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Influence of the killing method of the black soldier fly on its lipid composition

Augusta Caligiani[a,*], Angela Marseglia[a], Alessia Sorci[a], Francesca Bonzanini[a], Veronica Lolli[a], Lara Maistrello[b], Stefano Sforza[a]

[a] Department of Food and Drug, University of Parma, Parma, Italy
[b] Department of Life Sciences, University of Modena and Reggio Emilia, Reggio Emilia, Italy

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ABSTRACT

Black soldier fly (BSF, *Hermetia illucens*) represents a valuable source of biomolecules and it also constitutes an economic way to valorise residual biomasses. BSF prepupae contain high amounts of lipids (37% DM basis). The present investigation aimed at studying the composition of BSF lipids and the effect of killing/storage on their quality. The main fatty acid was lauric acid, sterols were represented primarily by beta-sitosterol and campesterol. Global fatty acid and sterol profiles, determined by GC–MS, were only slightly affected by the killing procedure, while lipid classes distribution, determined by ^{1}H NMR, strongly changed. Prepupae killed by freezing showed a drastic reduction of acylglycerols during storage and a relevant release of free fatty acids, likely due to activation of lipases. On the contrary, prepupae killed by blanching have a stable lipid fraction constituted mainly by triacylglycerols. Therefore, killing procedure strongly influences BSF oil composition and the potential applications.

1. Introduction

Entomophagy, the consumption of insects for food purposes, is conventionally practiced in many regions of the world, such as Asia, South America, Australia and Africa (Caparros Megido et al., 2013). In the last decades, the interest in insects as a food source strongly increased all over the world, primarily in connection with the perspectives of growing population. In 2050 the world population is estimated to reach 9 billion people, therefore there is a need to find sustainable food source for future food demands. Currently insects are not the main source of energy in any Countries, but they could complement in the future the traditional sources of protein and lipid.

Many insects, especially in the larvae/prepupae stages, have a high nutritive value, containing fats, proteins, vitamins, fibres and minerals (van Huis et al., 2013). The nutritional value is highly variable among the large number of edible insects (Kouřimská & Adámková, 2016). Certain insect species contain high proportions of fat (Barroso et al., 2014; Ramos-Elorduy, 1997), thereby opening interesting possibilities for food applications, but also for other industrial purposes, as biodiesel production. The Black Soldier Fly (BSF) *Hermetia illucens* (Linnaeus, 1758) (Diptera, Stratiomyidae) is one of the insect species richest in lipids (Ramos-Bueno, González-Fernández, Sánchez-Muros-Lozano, García-Barroso, & Guil-Guerrero, 2016.), and is currently also of particular interest because of its high efficiency as bio-converter of organic waste and agricultural by-products (Diener, Studt Solano, Gutiérrez, Zurbrügg, & Tockner, 2011). In the light of the new Regulation on Novel Foods (EU 2015/2283), edible insects are increasingly being introduced into the EU Countries. In parallel, several ways to process insects into food products and fractions are currently being explored, mainly to overcome the reluctance of some part of the population in eating whole insects. In fact, some studies evidenced that consumer have more willingness to eat insects when they are not visible in food (Balzan, Fasolato, Maniero, & Novelli, 2016). Transformation of whole insects into meals, protein and other fractions for food/feed ingredients can be viewed as a necessary step to enhance the acceptability and the diffusion of insects. To address this point, fractionation strategies were recently developed to separate lipid, protein and chitin from BSF prepupae: (Caligiani et al., 2018). It turned out that lipids can be easily extracted from BSF prepupae by common solvent-based extraction procedures, and the separation of the lipid fraction is often a preliminary step during the preparation of high protein meal. The main application currently exploited for the utilization of BSF fat is the production of biodiesel (Li et al., 2011; Li et al., 2015), but a deeper investigation of BSF fat composition and properties could support the

* Corresponding author at: Department of Food and Drug, University of Parma, Parco Area delle Scienze 27A, 43124 Parma, Italy.
E-mail address: augusta.caligiani@unipr.it (A. Caligiani).

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exploitation of higher added value uses in the food/feed sector. From this point of view, data about the behaviour of the lipid fraction and the lipid classes distribution during BSF processing and storage are almost completely lacking.

The insect pre-processing, stabilization/killing methods are also important points. The European animal welfare Directive 98/58/EC for animal farming does not apply to insects, as they are non-vertebrate animals, leaving room to the most convenient methodologies. The most common killing procedure used is freezing, eventually followed by freeze-drying to increase stabilization (Vandeweyer, Lenaerts, Callens, & Van Campenhout, 2017). Alternative ways for killing/stabilization still need to be explored, and thermal treatments look especially promising. For example, a common procedure employed mainly in vegetables and fruit processing is blanching, aimed at stopping enzymatic activity prior to further storage or processing (Fellows, 2009). Blanching was used for pre-treatment of *Tenebrio molitor* larvae, with the aim to reduce microbial load (Vandeweyer et al., 2017), but it has never been used for BSF killing/stabilization.

In this paper, two different killing methods (blanching or freezing) were compared to evaluate potential modifications in the lipid fraction. In particular, a detailed molecular profiling of lipid from BSF prepupae was assessed in terms of lipid classes distribution, fatty acid and sterol profiles.

2. Materials and methods

2.1. BSF sampling and pre-treatments

Samples of *H. illucens* prepupae were obtained by Smart Bugs s.s. (Ponzano Veneto, Italy). Their growing substrate consisted of vegetable residues: 50% corn flour zootechnical use (mill waste), 40% wheat bran, 10% alfa-alfa flour. Water was added to have 2/3 dry diet and 1/3 water. A first BSF sample was obtained frozen (BSF1 sample), half of it was stored frozen as whole prepupae at −20 °C (BSF1fr) and the other half was grinded and freeze-dried (Liophilizer LIO 5P, International PBI Milan, Italy) until 10% residual moisture, then stored at −20 °C (BSF1lio). A second sample of BSF prepupae (BSF2 sample) was provided alive. One kilogram was killed by blanching for 40 s in hot water at 100 °C (BSF2bl), and another kilogram by freezing at −20 °C (BSF2fr). All the samples were stored frozen at −20 °C in ziplock bags until usage. BSF2bl and BSF2fr were analysed immediately after the pre-treatment (T0) and after two months of frozen storage (T1).

2.2. Chemicals

Diethyl ether, petroleum ether (40–60 °C boiling point fraction), potassium hydroxide, dichloromethane were from Carlo Erba (Milan, Italy); hexane, methanol, methyl pentacosanoate, 5-alpha-Cholestan-3-beta-ol were purchased from Sigma-Aldrich (Milan, Italy), *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane were purchased from Fluka.

2.3. Lipid extraction

Different methods for lipid extraction were tested and the results compared. To determine crude fat content 2 g of BSF prepupae were finely grinded and extracted using an automatized Soxhlet fat extractor (SER 148/3 VELP SCIENTIFICA, Usmate Velate, Italy) with diethylether (60 mL) as extraction solvent (AOAC, 2006). The same Soxhlet extraction was performed using petroleum ether. Lipids were also extracted with the method of Folch, Lees, and Sloane Stanley (1957), using dichloromethane instead of chloroform, as suggested by Cequier-Sanchez, Rodriguez, Ravelo, & Zarate, 2008. BSF oil samples were kept at −20 °C and in the dark until analysis.

2.4. Determination of oil acidity

BSF oil acidity was determined by titration of the oil dissolved in a mixture of methylene chloride/acetic acid using KOH 0.1 N. Methods was adapted from official method for acidity determination in vegetable oils (Berner, 1989), by expressing results as percentage of lauric acid, because it represents the most abundant in BSF fatty acids profile.

2.5. Determination of fatty acids profile by GC–MS

BSF fat was subjected to acidic and basic transmethylations separately, performed according to ISO 12966-2 (ISO, 2017), with slight modifications. For basic transmethylation, 100 mg of the fatty residue were dissolved in hexane (5 mL), added to 2 mL of KOH 5% in methanol and mixed vigorously for 5 min. For acid-catalyzed transmethylation, 100 mg of BSF fat were added to 0,5 mL of H_2SO_4/methanol (1:15 v/v) and heated for 3 h at 100 °C. After cooling, 5 mL of hexane were added. For both samples, 500 μL of the hexane phase was withdrawn and added to 100 μL of internal standard methyl pentacosanoate. A dilution of this solution was performed to match the linearity range of the GC–MS instrument, by taking 50 μL and diluting with 1000 μL of n-hexane. The solutions were split-injected (1 μL) on a Thermo Scientific Trace 1300 gas-chromatograph (Thermo Scientific, Waltham, Massachusetts, USA) carrying a Supelcowax ms capillary column (Supelco, Bellafonte, USA) coupled to a Thermo Scientific Trace ISQ mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Content of each single fatty acid was calculated in relation to the concentration of the internal standard, after calculating the response factors using the Supelco® 37 Component FAME Mix (Sigma Aldrich, Saint Louis, MO, USA). Finally, results were expressed as relative percentage.

2.6. Determination of sterols and other compounds of unsaponifiable fraction

Unsaponifiable matter were extracted and silylated according to ISO 12228-1:2014 (ISO, 2014). 5 g of BSF oil and 100 mL of a 2.2 N potassium hydroxide solution in ethanol-water (8:2 v/v) were put into a 250 mL flask; saponification was carried out by boiling and stirring the sample for 1 h. After cooling, 100 mL of distilled water were added, and the sample transferred to a separating funnel and extracted 4 times with 50 mL of ethyl ether. The ether extracts were pooled into a separating funnel and washed with distilled water (50 mL each time), until the wash gave a neutral reaction. Then the wash water was removed, and the organic sample was dried with anhydrous sodium sulphate, filtered, taken to dryness and the residue weighed. The residue was dissolved in 100 μL of 5-alfa-cholestan-3-beta-ol solution (internal standard, 520 ppm in hexane) and silylated with 100 μL of BSTFA (1 h, 60 °C). The composition of the unsaponifiable was determined by injecting 1 μL of the silylated solutions in the GC–MS system. GC–MS analysis was performed on a 6890 N gas chromatograph coupled to an 5973 N mass selective detector (Agilent technologies, Santa Clara, CA) with a DB5 (J &W) capillary column (temperature: 80 °C for 2 min, 15 °C/min until 280 °C, 280 °C for 20 min). Injection mode: split. Acquisition mode: scan (*m/z* 40–550). Unsaponifiable components were quantified by means of the internal standard (5-alfa-cholestan-3-beta-ol), according to Caligiani, Bonzanini, Palla, Cirlini, & Bruni, 2010.

2.7. Determination of lipid class distribution by ^{1}H NMR

^{1}H NMR analysis was performed on the same samples analysed for fatty acid and sterol profiles and also on BSF1 and BSF1lyo samples after 6 months of storage at −20 °C. Lipids from BSF1 sample were extracted by Soxhlet from the whole insect (BSF1fr whole) or after grinding (BSF1fr). BSF lipids (50 mg) were diluted in 0.8 mL of deuterated chloroform ($CDCL_3$) in a 5 mm glass tube for NMR analysis. NMR spectra were registered on a Bruker Avance III 400 MHz NMR

Spectrometer (Bruker BioSpin, Rheinstetten, Karlsruhe, Germany) operating at a magnetic field-strength of 9.4 T. Spectra were acquired at 298 K, with 32 K complex points, using a 90° pulse length and 3 s of relaxation delay (d1). 128 scans were acquired with a spectral width of 9595.8 Hz and an acquisition time of 1.707 s. The relaxation delay and acquisition time allow the complete relaxation of the protons, allowing their integrals for quantitative purposes. Table S1 of the supplementary material reports the identification of the main signals assigned in the BSF lipid spectrum, grouped by classes, and highlighting with letters those integrated for the quantification. The whole zone ranging from 0.87 to 2.90 ppm plus signal centered at 5.35 ppm were used as determinant indicative of total fatty acid moles, both free and bound. For the glycerol esters, the specific signals listed in Table S1 were integrated. Integrals were normalized for the number of hydrogen contributing to the specific signal (see Table S1). In the case of fatty acid integral, the mean number of hydrogen in fatty acids was inferred from the mean fatty acid composition obtained by gas chromatographic analysis, and it was found as 27.7.

The normalized areas obtained were converted as relative molar percentages. To determine the molar percentage of free fatty acids, the total fatty acid molar percentages was subtracted of the contribution of fatty acids bound to TG, DG and MG, according to the simple relation:

$$[FFA] = [TFA] - (3[TG] + 2[DG] + 1[MG])$$

2.8. Data analysis

All data are presented in Tables 1, 2, 3, and 4 as mean and SD of three independent fat extractions and analyses. For each parameter, one-way ANOVA and post hoc Tukey-HSD tests were performed to detect statistically significant differences among the samples ($p < .05$). All the statistical analyses were performed utilizing SPSS statistical software (IBM SPSS Statistics 25, IBM, New York, USA).

3. Results and discussion

3.1. Yield of black soldier fly oil and oil acidity

Sample BSF1, consisting of frozen BSF prepupae having been killed by freezing before reception in the laboratory, was used to test the effect of different extraction systems on BSF lipids. Automated extraction based on Soxhlet system was performed with both diethyl ether and petroleum ether, giving not significantly different yields (12.5–12.6% fresh matter basis). These figures indicate a considerable lipid fraction, which confirms BSF as a good source of lipids, unlike other insect species with lower lipid content (Ramos-Bueno et al., 2016).

Fat extraction from BSF1 prepupae was also performed by the Folch method (Folch et al., 1957). Although it is generally recognized as a highly efficient method for extracting lipids from animal tissue, in this case it has low throughput, with a yield of lipid extracted significantly lower respect to Soxhlet methods (9.11% of lipid extracted, Table 1). Soxhlet method was then chosen as most suitable for BSF lipid extraction, and applied also to BSF2bl and BSF2fr samples (samples of BSF prepupae either killed by freezing or blanching). The total amount of lipids for these samples (13.0–13.3%) was not significantly different from BSF1 sample.

Table 1
Yield of lipid extraction obtained with three different extraction systems and corresponding oil acidity, determined by titration with KOH and expressed as percentage of free lauric acid.

	Method	Solvent	Lipid Yield (% fresh matter)*	Lipid acidity (% free lauric acid)*
BSF1	Soxhlet	Diethyl ether	12.52 ± 0.05 b	80 ± 1 c
	Soxhlet	Petroleum ether	12.6 ± 0.5 b	80 ± 1 c
	Folch	CH_2Cl_2/ CH_3OH (2:1)	9.1 ± 0.1 a	81.6 ± 0.9 c
BSF2fr	Soxhlet	Diethyl ether	13 ± 1 b	18.3 ± 0.5 b
BSF2bl	Soxhlet	Diethyl ether	13.3 ± 0.8 b	1.8 ± 0.2 a

* Values followed by different letters within one column are significantly different ($p < .05$); $n = 3$.

The low yield of lipids obtained by Folch method was tentatively explained by supposing the presence of acidic lipids (free fatty acids), which can be partly lost as fatty acid salts in the aqueous phase during Folch extraction. This hypothesis was tested by measuring the oil free acidity, and indeed, the free fatty acid amount was found to be surprisingly high for sample BSF1, reaching a striking 80% of free fatty acids. Also for BSF2fr the amount of free fatty acid was quite high (13%), even if at levels not comparable with the ones found in the previous sample. BSF1 and BSF2fr samples have in common the killing method by freezing. On the other side, for sample BSF2bl, where prepupae were blanched before freezing, the amount of free fatty acids was basically negligible (Table 1). To the best of our knowledge, the high acidity of BSF oil has never been reported previously in the literature, even if the killing by freezing is by far the most widespread method for BSF larvae/prepupae. These preliminary data called for a deeper investigation about the oil composition and, mostly, on the effect of prepupae killing method and storage on the lipid fraction. To this aim, the detailed distribution of the various lipid classes, the fatty acid profile and the sterol profile were studied.

3.2. Fatty acid profile

Fatty acid profiles were determined on BSF2 samples (both blanched and frozen) immediately after killing (T0) and after a period of two months of storage at −20 °C (T1). Due to the high amount of free fatty acids demonstrated by acidity determination, the total fatty acid profile was determined by GC–MS after esterification by acidic methylation. In parallel, BSF oil samples were also analysed after transesterification by alkali methylation, to obtain the profile of bound fatty acids and to highlight possible selective release of specific fatty acids from triacylglycerols. Results are reported in Table 2.

GC–MS analysis allowed to identify short, medium and long chain fatty acids, in the range 9:0–22:0. The main fatty acid was by far represented by lauric acid (12:0), a medium chain fatty acid characteristic of coconut oil, accounting in all samples for > 40% of total fatty acids, reaching a peak of 75% in BSF2fr sample after two months of storage. Other saturated fatty acids present in significant amount were myristic (14:0), palmitic (16:0), stearic (18:0) and capric (10:0) acids. The most abundant polyunsaturated fatty acids (PUFAs) was linoleic acid (9c12c-18:2) and minor amounts of its conjugated isomer (CLA, 9*cis*, 11*trans*) was also detected. This specific fatty acid composition can be influenced by the insect rearing substrate, but in general the data reported are in line with other studies, which also confirmed the abundance of medium chain saturated fatty acids (Leong, Kutty, Malakahmad, & Tan, 2016; Li et al., 2016; Surendra, Olivier, Tomberlin, Jha, & Khanal, 2016). Basic and acidic methylation for samples BSF2bl (T0 and T1) and for sample BSF2fr (T0) substantially gave similar relative profile of fatty acids, indicating that the distribution of glycerol-bound fatty acids and released (free) fatty acids are very similar. A completely different behavior was observed for sample BSF2fr after two months of storage (T1). This sample showed a dramatically different distribution of fatty acids when comparing the total with the bound fatty acid profile: the glycerol-bound fraction of BSF2fr T1 was found to be significantly richer in saturated fatty acids respect to the total one, (about 98% of saturated fatty acids vs 80%). In particular, the amount of bound lauric acid increased from 45.6 to 75.9%. These results indicate some selective hydrolytic reactions during storage of BSF2 sample not subjected to thermal treatment (blanching), suggesting a selective cleavage of unsaturated fatty acids by lipase enzymes. The lipase activity during frozen storage is a known phenomenon, for example in fish (Fidalgo,

Table 2
Profile of fatty acids (reported as relative percentages) of BSF2 sample stabilized by freezing (fr) or blanching (bl). Samples were analysed immediately after stabilization (T0) and after two months of frozen storage (T1). Fatty acids were determined as methyl esters after methylation in both acidic and alkali media. Coefficient of variations (CV%) ranged from 2% to 8%. Values followed by different letters within one row are significantly different ($p < .05$); n = 3.

	BSF2fr T0	BSF2fr T0	BSF2fr T1	BSF2fr T1	BSF2bl T0	BSF2bl T0	BSF2bl T1	BSF2bl T1
Methylation	Acid (total FA)	Basic (bound FA)	Acid (total FA)	Basic (bound FA)	Acid (total FA)	Basic (bound FA)	Acid (total FA)	Basic (bound FA)
Fatty acid*	1	2	3	4	5	6	7	8
9:0	0.17d	0.07 b	0.15d	0.02 a	0.11c	0.16d	0.01 a	0.10c
10:0	1.63b	2.45c	1.90b	0.74a	2.48c	2.60c	1.83b	2.44c
11:0	0.07b	0.03b	0.07b	0.01b	0.05b	0.07b	1.71a	0.05b
12:0	48.98	44.16b	45.60bc	75.93d	47.09c	40.17a	47.3c5	40.03a
14:0	14.43bc	15.79d	14.52bc	12.99a	15.84d	15.19cd	14.12b	14.87bc
12-Me-13:0	0.10de	0.07bc	0.12ef	0.03a	0.08 cd	0.14f	0.05ab	0.09 cd
9c-14:1	0.25b	0.15b	0.24b	0.03b	0.16b	0.29b	2.75a	0.18b
15:0	0.08c	0.05b	0.09cd	0.02a	0.05b	0.10d	0.03a	0.06b
12-Me-14:0	0.09d	0.06bc	0.11de	0.06a	0.06c	0.13e	0.09b	0.08c
13-Me-14:0	0.13d	0.08bc	0.15de	0.04a	0.10c	0.17e	0.07b	0.10c
9c-15:1	0.04c	0.02a	0.05d	n.d.	0.02a	0.04c	n.d.	0.03b
16:0	14.40cd	16.30e	15.54de	8.21a	14.26c	13.79bc	12.75b	16.62e
14-Me-15:0	0.22d	0.14b	0.25d	0.07a	0.13b	0.29e	n.d.	0.18c
9c-16:1	2.31b	2.58bc	2.23b	0.14a	2.39bc	3.42d	2.01b	2.99cd
17:0	0.07cd	0.04b	0.09ef	0.02a	0.08de	0.10f	n.d.	0.06c
14-Me-16:0	0.04d	0.02b	0.04d	0.01a	0.04d	0.05e	n.d.	0.03c
15-Me-16:0	0.09d	0.06bc	0.11e	0.03a	0.05b	0.12e	n.d.	0.07c
9c-17:1	0.07ab	0.04a	0.08b	n.d.	0.05ab	0.08b	0.36c	0.06ab
17:1	0.07abc	0.05a	0.09bc	n.d.	0.05a	0.10c	0.31d	0.06ab
18:0	1.31d	1.21cd	1.18cd	0.59a	0.96bc	1.45d	0.89b	1.27d
9c-18:1	6.57bc	7.98d	7.79d	1.06a	6.79c	9.88f	6.21b	9.12e
9c,12c-18:2	8.41b	8.36b	9.19c	n.d.	8.93bc	10.91d	7.46a	11.51d
9c,11t-18:2 (CLA)	0.38a	0.26a	0.32a	n.d.	0.20a	0.65b	2.01c	0.33a
19:0	0.01a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
19:1	0.04b	0.02a	0.06c	n.d.	0.01a	0.04b	n.d.	0.03b
9,10-P-19:0 (CPFA)	n.d.	0.01a	n.d	n.d.	n.d.	0.02b	n.d.	0.01a
20:0	0.02b	0.01a	0.03c	n.d.	0.01a	0.03c	n.d.	0.04d
22:0	0.01a	n.d.	0.01a	n.d.	0.01a	n.d.	n.d.	0.01a
Total saturated	81.86	80.55	79.96	98.77	81.41	74.58	78.90	75.73
Total monounsaturated	9.34	10.83	10.53	1.23	9.47	13.85	11.63	12.45
Total polyunsaturated	8.80	8.61	9.51	0.00	9.12	11.56	9.47	11.84

* The shorthand notation of fatty acids from AOCS lipid library was used.

Table 3
Sterol and policosanols content of BSF oil obtained from BSF2 samples (data are expressed as relative percentage on the unsaponifiable fraction except for the total, expressed as g/100 g fat).

	BSF2bl T0	BSF2bl T1	BSF2fr T0	BSF2fr T1
Policosanols	1,1a	1,5b	1,0a	1,9c
Cholesterol	1,3a	1,2a	1,2a	1,5a
Campesterol	22,5a	23,7a	23,7a	22,8a
Stigmasterol	6,3a	4,3b	6,2a	6,4a
Beta-sitosterol	61,1a	59,7a	60,1a	58,0a
Stigmastanol	7,2a	9,2b	8,1b	9,2b
Total (g/100 g fat)	0,46 ± 0.03b	0,33 ± 0.05c	0,52 ± 0.04b	0,81 ± 0.03a

Values followed by different letters within one row are significantly different ($p < .05$); n = 3.

Saraiva, Aubourg, Vázquez, & Torres, 2015), but it was never reported for insects and for BSF oil. It is likewise known that the lipolysis activities in foods is generally limited to a minor fraction of lipids. For example in some fishes, free fatty acids reach 3% after 12 months of frozen storage (Romotowska, Karlsdóttir, Gudjónsdóttir, Kristinsson, & Arason, 2016). In ripened cheeses, the content of free fatty acids is limited to 1–5%, according to the ripening time and production technology (Addis et al., 2015; McCarthy, Wilkinson, Kelly, & Guinee, 2017). Also in vegetable oils, the natural content of free fatty acids is generally limited to amount < 10%, for example, 2.3–6.7% in palm oil, according to Saad et al. (2006). Therefore, the free fatty acids content in BSF, reaching in some samples almost the total of the lipid fraction, represents an exceptional case, and this is the first report highlighting this strong lipolysis in insects.

Data seem to be in agreement with the high concentration (and thus likely the high enzymatic activity) of the lipase enzyme present in BSF

Table 4
Relative molar percentages determined by ^{1}H NMR of the different glycerides and free fatty acids present in BSF oil from BSF prepupae stabilized in different ways. Abbreviations: TG: triglyceride; 1,2-DG: 1,2-diglyceride; 1,3-DG: 1,3-diglyceride; 2-MG: 2-monoglyceride; 1-MG: 1-monoglyceride; FFA: free fatty acid. Lio: lyophilized; fr: frozen; bl: blanched. Values followed by different letters within one row are significantly different ($p < .05$); n = 3.

	BSF1lio	BSF1fr	BSF1fr whole	BSF2bl T0	BSF2bl T1	BSF2fr T0	BSF2fr T1
1,2DG	1.01a	1.47c	11.43e	0.54b	1.08a	5.52d	2.14c
2MG	0.11b	0.03b	0.47a	nd	0.23c	0.06b	0.13b
1,3DG	0.17d	0.34 cd	1.76a	0.46c	0.46c	0.79b	0.46c
1MG	1.5b	0.49c	2.1a	nd	0.23d	0.49c	0.44c
TG	0.14d	5.26c	5.6c	99.01a	97.97a	58.01b	6.38c
FFA	97.09a	92.42a	78.63b	tr	tr	35.14c	90.45a
Total	100	100	100	100	100	100	100

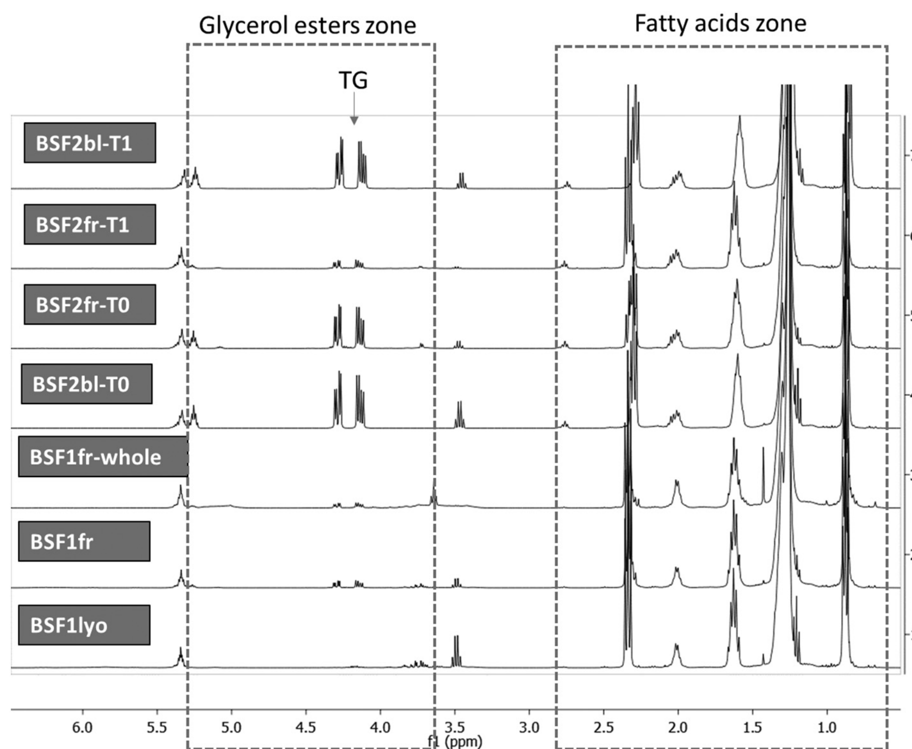

Fig. 1. ^{1}H NMR spectra ($CDCl_3$, 400 MHz) of lipid fraction of the BSF prepupae killed and stored in different ways.

(Kim et al., 2011).

3.3. Sterol and policosanol profile

Sterol and policosanols was determined in the unsaponifiable matter of the same samples characterized for fatty acid profile. Results are reported in Table 3 as percentage of each sterol and polycosanols respect to total sterol/policosanol. The absolute amount of the total sterols/policosanols of BSF fat was also reported as g/100 g fat.

Data showed that sterols normally characteristic of plants (phytosterols) are the main component of BSF sterol fraction, being β-sitosterol, and campesterol the most represented, while cholesterol is present in low amount. These results are consistent with what reported by Ushakova et al. (2016). These data suggest that larvae of black soldier fly grown on plant substrate do not metabolize completely plant sterols but store them as part of their lipid content. In fact, in animals phytosterols are not efficiently absorbed and their amount respect to cholesterol is normally around 5% (Ling & Jones, 1995). In this case the high amount of phytosterols in the insect body (deriving from the vegetable substrate on they were grown) suggests that they are probably absorbed, not metabolized and stored. The low cholesterol amount combined with high phytosterols levels make the BSF fat (grown on vegetable substrates) slightly healthier as compared to other animal fats, because phytosterols have been demonstrate to reduce LDL cholesterol (EFSA, 2008). Together with sterols, in the same GC–MS chromatographic run it was possible to identify six policosanols, i.e. long chain primary alcohols, characterized according to their mass spectra and by comparison with literature data (Caligiani et al., 2010). The most abundant policosanols in BSF fat are represented by C22 (docosanol) and C28 (octacosanol) followed by C24 (tetracosanol), C26 (hexacosanol), C23 (tricosanol) and C25 (pentacosanol). They are generally present in nature as ester with fatty acids, therefore their presence suggests a possible waxy component in BSF. Due to the analytical method adopted, involving the saponification step, it is not possible to know the ratio between free and esterified form. Policosanols are used as dietary supplements with some claimed physiological activities: reduction platelet aggregation, increased athletic performance, reduction in LDL cholesterol levels, and increased HDL cholesterol levels in blood. Therefore, their presence in BSF fat might contribute to a slight better lipid profile respect to animal fats. Their real efficacy, however, is still matter of questioning (Kassis, Marinangeli, & Jones, 2009).

Regarding the effect of the BSF killing method and storage on unsaponifiable profile, data were in perfect agreement with those obtained on fatty acid profile: the sterol content was similar in all the samples, except sample subjected only to freezing treatment after two months of storage (BSF2fr-T1), which showed significantly higher sterol amounts (0.81 g/100 g fat). The explanation for this result is still matter of investigation, but probably has to be searched as well in the global change occurring in the lipid profile of BSF prepupae not thermally treated. The difference observed is only quantitative, because the relative percentages of each sterol are very similar in all samples.

3.4. Lipid classes distribution determined by ^{1}H NMR

Due to the high amount of free acidity found in some BSF oil samples and the differences in fatty acids and sterols profiles, a deeper investigation on the lipid classes' distribution was finally performed. To obtain quantitative data on the relative amount of each lipid class, a simple procedure based on ^{1}H NMR spectroscopy was used, adapted for the identification and quantitation of different lipid species in BSF oil. The method offers a rapid assessment of content and composition of major lipid classes, including triacylglycerols (TG), diacylglycerols, (DG), monoacylglycerols (MG) and free fatty acid (FFA), as previously reported (Nieva-Echevarría, Goicoechea, Manzanos, & Guillén, 2014; Nieva-Echevarría, Manzanos, Goicoechea, & Guillén, 2017; Nuzzo et al., 2013).

The ^{1}H NMR spectra of the samples considered (see paragraph 2.7) are reported in Fig. 1. From a simple visual inspection of the spectra, it emerges the extremely different relative abundance of the triacylglycerol signals, which in some samples resulted to be much lower than in others. Beside triacylglycerols (TG), also the other classes of fatty acids

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esters (1-MG, 2-MG, 1,2-DG, 1,3-DG) showed diagnostic signals that could be selected as NMR markers with no interference by other compounds. The quantitative molar proportions of the different acyl groups and of some minor components present in the lipid extracts are reported in Table 4.

NMR data confirmed that in all cases where only freezing was applied, free fatty acids accounted for most of the lipid content. The only sample showing low free fatty acids and a stable lipid fraction during storage was BSF2bl, the one subjected to blanching treatment. This clearly indicates that thermal treatment inactivates endogenous lipase enzymes. Lipase are instead activated during the slow killing by freezing, and they remain active after the death. Data suggest also that lipase action occurs quickly during the killing by freezing: in fact, BSF2fr sample presents high molar percentages of free fatty acids (34.14%) also when analysed at T0, immediately after killing. After two months of storage the free fatty acids reached a molar percentage of > 90% (BSF2fr-T1), which was further slightly increased in prolonged storage (BSF1fr) and also when samples were stored after freeze-drying (BSF1lyo), due to the residual activities of lipase after death and during frozen storage. An effect of sample grinding was also observed: BSF1lyo sample, stored in grinded form, presented the higher amount of free fatty acids (97%) and negligible amounts of intact triacylglycerols (0.14%). The grinding effect seems to occur also instantaneously during sample preparation: lipids extracted from BSF1fr sample either after grinding or directly on the whole larva, showed a higher amount of free fatty acid in the former case (92.42% vs. 78.63%).

In general, the ^{1}H NMR results definitively confirmed that the BSF lipid fraction composition strongly depends on the BSF killing methods and on the storage conditions.

The lipid classes distribution is important to evaluate the possible uses of BSF oil.

Currently the most explored application for BSF oil is biodiesel, and it has then been proposed as a valid alternative to vegetal biodiesel (Dias et al., 2009; Fu et al., 2015; Leong et al., 2016). The high concentration of medium chain saturated fatty acids and the low concentration of polyunsaturated fatty acids make the BSF oil an ideal substrate for the production of high quality biodiesel with low viscosity and oxidative stability (Zheng et al., 2012). The production of biodiesel from vegetable and animal oils requests an acid-catalyzed esterification of free fatty acids (to reduce fatty acidity) and an alkaline-catalyzed trans esterification (Marchetti, Miguel, & Errazu, 2007; Meher, Vidya Sagar, & Naik, 2006). Due to this specific step of production, the lipolysis process demonstrated in this work on BSF larvae killed by freezing seems to be not critical in the case of the use of BSF oil for biodiesel production, as in any case a reaction of methylation of acylglycerol is needed. Of course, the knowledge of the ratio between glycerol-bound and free fatty acids is important to correctly define the reaction conditions for methylation.

On the other hand, a more economically advantageous use of BSF oil could be in the food/feed or pharmaceutical industries. The total lipid profile of BSF prepupae (Table 2) shows characteristics similar to some vegetable oils for food uses. In particular, the percentage of lauric acid and the global fatty acids profile make BSF oil comparable to coconut oil and palm kernel oil (Lira et al., 2017). These tropical oils, being highly saturated, have many applications in the alimentary industry (e.g. frying oils), but also in the soap industries, pharmaceuticals, cosmetics, plastics etc. (Gopala Krishna, Gaurav, Ajit Singh, & Prasanth Kumar, 2010). BSF oil could be used in similar applications. Food lipids (both of animal and vegetal origins) are mainly constituted by triacylglicerols and when the oil/fat acidity is higher than 2%, a neutralization step is needed. Therefore, the detailed lipid classes profiling obtained in this study suggests that a thermal pre-treatment on living larvae/prepupae is mandatory to inactivate lipase and preserve an intact lipid fraction suitable for eventual food applications.

4. Conclusions

The use of insects as feed is allowed in EU for aquaculture and with the new EU regulation on Novel Foods it is likely that in short times insects will be authorized also as food. Therefore, the use of insects as food, despite could be controversial, is a forthcoming event that cannot be neglected by food scientists. Insects as Novel Foods need authorization from EFSA, whose opinions are generally based on the examination of food composition data. In this context, the data collected in this paper about the lipid fraction gain particular importance. The detailed lipid profiling is the basis of new knowledge about BSF composition according to the killing method and storage. In particular, our data showed that a thermal pre-treatment (killing by blanching) is needed to inactivate lipase activity and preserve an intact lipid fraction to be used for eventual food applications. On the contrary, a strong lipolysis process seems to occur when killing by freezing is applied, which could be not critical in the case of the use of BSF oil for biodiesel production, as in any case a reaction of transmethylation of acylglycerol is needed.

The high rate of lipolisis in non-thermally inactivated BSF prepupe indicates a high lipase activity inside the insect biomass, possibly even stimulated by the cold environment. This aspect needs to be further investigated and the lipid detailed characterization extended to other insect species to verify if a similar behaviour occurs.

Role of authors

AC, conception and design, interpretation of data, drafting the article; AM, ^{1}H NMR analysis and interpretation of the data; AS, FB, VL, acquisition of GC–MS data, interpretation of the results, statistical analysis; LM, contribution to conception, critical revision; SS, contribution to conception and experiment design, critical revision and final approval of the version to be published.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2018.08.033.

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