the larvae were fed wheat bran moistened with deionized water (water content 75%), then switched to standardized chicken feed (PC-2 compound feed, Gatchinsky Feed Mill JSC, Leningrad region, Russia).

Chemicals

To contaminate the substrate with heavy metals, the chicken feed was thoroughly mixed with the appropriate volume of the salt solution of the toxicant until the desired concentration was reached. Copper salts ($CuSO_4$) were used in the experiment – 200 and 800 mg/kg of dry food, designated as Cu 200 and Cu 800; cadmium ($CdSO_4$) – 20 and 80 mg/kg of dry food, designated as Cd 20 and Cd 80; cobalt ($CoCl_2$) – 200 and 800 mg/kg of dry food, designated as Co 200 and Co 800; aluminium ($AlCl_3x6H_20$) – 20 and 80 mg/kg of dry food, designated as Al 20 and Al 80 and lead (PbOAc) – 50 and 200 mg/kg of dry food, designated as Pb 50 and Pb 200 (Sigma-Aldrich Pty Ltd., Darmstadt, Germany). Next, two special nutrient substrates were prepared to contain mixtures of metals with a minimum (Cu 200, Cd 20, Co 200, Al 20, Pb 50) – mix 200, and maximum (Cu 800, Cd 80, Co 800, Al 80, Pb 200) – mix 800 concentrations of each element. The selected metal concentrations for research are in the range of metal concentrations found in contaminated manure or commercial organic fertilizers **[19]**, **[20]**, **[27]**. In addition, the same volume of deionized water without adding heavy metals was mixed with chicken feed as a control (40, 60 and 80 mg/kg).

Instruments

The content of fatty acids in insect biomass was determined using gas chromatography with mass spectrometric detection, a Varian 450-GC gas chromatograph with CP-Wax 58 FFAP CB, 50 m x 0.32 mm x 0.50 µm column coupled with a Varian 240-MS mass spectrometric detector (Varian, USA).

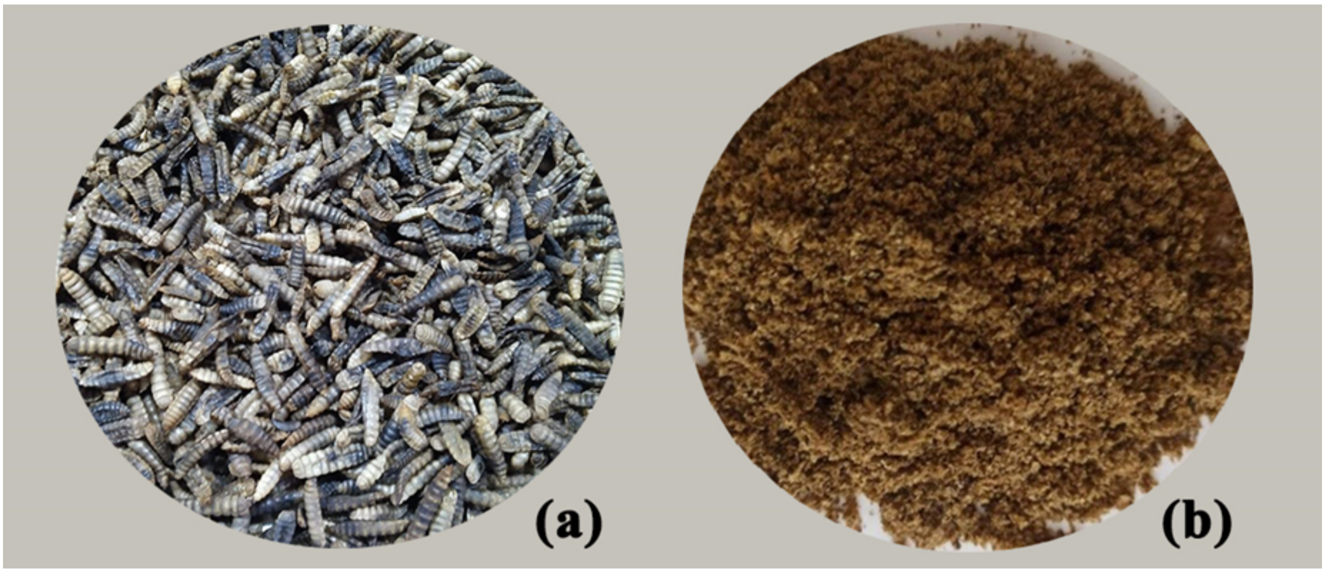

Figure 2 Dried larva of the Black soldier fly *Hermetia illucens*: (a) – whole; (b) – ground.

Analysis conditions

Carrier gas flow (Helium) rate 1 ml/min, injector temperature 250 °C, split 1:15, the start of chromatogram registration from 9 minutes. The temperature program is presented in Table 1.

Table 1 Temperature analysis program.

Temperature, °C	Heating rate, °C/min	Time at a given temperature, min	Total time, min
50	-	4	4
190	6	15	42.33
250	4	10	67.33

Description of the Experiment

Sample preparation: Each experiment was carried out in triplicate. For each repetition, 150 larvae were collected. Insects were placed in plastic containers (12×10×8 cm) located inside the insectarium and fed daily with wheat bran with or without the addition of metals. Feeding was stopped after 8 days. At the end of the experiment, the larvae and their faeces were separated by manual sieving using 3 mm sieves and then ground in a laboratory mill IKA A11 basic (IKA-Werke GmbH & Co. KG, Germany) for further analysis (Figure 2).

The resulting ground samples were evaporated to dryness in JEIO TECH vacuum oven OV-12 with cold trap bath CTB-10 coupled with Woosung Vacuum Co vacuum pump MVP 6 600 µl of a 15% sulfuric acid solution in methanol was added to the evaporated samples, and 600 µl of chloroform was added. Eppendorf was carefully sealed with Parafilm and placed in a heater for 1 hour at 65 °C. After the sample was cooled, 200 µl of deionized water was added and thoroughly mixed. The organic layer was analysed and injected directly into the chromatograph at 1 µl using a CPAL autosampler and a 10 µl Hamilton chromatographic syringe.

Statistical Analysis

The experimental data were subjected to statistical analysis using the R program (version 4.1.0, https://cran.r-project.org/bin/windows/base/) for Windows **[21]**, **[22]**. Statistical processing of the results of determining the profile of fatty acids in the samples was carried out using ANOVA analysis of variance and Fisher's test (F). Differences were considered significant, and the presence of a relationship between the indicators was recognized at a probability level not exceeding 0.05. Fractal portraits and indices of biosystematic determination (IF) of fatty acids were calculated based on the mathematical algorithm incorporated in the original computer program **[23]**.

RESULTS AND DISCUSSION

Biochemical tests showed that the composition of the larvae was dominated by linoleic (C18:2n-6), oleic (C18:1n-9) and lauric (C12:0) fatty acids $p < 0.01$ (Table 2). The latter is known for its antimicrobial activity against pathogenic Gram-positive bacteria **[18]**, **[24]**. For example, it has been shown that lauric acid can modulate gut health in humans **[28]** and mice **[29]**. Lauric Acid is an inhibitor of *Clostridium* - a gram-positive spore-forming anaerobic pathogen of the gastrointestinal tract **[30]**. Lauric acid also showed the best result in terms of inhibition of multiplication of the Junin arenavirus (JUNV) **[31]**.

Table 2 Average values of the content of fatty acids in the sample, %.

Variant	1	2	3	4	5	6
g/mol	172.26	200.3	228.37	226.36	242.4	256.43
Control	1.50 ±0.07	22.89 ±0.22	9.61 ±0.31	0.34 ±0.01	BDL	12.71 ±0.52
Al 20	1.75 ±0.09	24.76 ±0.91	7.93 ±0.29	0.38 ±0.02	0.44 ±0.01	9.12 ±0.36
Al 80	1.57 ±0.04	22.98 ±0.42	7.92 ±0.13	0.50 ±0.01	0.31 ±0.01	10.13 ±0.13
Cd 20	1.93 ±0.04	24.88 ±0.81	7.71 ±0.02	0.40 ±0.01	0.33 ±0.01	10.21 ±0.09
Cd 80	1.50 ±0.10	21.92 ±2.22	7.99 ±0.77	0.25 ±0.00	0.32 ±0.01	12.67 ±1.04
Co 200	2.29 ±0.05	23.62 ±0.38	7.82 ±0.11	0.22 ±0.01	0.32 ±0.01	9.40 ±1.56
Co 800	1.61 ±0.03	26.82 ±0.53	9.53 ±0.22	0.26 ±0.02	0.52 ±0.08	10.80 ±0.18
Cu 200	1.89 ±0.07	27.71 ±0.69	8.59 ±0.08	0.62 ±0.01	0.47 ±0.01	9.20 ±0.17
Cu 800	1.79 ±0.08	27.05 ±0.77	8.38 ±0.15	0.46 ±0.05	0.28 ±0.01	9.37 ±0.10
Pb 200	1.50 ±0.07	23.99 ±0.52	7.70 ±0.20	0.40 ±0.06	0.22 ±0.01	9.68 ±0.22
Pb 50	2.66 ±0.07	27.98 ±0.47	7.69 ±0.31	0.35 ±0.02	0.50 ±0.13	9.44 ±0.24
Mix 200	1.11 ±0.01	17.89 ±0.01	6.41 ±0.01	0.31 ±0.01	0.40 ±0.01	10.71 ±0.01
Mix 800	1.28 ±0.01	19.00 ±0.01	6.74 ±0.01	0.31 ±0.01	0.20 ±0.01	11.06 ±0.01
Variant	**7**	**8**	**9**	**10**	**11**	**12**
g/mol	254.41	270.45	284.48	282.46	280.45	278.44
Control	3.73 ±0.19	BDL	6.18 ±0.11	17.54 ±0.24	23.43 ±0.16	2.29 ±0.18
Al 20	3.23 ±0.09	0.45 ±0.01	6.6 ±0.27	14.59 ±1.88	27.57 ±0.68	3.17 ±0.10
Al 80	3.97 ±0.04	BDL	5.94 ±0.07	16.46 ±0.11	27.30 ±0.34	2.70 ±0.21
Cd 20	4.32 ±0.12	BDL	5.61 ±0.65	17.09 ±0.37	24.99 ±0.77	2.46 ±0.04
Cd 80	4.01 ±0.21	0.63 ±0.01	6.14 ±0.84	17.15 ±1.26	25.47 ±1.14	2.49 ±0.35
Co 200	3.72 ±0.12	0.36 ±0.03	7.32 ±0.29	16.23 ±0.52	25.96 ±0.79	2.78 ±0.14
Co 800	3.43 ±0.19	0.50 ±0.02	5.81 ±0.20	15.20 ±0.54	23.04 ±0.43	2.36 ±0.08
Cu 200	4.59 ±0.27	0.34 ±0.07	4.37 ±0.13	14.52 ±0.12	24.48 ±1.31	2.93 ±0.05
Cu 800	4.63 ±0.17	0.48 ±0.02	4.80 ±0.27	15.93 ±0.16	24.26 ±0.39	2.40 ±0.33
Pb 200	4.09 ±0.22	0.58 ±0.01	6.88 ±0.31	17.10 ±0.46	25.53 ±0.45	2.70 ±0.08
Pb 50	3.39 ±0.29	0.50 ±0.01	3.93 ±1.10	14.03 ±0.27	26.66 ±1.05	2.90 ±0.06
Mix 200	3.23 ±0.01	0.50 ±0.01	7.00 ±0.01	16.77 ±0.01	32.18 ±0.01	3.49 ±0.01
Mix 800	3.45 ±0.01	0.45 ±0.01	6.49 ±0.01	15.98 ±0.01	31.33 ±0.01	3.37 ±0.01

Note: Addendum: BDL* – below the detection limit. 1 – Capric acid; 2 – Lauric acid; 3 – Myristic acid; 4 – Myristoleic acid; 5 – Pentadecanoic acid; 6 – Palmitic acid; 7 – Palmitoleic acid; 8 – Heptadecanoic acid; 9 – Stearic acid; 10 – Oleic acid; 11 – Linoleic acid; 12 – Gamma-linolenic acid.

The initial values of fatty acids from percentages were converted into molar masses to approximate the obtained data and construct fractal portraits.

The visual difference between the data in the quantitative composition of the acid profiles for different experimentexperiment variants is shown on the heat map (Figure 3).

Further, based on these data, two-dimensional coordinate planes were constructed, on which point represents each acid represents each acid represents each acid represents each acid with the x-coordinate equal to the fractional part of $\log_2$ (ei/emax), and the y-coordinate equal to $\log_2$ (ei/emax), where ei, emax is an acid with a nominal, established serial number (i) and with a maximum intensity of synthesis. The entire field of the fractal portrait is divided into rectangular sectors by horizontal and vertical lines. The sectors highlighted in color contain dotted images of fatty acids inside. Further, on the basis of a decreasing power series of fatty acid indicators **[32]** for the selected sectors, IF calculations were made under the influence of metal-induced stress **[33]**, **[34]**, **[35]**. To calculate them, it suffices to count the total number of selected sectors in fractal portraits (N0) and the number of selected sectors (NF) located in the columns and diagonals of the fractal portrait grid and uniting groups consisting of three or more sectors. As a result, all IF calculations are reduced to the formula (1):

$$IF = NF/N0 \quad (1)$$

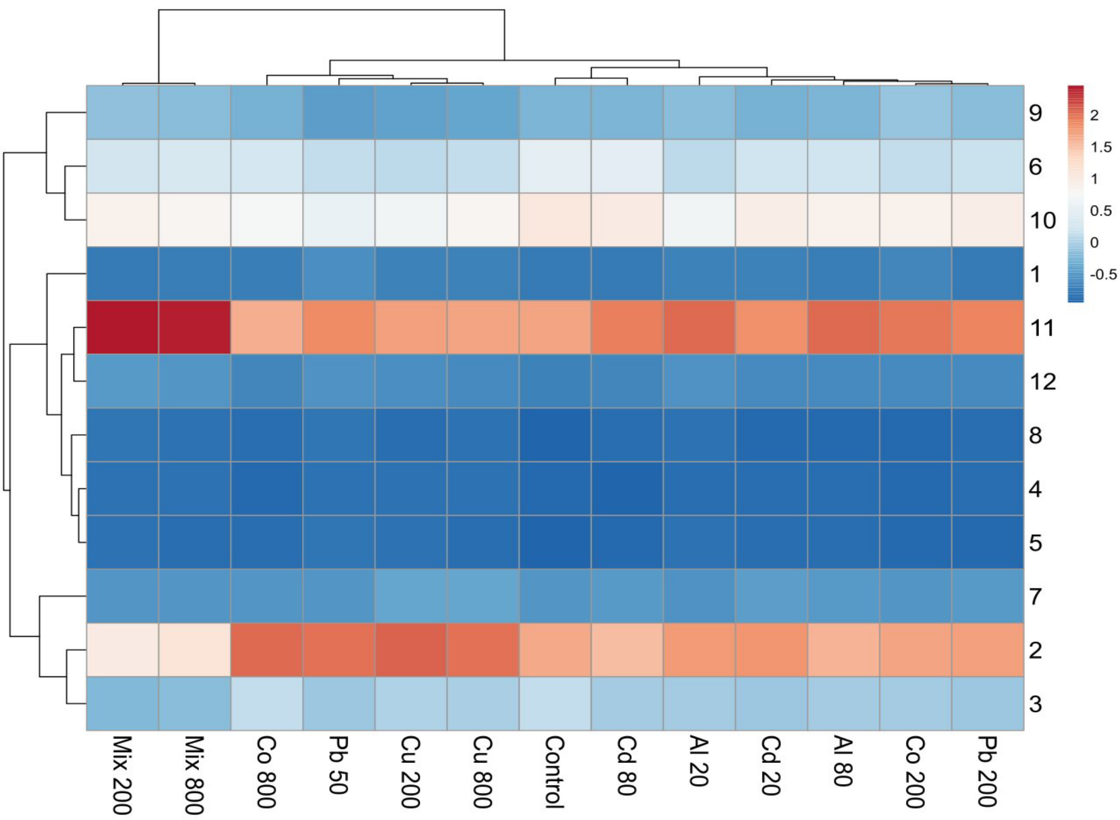

Figure 3 Heat map of changes in the composition of fatty acids in the larvae of the black soldier fly *Hermetia illucens* under metal-induced stress. Red indicates a higher concentration; blue indicates a lower concentration of each component. 1 – Capric acid; 2 – Lauric acid; 3 – Myristic acid; 4 – Myristoleic acid; 5 – Pentadecanoic acid; 6 – Palmitic acid; 7 – Palmitoleic acid; 8 – Heptadecanoic acid; 9 – Stearic acid; 10 – Oleic acid; 11 – Linoleic acid; 12 – Gamma-linolenic acid.

Also, the Simpson and Shannon indices were calculated according to the data obtained **[36]**, **[37]**, **[38]**, most commonly used by ecologists to measure the diversity of communities **[39]**. The results of all calculations are shown in Table 3.

Table 3 Average indices of various indices of systemic organization of fatty acids in insect larvae of *Hermetia illucens.*

No. p/n	Option	IF	Index Simpson	Index Shannon
1	Control	0.51 ±0.05	0.08 ±0.01	0.85 ±0.01
2	Al 20	0.56 ±0.06	0.11 ±0.01	0.80 ±0.01
3	Al 80	0.67 ±0.02	0.10 ±0.01	0.81 ±0.01
4	Cd 20	0.71 ±0.01	0.10 ±0.01	0.80 ±0.01
5	Cd 80	0.53 ±0.12	0.09 ±0.01	0.82 ±0.01
6	Co 200	0.55 ±0.03	0.10 ±0.01	0.80 ±0.01
7	Co 800	0.71 ±0.07	0.11 ±0.01	0.78 ±0.01
8	Cu 200	0.68 ±0.04	0.12 ±0.01	0.78 ±0.02
9	Cu 800	0.66 ±0.10	0.12 ±0.01	0.77 ±0.01
10	Pb 200	0.68 ±0.05	0.10 ±0.01	0.81 ±0.01
11	Pb 50	0.65 ±0.02	0.12 ±0.01	0.77 ±0.01
12	Mix 200	0.72 ±0.01	0.11 ±0.01	0.78 ±0.01
13	Mix 800	0.53 ±0.01	0.11 ±0.01	0.78 ±0.01

As we can see, the Cd 20, Co 800 and Mix 200 samples had the greatest impact on the change in the biochemical fatty acid composition of the larvae. The overall variation in IF values ranged from 0.41 to 0.82. Their fractal portraits and chromatograms are shown in Figure 4.

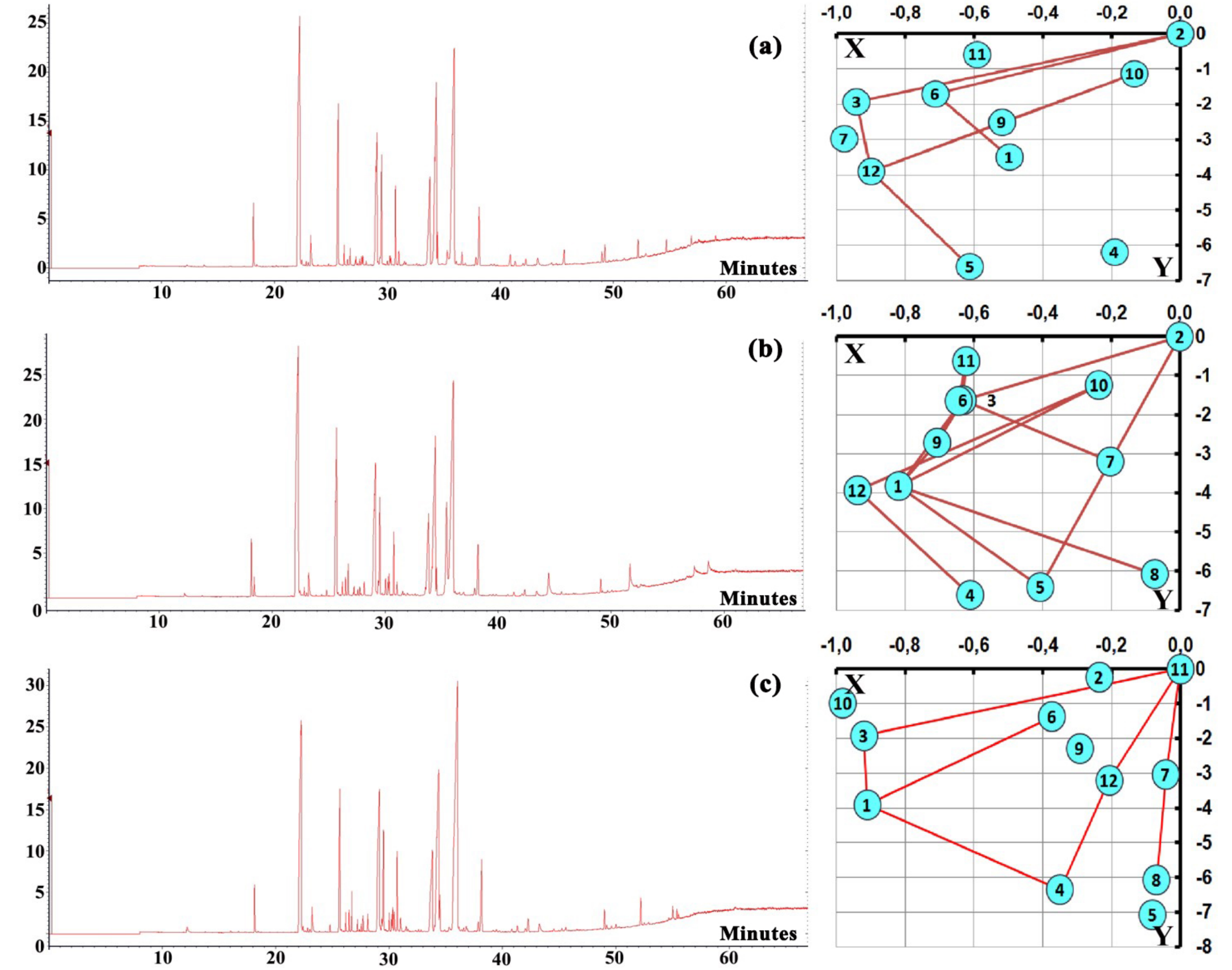

Figure 4 Chromatograms and fractal portraits of the biocomposition of fatty acids in the body of *Hermetia illucens* insects: a – sample Cd 20; b – sample Co 800 and c – sample Mix 800.

So far, only few data have been published on contaminants identified in commercially available insects and insect-based products for human consumption or animal feed. It seems clear that the substrate has an effect on the fatty acid composition, which are the source of energy in insects **[40]**, **[41]**. A study by a fully accredited laboratory in the UK studying the chemical safety of four different species of fly larvae, including *Hermetia illucens*, as a protein source for animal feed showed that only cadmium was above the maximum EC limit in animal feed of 0.5 mg/kg (three of nine samples analyzed) **[42]**. This finding confirms the cadmium accumulation shown in feeding studies under controlled lab conditions **[43]**, **[44]**. In addition to cadmium, the influence of chromium, lead and arsenic has also been studied **[45]**, **[46]**, **[47]**. Pimpla turionellae showed a striking decrease in lipid content, when exposed to Cd **[48]**. Also was demonstrated that whole body lipid concentration of day-3 4th instar larvae Lymantria dispar were significantly reduced in Cd-contaminated **[49]**.

Our results showed that the composition of the larvae was dominated by linoleic, lauric and oleic fatty acids. Insect hemolymph composition is known to change with developmental stage and within one stage **[50]**. Hence, the interpretation of lipid and fatty acid composition in the insects may be difference. In spite of that, this study lends further support to the observation that *Hermetia illucens* a is sensitive to Cd and these results suggest that the whole body lipid concentration are affected directly by Cd concentrarion in nutrient substrate.

CONCLUSION

The use of the mathematical method of fractal analysis based on the data on the profile distribution of fatty acid components in the insect body according to the variants of the experiment showed that samples with metal concentrations of 20 mg of Cd, 800 mg of Co and Mix (200 mg of Cu , 20 mg Cd, 200 mg Co, 20 mg Al and 50 mg Pb) per kilogram of dry food. Since the Mix 200 mixture also contains cadmium ions, and the mean error in the Co 800 variant exceeds those for the other two IF values selected based on the calculation results, it can be concluded that this pollutant, to a greater extent, compared to all other heavy metals, takes part in the launch of typical reactions of fatty acid homeostasis involved in the implementation of the internal program of adaptive lability in this insect species.

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Ethical Statement:

The study titled "Changes in the level of consolidation of the fatty acid profile of Hermetia illucens larvae grown on a substrate contaminated with heavy metals" conducted by the All-Russian Research Institute for Agricultural Microbiology in Pushkin, St. Petersburg, Russia, upholds the principles of ethical research. The welfare of Hermetia illucens larvae was given utmost consideration throughout the study. All necessary measures were taken to ensure their well-being and minimize any potential harm or discomfort caused during the experiment.

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