1 Optimisation of the Lipid Extraction of Fresh Black Soldier
2 Fly Larvae (*Hermetia illucens*) with 2-Methyltetrahydrofuran
3 by Response Surface Methodology[†]

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14
15 [†] Supplementary material available

Abstract

While black soldier fly larvae (BSFL, *Hermetia illucens*) have the potential to become a renewable, industrial feedstock; lipid extractions are typically performed with hexane. Green solvents, however, are gaining more popularity to replace these harmful solvents such as hexane. In this study, the green solvent 2-methyltetrahydrofuran (2-MeTHF) was compared with hexane for extracting lipids from fresh BSFL at moderate temperature. Response surface methodology was used to investigate these wet extractions with a Soxhlet extraction as benchmark. Two sets of optimal process settings were predicted and validated for (1) maximising solvent efficiency and (2) maximising lipid recovery. The resulting extracts were characterised by their fatty acid profile and lipid class composition. The results of the benchmark extraction (Soxhlet) showed that 2-MeTHF could extract significantly more lipids from dried BSFL than hexane (43.23 vs. 40.34 g lipids/100 g DM). No significant difference was found between the maximum solvent efficiencies of hexane and 2-MeTHF. By performing the wet extraction with 2-MeTHF at 45 °C with 15 w/w % fresh BSFL, 40 w/w % 2-MeTHF, and 45 w/w % water; the highest lipid recovery was obtained (94.9 ± 1.8 % of total BSFL lipids). These results indicated that the wet extraction was able to extract a similar amount of lipids compared to the Soxhlet extraction. Although the fatty acid profiles did not differ between extraction conditions, 2-MeTHF extracted a higher quantity of free fatty acids and valuable phospholipids compared to hexane. Consequently, the wet lipid extraction with 2-MeTHF can be considered a suitable, more ecologic alternative for the conventional extraction of dried BSFL with hexane.

Key words

Lipid extraction; response surface methodology; *Hermetia illucens*; hexane; 2-methyltetrahydrofuran

1. Introduction

Hermetia illucens, also known as the black soldier fly, is an insect with a large potential. Many researchers have already described the usefulness of its larval stage as the larvae are able to convert low-value organic waste or by-products from industry into high-value biomass[1,2]. By doing so, they have the potential to play a substantial role in the circular bio-economy[3]. Black soldier fly larvae (BSFL) contain high amounts of lipids, with values ranging from 18 to 48 % on dry matter (DM) basis depending upon the substrate in which the larvae were reared[4–8], making them promising as renewable feedstock for various industries. To this end, significant effort has already been made to investigate the usefulness of BSFL lipids[9–14]. The defatted, solid residue can then be valorised further by extracting proteins and chitin [15,16].

In nearly all previously mentioned research articles, as well as in other articles concerning the chemical composition of *H. illucens*, lipid extraction is almost always performed using either hexane or petroleum ether. Although the price of petroleum ether is lower compared to hexane, its composition is less defined, making hexane more suitable for the purpose of this research. Additionally, hexane is already commercially applied for the extraction of lipids from food- or feedstuffs such as soybeans, sunflower seeds, and olives[17,18]. Although hexane has the advantage of being able to extract nearly all of the oil in the aforementioned products, it has several limitations and drawbacks[19]. Firstly, hexane is acute toxic to aquatic life and suspected of damaging fertility according to the European Chemicals Agency. A second drawback is that chronic exposure might damage organs, causes nerve disorders, and results in irritation of skin and lungs[20]. Thirdly, hexane also has some concerns from a safety perspective as it is highly flammable. Finally, the availability and price of hexane varies due to fluctuations in the fossil fuel market[20].

Because of these drawbacks towards health and environment, research towards greener, biodegradable and non-dangerous solvents is becoming increasingly important. COSMO-RS (COnductor-like Screening MOdel for Real Solvents) is a predictive model which has already

been used by several researchers to compare solvents by determining the relative solubility of model compounds such as certain triacylglycerols and phospholipids[21–23]. This has led to a number of greener solvents receiving more attention over the past few years, one solvent being 2-methyltetrahydrofuran (2-MeTHF)[16,21–27]. This is a green, sustainable, biomass-derived solvent that can be produced from furfural or levulinic acid, which in turn are derived from simple C5 or C6 sugars[24,27]. Modelling showed that 2-MeTHF could perform better than hexane, which was also confirmed experimentally on rapeseed and *Pistacia lentiscus*[21,22]. Additionally, previous studies already showed the advantages of 2-MeTHF over other green solvents and its potential for industry. For example, ethanol was also analysed with COSMO-RS, yet it was clear that this solvent was inferior to hexane and 2-MeTHF[21–23]. Other solvents such as ethyl lactate, *p*-cymene, and *d*-limonene, could perform equivalently to 2-MeTHF, though these solvents are characterised by a high boiling point (resp. 154 °C, 158 °C, and 175 °C) and a high enthalpy of vaporisation (resp. 49.2 kJ/mol; 49.2 kJ/mol; and 49.6 kJ/mol), making them less desirable for industry because of the high cost of solvent evaporation[22,23,28]. In comparison, while hexane has a boiling point of 69 °C and a vaporisation enthalpy of 31.0 kJ/mol, these values are only slightly higher for 2-MeTHF being 79 °C and 34.0 kJ/mol[28]. Recuperation of 2-MeTHF can therefore be achieved through a conventional batch distillation at atmospheric pressure as water and 2-MeTHF form a favourable azeotrope[29]. In another study, 2-MeTHF was deemed particularly promising in terms of process efficiency compared to hexane as higher crude lipid yields were obtained which resulted in an increased biodiesel yield[26]. Other researchers[23] also found that 2-MeTHF extracts less protein compared to ethyl acetate, which is an advantage when the extraction of only lipids is desired. In a more recent article, 2-MeTHF has been brought up as an alternative solvent for the lipid extraction of dried BSFL[16]. In their study, freeze-dried larvae powder was used to evaluate the suitability of 2-MeTHF for a lipid extraction at 55 °C.

In addition, they also found that the protein quality parameters of BSFL powder defatted with 2-MeTHF were slightly better in comparison to hexane [16]. Since freeze drying is a costly operation and conventional drying techniques at elevated temperatures may result in increased lipid oxidation and a high amount of free fatty acids, it would be preferable if the preceding drying step is omitted and that the lipid extraction is performed on fresh material[30].

As such, the feasibility of replacing hexane with 2-MeTHF for the lipid extraction of fresh BSFL through a simple wet extraction procedure at moderate temperature, was investigated in the present study. As pointed out by Chemat and colleagues [31], the solid-liquid extraction is the most crucial step in the entire extraction process and should be performed under optimised conditions. To achieve this, Design of Experiments (DoE) was employed in order to study the wet lipid extraction process and to determine the optimal extraction parameters[32]. Additionally, by using this approach, both linear and quadratic factor effects as well as interactions between factors can be identified[33]. To the authors' knowledge, this is the first time the lipid extraction of fresh BSFL is investigated in such detail. Additionally, a blanching step was included in the proposed wet extraction procedure to lower the microbial load and to inhibit endogenous enzymatic hydrolysis[34]. This lipolysis is suspected to be responsible for the high quantities of free fatty acids observed in extracted BSFL lipids[13,16,34]. Through the DoE approach, optimal extraction conditions to maximise the (1) lipid recovery and (2) solvent efficiency (i.e., mass lipids extracted with a certain amount of solvent) are identified for both hexane and 2-MeTHF and these predictions are validated experimentally. Finally, the lipids extracted with hexane and 2-MeTHF are compared in terms of their fatty acid profile as well as their lipid class composition.

2. Experimental setup

2.1.Characterisation of the raw material

Larvae of *Hermetia illucens* were purchased from RADIUS (Thomas More, Geel, Belgium) and fasted for 48 hours prior to inactivation by freezing at -20°C . A portion of the larvae was ground cryogenically and freeze dried for 48 hours (Büchi, Lyovapor L-200). The dry matter content of the freeze-dried larvae ($w_{\text{dry matter freeze dried sample}}$) and blached larvae paste ($w_{\text{dry matter paste}}$) was determined in triplicate by drying in a forced-air oven (Memmert, UF 500) for 17 hours[15]. The dry matter content was calculated as the residual sample weight after drying divided by the original sample weight.

A lipid extraction according to Soxhlet with slight modifications was used as a reference for the proposed wet extraction procedure[15]. This extraction was performed in triplicate with both hexane (Chemlab, 99 %) and 2-MeTHF (Merck, Emplura 99 %). In brief, about 5 grams of freeze-dried sample was weighed to the nearest 0.1 mg in a cellulose thimble (Machery Nagel, 30 mm x 100 mm). The thimble was covered with glass wool and put in a Soxhlet apparatus attached to a round-bottom flask containing 120 mL of solvent. The heating of the apparatus was adjusted in such a way that for both solvents, a cycling rate of 10 – 12 cycles per hour was achieved. After 18 hours, the solvent was removed using a rotary evaporator (Büchi, R-300) with a vacuum pump (Büchi, V-300) and a recirculating cooler (Julabo, F25-MA) attached. The temperatures of the heating bath and cooler were maintained at 45°C and -10°C respectively. For hexane, the solvent was removed by setting the initial vacuum to 260 mbar. For 2-MeTHF, the initial vacuum was set at 150 mbar. After 15 minutes at the initial vacuum, the receiving flask was emptied and the pressure was lowered to 50 mbar for 30 minutes to ensure the total evaporation of solvents and traces of water. By differential weighing of the round-bottom flask, the mass of extracted lipids was calculated. The lipid content on dry matter of the larvae could be calculated as follows:

$$w_{\text{lipids, Soxhlet}} [\%] = m_{\text{extracted lipids}} [\text{g}] / (m_{\text{freeze dried sample}} [\text{g}] \times w_{\text{dry matter freeze dried sample}} [\%]) \text{ (Eq. 1)}$$

2.2. Experimental design for the lipid extraction

In order to investigate the wet lipid extraction process, two central composite designs (CCD) were used[35]. The influence of three factors on the lipid recovery (Y_R) and solvent efficiency (Y_E) for both hexane and 2-MeTHF was investigated. These three factors were the following: the amount of insect (X_1 , w/w %), the amount of extraction solvent (X_2 , w/w % of the liquid fraction), and the extraction temperature (X_3 , °C). They were chosen based on previous research which has already shown that the solid-solvent ratio and the extraction temperature both have a pronounced effect on the lipid recovery from BSFL powder[12]. Factor ranges were selected based on the results from a screening study (data not shown). Since a 3-factor design was used, a total of 8 different combinations of the high and low factor levels are required. In addition, 3 center points (i.e., all factor levels set halfway between the high and low value) and 6 axial points were included as well. For the axial points one variable was set at an extreme level ($\alpha = 1.682$) while the other two variables were set at the center level. Each of these 14 factor combinations are further referred to as “a run”. As such, each run of the design was a wet lipid extraction with a certain amount of insect, extraction solvent, and extraction temperature. All runs were performed in a randomized order to minimize the effect of external factors[36,37]. Because of the small sample size (1 – 10 g of fresh BSFL), each run of the designs was performed in triplicate. An overview of the designs for both hexane and 2-MeTHF with the used factor levels or settings for Y_R and Y_E is shown in Table 2. While the variables are coded by the [-1; +1] interval in DoE[35–38], only the un-coded levels are shown in the table for the purpose of clarity.

A full quadratic response surface model (Eq. 2) was fitted to the experimental data using standard least squares to identify significant ($p < 0.05$) effects. In Eq. 2, Y is the predicted response (Y_R : recovery, or Y_E : efficiency); β_0 is the intercept of the linear function; β_i is the main effect of X_i ; β_{ii} is the quadratic effect of X_i ; and β_{ij} is the interaction effect between X_i and X_j . After reducing the model for both hexane and 2-MeTHF by removing non-significant

coefficients while preserving effect hierarchy, the resulting models were evaluated in terms of their values for R-squared (R^2) and R-squared adjusted (R^2_{adj}). While R^2 measures how well the experimental data fits the model, R^2_{adj} can indicate overfitting of the data if it is much lower than R^2 . As such, the use of R^2_{adj} is preferred over R^2 in multiple linear regression analysis[39]. After evaluation, the resulting prediction expressions were used for predicting the optimal process settings. Two sets of optimal process settings were predicted, one with the focus on maximizing the lipid recovery and another with the emphasis on achieving the highest solvent efficiency possible. Subsequently, the predictions of the optimal settings for both hexane and 2-MeTHF were validated experimentally.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 + \beta_{11}X_1^2 + \beta_{22}X_2^2 + \beta_{33}X_3^2 \text{ (Eq. 2)}$$

2.3. Wet extraction procedure

For the proposed wet extraction of BSF lipids, the frozen larvae were first blanched in boiling water for 40 seconds to decrease the microbial load as well as to stop enzymatic activity. This time/temperature combination was chosen based on the results of previous research[40]. The positive influence of blanching on the nutritive and sensory quality of the protein and lipid extracts from BSF has also been documented before[2,34]. After blanching, the larvae were cooled with running tap water for 60 seconds and left to drain in a sieve for another 60 seconds. Subsequently, the larvae were ground using a kitchen blender (Moulinex AR110830). At this moment, the dry matter content of the resulting BSFL paste ($W_{dry\ matter\ paste}$) was determined. Since not all runs of the experimental designs could be performed on the same day, a fresh batch of blanched larvae paste was prepared daily. As such, the dry matter content of the paste was determined every day.

In the second step, the BSFL paste was mixed with solvent and demineralised water for a total mass of 20.0 g in a 50 mL centrifuge tube (VWR International). The addition of water was necessary as further processing was otherwise impeded due to the viscosity of the mixture. The

mass fractions (w/w %) of X_1 and X_2 shown in Table 2 were used to calculate the required amounts of BSFL paste and solvent for each run of the designs. The required amount of water could be calculated based on the amounts of BSFL paste and solvent. The BSFL paste, solvent, and water were then accurately weighed in the centrifuge tube up to the nearest 0.001 g (Alessandrini Exacta). The centrifuge tube was then immersed in a water bath while being agitated at regular intervals to homogenize the contents. The temperature of the water bath was varied according to the experimental design shown in Table 2. After 30 minutes, the sample was taken out of the water bath and centrifuged at 4000 g for 15 minutes in a pre-heated centrifuge (Heraeus Multifuge 3S) to separate the aqueous and organic layer. After phase separation, the organic layer was transferred to a tarred round-bottom flask and the mixture of BSFL and water was extracted two more times with the same amount of solvent to increase the lipid recovery[41]. The three extracts were combined in the same round-bottom flask. Finally, the solvent was removed using a rotary evaporator as described previously. By differential weighing of the round-bottom flask, the mass of extracted lipids was calculated.

Because of the elevated temperature and prolonged extraction time of the Soxhlet extraction, it was expected that all lipids were extracted from the sample. As such, the results of the Soxhlet extraction were used as a reference to calculate the recovery of the proposed lipid extraction procedure (Eq. 3). Additionally, the solvent efficiency (i.e., the amount of lipids extracted with a certain volume of solvent) was calculated using Eq. 4. For the experimentally validated predictions, the actual lipid yield (i.e., the mass of lipids extracted on dry matter basis) was calculated by means of Eq. 5.

$$\text{Lipid recovery [\%]} = m_{\text{extracted lipids}} [\text{g}] / (m_{\text{sample}} [\text{g}] \times w_{\text{lipids, Soxhlet}} [\%] \times w_{\text{dry matter paste}} [\%]) \times 100 \%$$

(Eq. 3)

$$\text{Solvent efficiency [g lipids/100 mL of solvent]} = (m_{\text{extracted lipids}} [\text{g}] \times \rho_{\text{solvent}} [\text{g/mL}] \times 100) / m_{\text{solvent}} [\text{g}]$$

(Eq. 4)

$$\text{Absolute lipid yield [g lipids/100 g dry matter]} = m_{\text{extracted lipids [g]} / (m_{\text{sample [g]} \times W_{\text{dry matter paste}} [\%]) \quad (\text{Eq. 5})$$

2.4.Characterisation of extracted lipids

In addition to comparing the recovery and solvent efficiency of hexane and 2-MeTHF, the extracted lipids were also characterised by their fatty acid profile and lipid class composition. For this purpose, three replicate extractions according to Soxhlet were performed for both hexane and 2-MeTHF. Lipids were also extracted in triplicate using to the wet extraction procedure with the two sets of predicted optimal process settings (resp. for maximising recovery and solvent efficiency) for both hexane and 2-MeTHF. The resulting 18 extracted lipid samples were then characterised by the procedures described in the following sections.

2.4.1. Fatty acid analysis

The fatty acid profile was determined using the protocol from a previous study[42]. First, fatty acid methyl esters (FAMES) were prepared from the extracted lipids through esterification and transesterification according to Joseph and Ackman[43]. In brief, 25.0 mg of the extracted lipids were heated in the presence of 1.5 mL methanolic KOH (0.500 M, Sigma-Aldrich) and 2.4 mL 20 % BF_3 in methanol (Sigma-Aldrich) to produce the FAMES which were then extracted quantitatively with *iso*-octane (technical, VWR Chemicals). Using anhydrous MgSO_4 (USP, VWR Chemicals), the last traces of water were removed before filtrating (MN 640, Machery Nagel) the solution in a volumetric flask of 5.0 mL. Appropriate dilutions were made in *iso*-octane when necessary. The resulting FAMES were analysed with GC-MS (Agilent, 7820A-5977E GC-MSD) with the following settings (Table 1). Calibration was performed with commercially available standards (Sigma-Aldrich) and methyl tricosanoate (Sigma-Aldrich) was used as internal standard for the analysis.

Table 1 Parameter settings of the GC-MS for the fatty acid analysis

Parameter	Setting
Column	Sigma-Aldrich SLB-IL60 Capillary Column (30 m x 0.25 mm, 0.20 μ m)
Carrier gas	Helium (12 psi) at 40 °C
Sample volume	1.0 μ L
Ratio split injector	10:1
Oven program	Initial temperature 40 °C Hold 40 °C for 4 minutes Ramp 5 °C/min to 280 °C
Ion source MS	Electron impact (EI)
Scan type MS	Selected ion monitoring (SIM)

2.4.2. Lipid class determination

High-performance thin-layer chromatography (HPTLC) was used for the relative quantification of the lipid classes present in the extracted lipids. The following analytical standards, each representing a certain lipid class (mentioned between brackets), were used: palmitic acid (free fatty acids), DL- α -palmitin (monoacylglycerols), dipalmitin (diacylglycerols), tripalmitin (triacylglycerols), cholesterol (sterols), and cholesteryl butyrate (sterol esters). Analytical standards of L- α -phosphatidylcholine (PC) and L- α -phosphatidylethanolamine (PE) were also used to quantify the polar lipids. All standards, reagents, and solvents used for the determination of the lipid classes were purchased from Sigma-Aldrich. The procedures for the separation of neutral and polar lipids were slightly adapted from previous research[16,21]. Samples were applied in duplicate onto the plates (HPTLC Silica gel 60 F₂₅₄ 20 x 10 cm, Merck) by means of an Automatic TLC Sampler 4 (CAMAG) after which the plates were developed in an Automatic Developing Chamber (CAMAG). For the separation of the neutral lipids, a solvent mixture of hexane/diethyl ether/acetic acid with a volumetric ratio of 78/20/2 was used. Polar lipids were separated with a 25/25/25/10/9 mixture (V/V) of chloroform/methyl acetate/isopropyl alcohol/methanol/KCl (0.25 % *m/V*). After development of the HPTLC plates, iodine vapour was used for the visualisation of all lipid classes except cholesterol. Cholesterol was visualised by dipping the HPTLC plate in a 4-anisaldehyde solution (4-anisaldehyde/acetic acid/methanol/sulphuric acid in a volumetric ratio of 1/20/170/10). The lipid classes were then

measured by scanning densitometry (CAMAG TLC Scanner 3) and processed using the visionCATS software (CAMAG). Since only one analytical standard was used for each lipid class, only the lipid class distribution could be calculated[16,21,44]. Consequently, the resulting distributions were multiplied with the actual amounts of lipids extracted, to allow for a better comparison of the two different extraction solvents. In order to compare the results from the wet extraction with the Soxhlet extraction, the results were also calculated on dry matter basis.

2.5. Statistical analysis

All analyses were performed in triplicate unless specified otherwise. The JMP Pro 14.0.0 software package from SAS was used for the generation of the experimental design. All other statistical evaluations, including the fitting of the response surface models, were also performed using JMP Pro 14.0.0. In case of equal variances (Levene's test), one-way ANOVA and Tukey HSD post hoc test were used to evaluate the fatty acid profiles and lipid classes of the extracted lipids. Otherwise, Welch's ANOVA was used with the Steel-Dwass All Pairs post hoc test[45].

3. Results and discussion

3.1. Proximate composition

The dry matter content of the freeze-dried, ground larvae was found to be 92.96 ± 0.60 % which was sufficiently high for performing Soxhlet extractions, as high moisture contents (> 10 %) are found to interfere with solvent penetration and oil diffusion, resulting suboptimal lipid extractions[46,47]. A new batch of blanched larvae paste was prepared daily because not all experimental runs could be performed on the same day. However, there was not much day-to-day variation in the dry matter content of the larvae paste with the total average being 32.33 ± 0.35 % ($n = 9$) on wet weight basis. The dry matter content of the blanched larvae paste was very similar to the dry matter content of fresh BSFL[4,48–50], indicating that little water is taken up by the larvae during the blanching process.

Resulting from the Soxhlet extractions, lipid contents of $40.34 \pm 0.05 \%$ and $43.23 \pm 0.13 \%$ were obtained for extraction with hexane and 2-MeTHF respectively. This significantly higher lipid content ($p < 0.01$) obtained with 2-MeTHF was anticipated since this was also found in previous research on microalgae[26]. Moreover, the recent study on the lipid extraction of dried BSFL also confirmed the results obtained here [16]. This higher content may be explained by the difference in solvent characteristics between hexane and 2-MeTHF. The partition coefficient of hexane is 3.9 whereas for 2-MeTHF it is 0.8, indicating that the latter is a more polar solvent[22]. Another difference can be observed in the Hansen Solubility Parameters (HSP), which are values that can aid in understanding the solubility of solutes in a certain solvent or the miscibility of two compounds[16,22,51]. The three HPS are correlated with (1) atomic dispersion forces (δD); (2) dipole moments (δP); and (3) hydrogen bonds (δH). If the HSP values of solute and solvent are highly similar, then the solute will likely dissolve in the solvent. The HSP values of hexane are all zero except δD , while all HSP values of 2-MeTHF are greater than zero, indicating that 2-MeTHF will likely also dissolve more polar lipids. Consequently, based on the differences in solvent characteristics, 2-MeTHF is likely able to extract a higher fraction of the more polar lipids.

3.2. Experimental designs

3.2.1. Evaluation of the designs

As stated before in the materials and methods section, two central composite designs were used to study the wet lipid extraction process. One design was used to determine which main effects, quadratic effects, and interaction effects were significant for the lipid extraction with hexane, while the second design was used for 2-MeTHF. For both extractions, the influence on lipid recovery (Y_R) and solvent efficiency (Y_E) was investigated. The results of all extractions and an overview of the statistical evaluation of both designs are displayed in Table 2 and Table 3, respectively. All non-significant effects have been removed from the model, while preserving

the hierarchy[33]. The global p-value of all four models was lower than 0.0001 indicating a strong significance and all models had high R^2 and R^2_{adj} values meaning that they adequately represent the experimental data[32,37,52]. Additionally, the differences between R^2 and R^2_{adj} are less than 0.05, indicating that there was no overfitting of the experimental data[53]. Therefore, the models were deemed suitable for making predictions of the extraction processes with hexane and 2-MeTHF.

Table 2 The two central composite designs with the factor settings of all investigated factors: amount of insect (X_1 , w/w %), amount of solvent (X_2 , w/w %), and extraction temperature (X_3 , °C). The responses lipid recovery (Y_R , %) and solvent efficiency (Y_E , g lipids/100 mL solvent) are presented as the average of three replicates $\pm$ standard deviation.

Run	Hexane					2-Methyltetrahydrofuran				
	Factors			Responses		Factors			Responses	
	X_1	X_2	X_3	Y_R	Y_E	X_1	X_2	X_3	Y_R	Y_E
1	15.0	15.0	30.0	66.5 $\pm$ 0.6	2.51 $\pm$ 0.01	29.5	29.5	37.5	79.0 $\pm$ 0.3	5.03 $\pm$ 0.04
2	29.5	29.5	37.5	63.7 $\pm$ 0.8	2.89 $\pm$ 0.04	29.5	29.5	50.1	83.9 $\pm$ 0.4	5.33 $\pm$ 0.04
3	29.5	53.9	37.5	64.4 $\pm$ 0.5	1.59 $\pm$ 0.01	53.9	29.5	37.5	60.8 $\pm$ 6.8	10.7 $\pm$ 1.2
4	5.1	29.5	37.5	64.8 $\pm$ 1.3	0.38 $\pm$ 0.02	29.5	29.5	37.5	82.2 $\pm$ 1.7	5.24 $\pm$ 0.11
5	44.0	44.0	45.0	66.6 $\pm$ 0.2	3.80 $\pm$ 0.01	44.0	15.0	45.0	56.8 $\pm$ 4.9	13.3 $\pm$ 1.2
6	29.5	29.5	50.1	72.2 $\pm$ 2.2	3.27 $\pm$ 0.10	15.0	15.0	30.0	69.0 $\pm$ 0.4	3.70 $\pm$ 0.04
7	53.9	29.5	37.5	59.8 $\pm$ 1.1	7.55 $\pm$ 0.12	15.0	15.0	45.0	79.1 $\pm$ 0.5	4.18 $\pm$ 0.11
8	29.5	29.5	37.5	66.1 $\pm$ 3.9	2.99 $\pm$ 0.17	29.5	29.5	24.9	78.5 $\pm$ 0.5	4.99 $\pm$ 0.03
9	15.0	15.0	45.0	61.1 $\pm$ 0.1	2.35 $\pm$ 0.03	29.5	29.5	37.5	83.8 $\pm$ 0.2	5.34 $\pm$ 0.01
10	29.5	29.5	24.9	57.9 $\pm$ 0.2	2.62 $\pm$ 0.01	44.0	44.0	30.0	77.9 $\pm$ 1.0	6.24 $\pm$ 0.07
11	44.0	44.0	30.0	56.3 $\pm$ 1.0	3.21 $\pm$ 0.06	44.0	44.0	45.0	86.6 $\pm$ 1.2	6.90 $\pm$ 0.12
12	29.5	29.5	37.5	68.2 $\pm$ 0.8	3.10 $\pm$ 0.04	15.0	44.0	45.0	95.3 $\pm$ 1.1	1.73 $\pm$ 0.03
13	44.0	15.0	30.0	53.6 $\pm$ 0.6	8.97 $\pm$ 0.13	29.5	5.1	37.5	19.8 $\pm$ 4.6	7.21 $\pm$ 1.6
14	44.0	15.0	45.0	59.3 $\pm$ 0.5	9.90 $\pm$ 0.07	5.1	29.5	37.5	91.6 $\pm$ 3.1	0.76 $\pm$ 0.02
15	15.0	44.0	30.0	69.7 $\pm$ 1.1	0.93 $\pm$ 0.07	29.5	53.9	37.5	87.1 $\pm$ 0.1	3.03 $\pm$ 0.01
16	29.5	5.1	37.5	51.1 $\pm$ 0.3	13.3 $\pm$ 0.1	44.0	15.0	30.0	56.9 $\pm$ 1.1	13.3 $\pm$ 0.2
17	15.0	44.0	45.0	65.5 $\pm$ 1.5	0.84 $\pm$ 0.02	15.0	44.0	30.0	85.7 $\pm$ 1.3	1.55 $\pm$ 0.03

Table 3 Analysis of variance and regression analysis of the lipid recovery (Y_R) and solvent efficiency (Y_E) of hexane and 2-methyltetrahydrofuran. All non-significant factors and interactions have been removed from the model, while maintaining effect hierarchy ($\alpha = 0.05$).

Hexane				2-Methyltetrahydrofuran			
Y_R		Y_E		Y_R		Y_E	
Factor	p-value	Factor	p-value	Factor	p-value	Factor	p-value
X_1 (amount of insect)	< 0.0001	X_1	< 0.0001	X_1	< 0.0001	X_1	< 0.0001
X_2 (amount of solvent)	< 0.0001	X_2	< 0.0001	X_2	< 0.0001	X_2	< 0.0001
X_3 (temperature)	< 0.0001	X_1X_2	< 0.0001	X_3	0.0191	X_1X_2	< 0.0001
X_1X_3	< 0.0001	X_2^2	< 0.0001	X_2^2	< 0.0001	X_1^2	0.0309
X_1^2	0.0026			X_3^3	0.0235		
X_2^2	< 0.0001						
Model	< 0.0001	Model	< 0.0001	Model	< 0.0001	Model	< 0.0001
R^2	0.816	R^2	0.939	R^2	0.896	R^2	0.937
R^2 adjusted	0.791	R^2 adjusted	0.934	R^2 adjusted	0.888	R^2 adjusted	0.933

3.2.2. Extraction with hexane

For the lipid recovery (Y_R) with hexane, the main effects of all three factors were found to be significant. The same goes for the interaction effect between X_1 and X_3 , and for the quadratic effects of X_1 and X_2 . The resulting prediction expressions for the lipid recovery (Y_R) and solvent efficiency (Y_E) are given in Eq. 5 and Eq. 6 respectively.

$$Y_{R, \text{hexane}} = 66.01 - 2.64 X_1 + 2.58 X_2 + 2.22 X_3 + 3.44 X_1 X_3 - 1.28 X_1^2 - 2.44 X_2^2 \quad (\text{Eq. 5})$$

$$Y_{E, \text{hexane}} = 4.51 + 3.37 X_1 - 3.79 X_2 - 1.66 X_1 X_2 + 1.99 X_2^2 \quad (\text{Eq. 6})$$

As can be deduced from the signs of the coefficients in Eq. 5, increasing the amount of insect (X_1) results in a lower lipid recovery. The recovery can be increased by using a higher amount of solvent (X_2) or a higher temperature (X_3). Additionally, X_1 and X_2 also had a significant quadratic effect and an interaction between X_1 and X_3 was observed as well. The latter means that the effect of the changes in X_1 depends on the value of X_3 and vice versa[35]. Such interactions are quite common[32,33,36,52,53]. While the significance of the three main effects was expected, as this is in agreement with previously published data[12], to the authors' knowledge, this is the first time that quadratic effects and interactions are identified for the lipid extraction of BSFL.

An overview of all the significant factor effects and interactions on the lipid recovery is shown in Figure 1. As already mentioned, increasing X_1 resulted in a lower lipid recovery. One possible explanation for this result could be that the amount of extractable lipids was too high for hexane to dissolve, leaving a portion behind in the residue. As the data shows, this will not be the case for low quantities of insect. It could also be observed from the graph, that the effect of X_2 was more pronounced at low values because of the curvature being much steeper. A possible reason for this is that a low amount of hexane will likely extract less lipids, resulting in a lower recovery. Yet, increasing the amount of solvent beyond 30 % no longer has a positive

impact on the recovery, as can be seen in Figure 1. In previous research[46], it is theorised that lipid recovery can have a limit as the concentration gradient decreases when the ratio solid:liquid keeps decreasing (e.g., larger amounts of solvent).

Figure 1 Interaction plots of the lipid recovery for the extraction with hexane. The horizontal axis is scaled for the factor displayed in each column (X₁: amount of insect, X₂, amount of solvent, X₃: extraction temperature). Coloured line segments display the interaction of that factor with the factor in the corresponding row (low level: red; high level: blue). Values for the factor levels are also plotted next to the line segments. Parallel lines indicate the absence of an interaction, while diverging lines indicate the presence of a (non)-significant interaction. Significant interactions ($p < 0.05$) are marked by an asterisk.

The interaction between X₁ and X₃ is also visualised as response surface plot in Figure 2. When the extraction was carried out at low temperature (30 °C), the highest recovery was obtained for a small amount of insects. In contrast, when extracting BSF lipids at an elevated temperature (45 °C), the lipid recovery could be increased by increasing the amount of insect. Increasing the extraction temperature has already proven to result in higher recoveries in the literature[12]. However, since the goal of this research was to optimise a wet lipid extraction at moderate

temperature, the temperature was not increased beyond the design space. It was likely that increasing the temperature further, would increase the lipid recovery considering that the Soxhlet extraction, being the reference, was also carried out at a higher temperature.

Figure 2 Response surface plot depicting the interaction between amount of insect (X₁, w/w %) and extraction temperature (X₃, °C) on the lipid recovery (Y_R, %) of the extraction with hexane.

As for the solvent efficiency, only X₁ and X₂ had a significant influence. Both their individual effects as well as their interaction was shown to have an impact on the solvent efficiency. Moreover, X₂ also had a quadratic effect. An overview of the factor and interaction effects is displayed in Figure S1 of the supplementary material. As can be deduced from Eq. 6, increasing X₁ had a positive impact on the solvent efficiency. The presence of a higher amount of extractable lipids likely resulted in a saturation of hexane, meaning that as much lipids were dissolved as possible, resulting in a high efficiency. When the amount of hexane (X₂) was

increased, the efficiency decreased. This was as expected since the amount of solvent was part of the calculation of the solvent efficiency (Eq. 4). Nevertheless, the solvent efficiency can be a noteworthy response to keep in mind and to consider when designing large scale extractions. Though, it is likely that a compromise will have to be made between maximising the lipid recovery and the solvent efficiency.

3.2.3. Extraction with 2-MeTHF

Concerning the lipid recovery of the extraction with 2-MeTHF, some differences were found compared to hexane. The prediction expressions for the lipid recovery (Y_R) and solvent efficiency (Y_E) for the extraction with 2-MeTHF are displayed in Eq. 7 and Eq. 8 respectively.

$$Y_{R, 2\text{-MeTHF}} = 80.20 - 7.43 X_1 + 14.76 X_2 + 2.40 X_3 - 9.26 X_2^2 + 2.37 X_3^3 \quad (\text{Eq. 7})$$

$$Y_{E, 2\text{-MeTHF}} = 6.23 + 3.77 X_1 - 2.05 X_2 - 1.55 X_1 X_2 + 0.33 X_1^2 \quad (\text{Eq. 8})$$

All main effects were found to have a significant effect on the lipid recovery. No interactions were observed though both the amount of solvent (X_2) and temperature (X_3) had a significant quadratic effect. The overview of all factor effects on the lipid recovery with 2-MeTHF is shown in Figure 3. Although the blue and red lines of X_1 and X_3 slightly diverge in this figure, which might indicate the presence of an interaction, this interaction was not significant. As with hexane, decreasing X_1 (amount of insect) or increasing X_2 or X_3 resulted in a higher lipid recovery. For the given range, the impact of X_1 and X_2 was, however, larger for 2-MeTHF than for hexane, which could be derived from their coefficients in Eq. 7 and Eq. 5, respectively. Consequently, it might be that the lipid extraction with hexane is more robust in case of variation in the process parameters X_1 and X_2 . The positive effect of X_3 might be attributed to the inverse solubility of 2-MeTHF in water[25]. As the temperature increases, the solubility of 2-MeTHF in water decreases. Considering that the fraction of 2-MeTHF dissolved in water will likely not contribute to the lipid extraction, a higher fraction of 2-MeTHF can take part in the

extraction at elevated temperatures, which might be a possible explanation for the slightly positive impact of temperature on the lipid extraction. Although the effects of X_3 were significant, they could be considered negligible compared to the effects of X_1 and X_2 based on the p-values and the coefficients in Eq. 7. Like the lipid recovery of hexane, assigning higher levels to X_2 (amount of 2-MeTHF) results in a maximum lipid recovery, as displayed in Figure 3. As mentioned before, this may be attributed to the decreasing concentration gradient when the amount of solvent increases[46].

Figure 3 Interaction plots of the lipid recovery for the extraction with 2-methyltetrahydrofuran. The horizontal axis is scaled for the factor displayed in each column (X_1 : amount of insect, X_2 , amount of solvent, X_3 : extraction temperature). Coloured line segments display the interaction of that factor with the factor in the corresponding row (low level: red; high level: blue). Values for the factor levels are also plotted next to the line segments. No significant interactions ($p < 0.05$) were found.

For the solvent efficiency, it was found that the main effects of X_1 and X_2 were significant, as well as their interaction, just as it was the case for hexane. However, there was also a significant

quadratic effect for X_1 , which was not the case for hexane. Figure S2 of the supplementary material shows an overview of the factor and interaction effects. The reasoning behind the increased solvent efficiency by increasing X_1 and decreasing X_2 may be similar to hexane.

3.2.4. Predictions

Using the prediction expressions described in the previous sections, optimal factor settings have been predicted in order to (1) achieve the highest lipid recovery and (2) obtain the highest solvent efficiency for both hexane and 2-MeTHF. Table 4 shows the optimal predicted factor settings for both solvents, the expected responses as a 95 % confidence interval, as well as the experimentally obtained responses from confirmatory tests using the predicted optimal settings. A noteworthy observation was that the optimal extraction temperature was estimated at 45.0 °C for both hexane and 2-MeTHF, which was on the edge of the design space. As already mentioned before, it is possible that the lipid recovery might still be improved by increasing the temperature beyond 45.0 °C. For this present research, however, the upper value for the extraction temperature was fixed at 45.0 °C in order to minimise potential lipid oxidation^[47] and to obtain a lipid extraction procedure which can be performed at a moderate temperature. Because it was found that the temperature did not have a significant influence on the solvent efficiency for both solvents, no temperature setting could be predicted by the software. Therefore, the temperature for maximising the solvent efficiency was set the same as the predicted temperature for maximising the lipid recovery, being 45.0°C.

Table 4 Predicted optimal factor settings (X_1 , amount of insect w/w %; X_2 , amount of solvent w/w %; X_3 , temperature °C) for the lipid extraction with hexane and 2-methyltetrahydrofuran, the predicted responses (95 % confidence interval), and the actual, experimentally validated responses. The absolute lipid yield of the four extractions is displayed as well. All results are presented as the average of three replicates $\pm$ standard deviation. Means in the same row bearing a different superscript differ significantly from each other ($p < 0.05$).

	Hexane		2-Methyltetrahydrofuran	
	Maximising recovery	Maximising efficiency	Maximising recovery	Maximising efficiency
Predicted X_1 (w/w %)	35.5	44.0	15.0	44.0
Predicted X_2 (w/w %)	37.5	15.0	40.0	15.0
Predicted X_3 (°C)	45.0	45.0	45.0	45.0
Predicted recovery (%)	[67.8 ; 70.4]	[60.0 ; 64.5]	[93.5 ; 101.8]	[50.13 ; 56.94]
Actual recovery (%)	70.3 $\pm$ 1.2 ^a	60.4 $\pm$ 1.3 ^b	94.9 $\pm$ 1.8 ^c	45.7 $\pm$ 1.9 ^{d*}
Absolute lipid yield [†]	28.36 $\pm$ 0.47 ^a	24.35 $\pm$ 0.53 ^b	41.02 $\pm$ 0.76 ^c	19.76 $\pm$ 0.83 ^d
Predicted efficiency [‡]	[2.73 ; 3.16]	[14.48 ; 16.15]	[1.73 ; 2.99]	[13.93 ; 14.51]
Actual efficiency [‡]	3.29 $\pm$ 0.05 ^{a*}	10.07 $\pm$ 0.22 ^{b*}	1.86 $\pm$ 0.03 ^c	10.56 $\pm$ 0.53 ^{b*}

[†] g lipids/100 g dry matter; [‡] g lipids/100 mL solvent; * not within the 95 % confidence interval

For the lipid recovery, it was shown that all but one actual recovery was located in the confidence interval reported by the software. The lower recovery obtained with 2-MeTHF (45.7 $\pm$ 1.9 %) might have originated from the low amount of solvent used in the extraction, which made quantitative removal of the solvent from centrifuge tube difficult. In contrast, three actual solvent efficiencies were located either below or above the predicted intervals. For one value (3.29 $\pm$ 0.05 %) this difference was rather small and therefore not deemed an issue. Two actual solvent efficiencies of hexane and 2-MeTHF (resp. 10.07 $\pm$ 0.05 % and 10.56 $\pm$ 0.53 %) were, however, lower than the predicted values. Although these three deviations from the predicted optima suggested that models for solvent efficiency were imperfect, both models were found to have a high significance ($p < 0.0001$) and high values for R^2 and R^2_{adj} . Such deviations from predicted optima are not entirely uncommon in DoE[54,55], though they further highlight the importance of confirmatory runs of response surface models as these models can have considerable variance[56]. Because of these deviations, the predictions of the solvent efficiency should be interpreted with caution.

Concerning the lipid recoveries, it was observed that 2-MeTHF resulted in a higher lipid recovery than hexane should the recovery be maximised. On the other hand, only around 46 % of lipids could be extracted with 2-MeTHF when the solvent efficiency was maximised, in

comparison to around 60 % with hexane. Nonetheless, by using the appropriate factor settings, 2-MeTHF was able to extract around 95 % of the lipids present in the blanched BSFL paste, compared to only 70 % for hexane. Based on these results, it was hypothesised that a small amount of polar lipids must have been extracted with hexane during the Soxhlet extraction because of the high temperature and amount of extraction cycles (18 hours, 10 – 12 cycles/hour). Less polar lipids may have been extracted during the wet extraction with hexane, resulting in a lower lipid recovery since this value was calculated with the Soxhlet extraction as reference. Since 2-MeTHF is a more polar solvent than hexane[16,22,51], this effect was less pronounced. Moreover, it must be stressed that these recoveries are expressed relatively to the results of the Soxhlet extraction with hexane and 2-MeTHF. Consequently, the absolute difference in the amount of lipids extracted between hexane and 2-MeTHF was even larger, as can be observed from the absolute lipid yield in Table 4. It was clear that by using 2-MeTHF instead of hexane for a wet lipid extraction, an additional 12.66 g of lipids per 100 g dry matter could be extracted when the settings for maximising the lipid recovery were used. Moreover, considering that the Soxhlet extraction with hexane extracted 40.34 ± 0.05 g lipids on dry matter; it could be concluded that 2-MeTHF is able to extract a similar amount of lipids (41.02 ± 0.76 g/100 g DM) by just three wet extraction cycles. Further optimisation might be achieved by applying ultrasound or microwaves during the extraction, enhancing the mass transfer and reducing the particle size, potentially lowering the required amount of solvent even further [57]. It should, however, be noted that 2-MeTHF was already able to extract 95 % of the BSFL lipids under relatively mild conditions.

When looking at the solvent efficiencies of the extractions, it was clear that there was a big difference between the results for achieving the highest recovery and the highest efficiency. Though, when extracting BSFL lipids with the appropriate settings for maximising the solvent efficiency, there was no statistically significant difference between the actual solvent

efficiencies of hexane (10.07 ± 0.22) and 2-MeTHF (10.56 ± 0.53). Depending on the desired application, it might be more suitable to go for the highest recovery possible. Yet, the solvent usage will be higher by doing so, as can be interpreted from the values of X_2 in Table 4. It was also shown that, if the recovery was maximised, the solvent efficiency of 2-MeTHF was significantly lower ($p < 0.05$) compared to hexane. However, by using the prediction profiler of JMP Pro, the desired lipid recovery could be altered as required. If the desired lipid recovery with 2-MeTHF was set at 70 % (i.e., the highest recovery obtained with hexane), a possible combination of new process settings (e.g., 32% insect; 20 % solvent; 45 °C) should result in a solvent efficiency of around 8.40 g lipids/100 mL solvent (Figure S3 of the supplementary material).

As such, the results of this work clearly show that the recovery of the wet lipid extraction at moderate temperature can be improved by a significant amount (i.e., more than 20 %) by using 2-MeTHF instead of hexane and applying the appropriate factor or process settings. Furthermore, these results of 2-MeTHF also indicated that the wet extraction at moderate temperature could match the lengthy Soxhlet extraction in terms of lipid recovery. Even though 2-MeTHF requires slightly more energy than hexane to evaporate (0.126 vs. 0.121 kWh/kg solvent) thus making 2-MeTHF somewhat more costly to recuperate[44], not having to dry the fresh BSFL (± 67 % moisture) will more than likely compensate this slightly higher energy cost. Moreover, it might even result in an overall reduction of the energy cost of the process as drying techniques are highly energy consuming[30]. Consequently, it could be concluded that the conventional lipid extraction of dried BSFL with hexane may be replaced with a wet extraction with 2-MeTHF. Nevertheless, a complete economic and energetic evaluation thereof is certainly worth further research.

3.3.Characterisation of extracted lipids

3.3.1. Fatty acid profile

The results of the fatty acid analysis of the extracted lipids obtained via the Soxhlet extraction and the proposed wet extraction procedures utilising the optimised factor settings, are shown in Table 5 for both hexane and 2-MeTHF. Overall, it could be concluded that the fatty acid profiles of all six lipid extracts are comparable to each other. Only the stearic acid (C18:0) content differed significantly ($p = 0.001$) between extraction procedure. While the Soxhlet extraction was able to extract slightly more stearic acid compared to the wet extraction procedures, the differences could be considered negligible as the biggest difference is only 0.6 %. Additionally, the solvent did not seem to have an impact on the amount of stearic acid extracted. The total amount of extracted saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA) also did not seem to differ greatly between extraction solvent and procedure. Previous research by Ramos-Bueno and colleagues also showed that the fatty acid profile of lipid extracts from seven insect species differed only slightly when extraction solvents with different polarities (e.g., hexane, ethanol, acetone) were used[14].

The fatty acid quantities obtained were comparable to those previously reported in the literature, with lauric acid (12:0) being the most predominant fatty acid, followed by oleic (C18:1), myristic (C14:0), palmitic (C16:0), and linoleic acid (C18:2)[6,14,16,50]. It has already been proven that the composition of the rearing substrate has a fairly large impact on the fatty acid composition of BSFL[4,8]. As such, the observed differences between these results and those reported in the literature can be attributed to differences in the rearing substrate. As the extracted lipids contain high amounts of lauric acid, they might be used for the production of soaps and shower gel [9]. Another possible application of the extracted lipids is the production of biodiesel as previous research already proved that the resulting biodiesel of BSFL lipids exhibited similar fuel properties compared to biodiesel produced from rapeseed oil[10–13].

Table 5 Fatty acid profiles (% of total fatty acids) of the extracted lipids with hexane and 2-methyltetrahydrofuran obtained with the Soxhlet extraction and wet extraction procedures with optimal factor settings for the highest recovery (HR) and highest solvent efficiency (HE). Results represent the mean $\pm$ SD of three measurements. Means in the same row with a different superscript differ significantly from each other ($p < 0.05$).

	Hexane			2-Methyltetrahydrofuran		
	Soxhlet	HR	HE	Soxhlet	HR	HE
Decanoic acid (C10:0)	0.97 $\pm$ 0.08	0.87 $\pm$ 0.06	0.86 $\pm$ 0.02	0.96 $\pm$ 0.06	0.90 $\pm$ 0.04	0.88 $\pm$ 0.07
Lauric acid (C12:0)	34.82 $\pm$ 0.73	34.28 $\pm$ 1.38	34.63 $\pm$ 0.48	33.09 $\pm$ 1.09	34.91 $\pm$ 1.04	34.39 $\pm$ 1.28
Myristic acid (C14:0)	10.57 $\pm$ 1.99	9.84 $\pm$ 0.87	10.14 $\pm$ 0.30	10.58 $\pm$ 1.47	10.35 $\pm$ 0.50	8.76 $\pm$ 0.70
Myristoleic acid (C14:1)	0.49 $\pm$ 0.06	0.55 $\pm$ 0.02	0.52 $\pm$ 0.02	0.54 $\pm$ 0.01	0.50 $\pm$ 0.02	0.56 $\pm$ 0.03
Pentadecanoic acid (C15:0)	0.44 $\pm$ 0.20	0.43 $\pm$ 0.02	0.40 $\pm$ 0.02	0.46 $\pm$ 0.02	0.39 $\pm$ 0.02	0.46 $\pm$ 0.03
Palmitic acid (C16:0)	13.03 $\pm$ 1.28	13.00 $\pm$ 0.44	12.50 $\pm$ 0.28	13.75 $\pm$ 0.54	12.96 $\pm$ 0.31	13.12 $\pm$ 0.36
Palmitoleic acid (C16:1)	3.01 $\pm$ 0.44	3.04 $\pm$ 0.21	3.07 $\pm$ 0.13	2.98 $\pm$ 0.21	3.16 $\pm$ 0.18	2.84 $\pm$ 0.17
Heptadecanoic acid (C17:0)	0.40 $\pm$ 0.10	0.43 $\pm$ 0.01	0.40 $\pm$ 0.03	0.46 $\pm$ 0.03	0.39 $\pm$ 0.02	0.47 $\pm$ 0.03
<i>Cis</i> -10-heptadecanoic acid (C17:1)	0.48 $\pm$ 0.12	0.54 $\pm$ 0.01	0.50 $\pm$ 0.03	0.54 $\pm$ 0.04	0.47 $\pm$ 0.03	0.59 $\pm$ 0.03
Stearic acid (C18:0)	2.38 $\pm$ 0.17 ^a	2.02 $\pm$ 0.12 ^b	2.01 $\pm$ 0.10 ^b	2.56 $\pm$ 0.15 ^a	2.31 $\pm$ 0.05 ^{a,b}	1.99 $\pm$ 0.08 ^b
Oleic acid (C18:1)	19.38 $\pm$ 2.48	21.49 $\pm$ 0.40	20.66 $\pm$ 0.50	20.15 $\pm$ 1.23	19.82 $\pm$ 0.21	22.15 $\pm$ 0.52
Linoleic acid (C18:2)	11.43 $\pm$ 2.51	10.66 $\pm$ 0.80	11.59 $\pm$ 0.79	11.07 $\pm$ 0.99	11.22 $\pm$ 0.41	10.77 $\pm$ 0.6
Linolenic acid (C18:3)	1.48 $\pm$ 0.01	1.56 $\pm$ 0.08	1.58 $\pm$ 0.08	1.61 $\pm$ 0.05	1.52 $\pm$ 0.05	1.63 $\pm$ 0.07
Arachidic acid (C20:0)	0.49 $\pm$ 0.15	0.55 $\pm$ 0.01	0.50 $\pm$ 0.04	0.56 $\pm$ 0.05	0.47 $\pm$ 0.03	0.61 $\pm$ 0.04
5,8,11,14,17-Eicosapentaenoic acid (C20:5)	0.62 $\pm$ 0.15	0.73 $\pm$ 0.03	0.66 $\pm$ 0.02	0.70 $\pm$ 0.07	0.64 $\pm$ 0.03	0.79 $\pm$ 0.04
Saturated fatty acids	53.02 $\pm$ 2.17	52.13 $\pm$ 1.58	51.82 $\pm$ 0.76	52.36 $\pm$ 1.47	52.82 $\pm$ 1.12	52.48 $\pm$ 1.34
Monounsaturated fatty acids	33.44 $\pm$ 0.20	34.91 $\pm$ 0.69	34.36 $\pm$ 0.28	34.26 $\pm$ 0.58	33.79 $\pm$ 0.66	34.33 $\pm$ 0.80
Polyunsaturated fatty acids	13.54 $\pm$ 2.34	12.96 $\pm$ 0.90	13.82 $\pm$ 0.84	13.38 $\pm$ 0.97	13.39 $\pm$ 0.46	13.19 $\pm$ 0.64

Resulting from comparison of the fatty acid profiles, 2-MeTHF can be considered a suitable replacement solvent for the lipid extraction of BSFL paste. Not only were there only small differences observed between the fatty acid profiles obtained with 2-MeTHF and hexane, the used extraction procedure (e.g., Soxhlet, highest recovery, or highest solvent efficiency) did not have a significant influence on the fatty acid profile.

3.3.2. Lipid classes

The results of the lipid class quantification of the extracted lipids are displayed in Figure 4 for both hexane (yellow background) and 2-MeTHF (green background). It should be noted that for 2-MeTHF, the results of the highest efficiency ('2-MeTHF HE') were much lower compared to those of the highest recovery ('2-MeTHF HR') because of the lower lipid recovery. An overview of the results with the statistical evaluation can also be found in Table S1 in the supplementary material. As can be observed from the graph, the main constituents of all extracted lipids were triacylglycerols (TAG), free fatty acids (FFA) and monoacylglycerols (MAG). All extracts also contained diacylglycerols (DAG), phosphatidylethanolamine (PE),

550 and smaller amounts of cholesterol and phosphatidylcholine (PC). Sterol esters, represented by
 551 cholesteryl butyrate, were not detected in any of the analysed samples.

552
 553 Figure 4 Amounts (on dry matter basis) of the different lipid classes that were extracted according to
 554 Soxhlet and according to the wet extraction procedures with the optimal factor settings for achieving
 555 highest recovery (HR) and highest solvent efficiency (HE). The three bars on the left display the results
 556 of the extraction with hexane (yellow background) whereas the three bars on the right are the results of 2-
 557 methyltetrahydrofuran (green background). Results are shown as the mean of six replicates $\pm$ standard
 558 deviation. Different, capital letters above each stacked column display statistically significant ($p < 0.05$)
 559 differences between the total amount of lipids extracted according to the respective procedure.

560 When comparing the results from the six extractions, several differences can be observed.

561 Firstly, although the Soxhlet extractions were able to extract a higher amount of TAG compared
 562 to the wet extractions, this difference was smaller for '2-MeTHF HR'. Secondly, it was found
 563 that for both solvents, the wet extractions yielded a significantly higher ($p < 0.05$) amount of
 564 DAG than the Soxhlet extraction, except for '2-MeTHF HE'. This increase might be attributed
 565 to the hydrolysis of TAG into DAG and MAG during the wet extraction process [47] and/or
 566 during frozen storage [34]. The increase was more pronounced for 2-MeTHF, which may be
 567 due to its higher polarity and subsequently the more efficient extraction of more polar lipid
 568 classes [58]. Considering that MAG are slightly more polar than TAG, it was plausible that not
 569 all MAG were extracted during the wet extraction with hexane [16]. Thirdly, the extract '2-

MeTHF HR' contained higher amounts of FFA compared to hexane. It was also observed that for 2-MeTHF, the FFA content of the wet extraction HR was similar compared to Soxhlet. In contrast, the wet extractions with hexane extracted significantly less FFA (8.37 – 9.69 g/100 g DM) compared to the Soxhlet extraction (11.94 g/100 g DM). These high amounts of FFA might be attributed to the hydrolysis of TAG during extraction and/or during frozen storage, hereby liberating FFA. It may be that the higher polarity of 2-MeTHF resulted in a more efficient extraction of FFA, both at moderate temperature (i.e., 45 °C during the wet extraction) and at elevated temperature (Soxhlet extraction).

A fourth difference was observed in the extracted amounts of phosphatidylcholine (PC) and phosphatidylethanolamine (PE). While hexane could only extract 0.02 – 0.09 g PC/100 g DM and 0.10 – 0.90 g PE/100 g DM, 2-MeTHF was able to extract higher amounts of PC and PE, being 0.04 – 0.20 and 0.21 – 1.58 g/100 g DM respectively. When the results from the wet extractions are compared with those of the Soxhlet extraction, it can be noticed for both solvents that the Soxhlet extraction is able to extract a higher percentage of phospholipids. This was as expected because it was assumed that the lipid extraction would be complete after 18 hours at a cycling rate of 10 – 12 cycles per hour. As such, the results from the Soxhlet extraction can be considered as reference. Moreover, it can also be deduced from the graph that the wet extractions with 2-MeTHF are able to extract a higher amount of the phospholipids relative to the Soxhlet extraction, compared to hexane. Although phospholipids are generally not desired in the oil industry, they are a valuable resource and can easily be refined [18,47]. As such, the increased phospholipid extraction with 2-MeTHF might be an advantage depending on the application.

When the lipid class compositions obtained in this study are compared with those found in the recent article on the lipid extraction of dried BSFL with 2-MeTHF, several differences can be seen [16]. In their study, they only determined the relative content of TAG, DAG, MAG, FFA,

ergosterol and phospholipids via HPTLC. A first difference was that much lower quantities of TAG were found (5 – 10 %) in their study, compared to the results in this study. Additionally, although high amounts of FFA were present in all the extracted lipids obtained in this study, these values are considerably lower compared to those found in their research as the relative FFA content was around 50 % and around 65 % for their extracts with hexane and 2-MeTHF, respectively [16]. This higher amount of FFA may be attributed to the fact that they used freeze-dried BSFL without blanching step as it already known that the pre-treatment of the BSFL prior to processing can have a high impact on the lipid composition [34]. Although phospholipids were also analysed by Ravi and colleagues [16], it was not possible to compare the results obtained in this study with theirs since they are displayed as a fraction of the total phospholipid content.

As already mentioned in Sections 3.1 and 3.2.4, the significantly higher lipid content obtained with 2-MeTHF could be explained by its higher polarity and consequently its ability to also extract more polar lipids as well. As can be seen on Fig. 4 and in Table S1, the results of the lipid class determination also confirmed this hypothesis since higher amounts of phospholipids and free fatty acids were extracted. It was also observed that the wet extraction with 2-MeTHF showed a higher similarity to the Soxhlet extraction, in comparison to hexane. This may also be an explanation for the fact that the lipid recovery of 2-MeTHF was always higher than the lipid recovery of hexane, as can be seen in Table 4 in Section 3.2.4.

4. Conclusions

Black soldier fly larvae (BSFL) can be reared efficiently on low-value organic side streams, though valorisation of their lipids is typically performed using solvent extractions that are harmful for the environment. Although hexane is still commonly used as extraction solvent in industry, greener solvents such as 2-methyltetrahydrofuran (2-MeTHF) have been receiving more attention over the past few years. Using the design of experiments (DoE) approach, the

procedures for extracting lipids from fresh BSFL with hexane and 2-MeTHF were studied in detail and two sets of optimal conditions have been identified. Using the process conditions determined via response surface methodology, either the lipid recovery or the solvent efficiency could be maximised. When maximising the lipid recovery, the wet extraction with 2-MeTHF was able to extract 95 % of the BSFL lipids present. More specific, around 41 g lipids/100 g DM could be extracted by using these conditions, indicating that this wet extraction procedure with 2-MeTHF at a moderate temperature of 45 °C may be a promising alternative to the conventional extraction of dried BSFL (± 40 g lipids/100 g DM). In contrast, the wet extraction with hexane was only able to extract 60 – 70 % of the lipids present. When maximising the solvent efficiency, there was no significant difference between the efficiencies of hexane and 2-MeTHF, being 10.07 ± 0.22 and 10.56 ± 0.53 g/100 mL of solvent respectively. It was also shown that the fatty acid profiles of the different lipid extracts were highly similar. Concerning the lipid classes of the extracted lipids, 2-MeTHF was able to extract more phospholipids and free fatty acids compared to hexane. However, depending on the application, it may be preferential to extract all lipids, both polar and nonpolar, especially since phospholipids are considered a valuable resource. Additionally, it was also shown that the wet extractions with 2-MeTHF had a similar composition to the Soxhlet extraction. In contrast, significantly lower amounts of triacylglycerols, free fatty acids, and phospholipids were obtained using the wet extraction with hexane. As such, the biomass-derived solvent 2-methyltetrahydrofuran can be considered a suitable replacement for hexane for the lipid extraction of black soldier fly larvae. Moreover, the wet extraction procedure may even replace the conventional lipid extraction from dried BSFL, hereby lowering the energy cost of the process.

CRediT authorship contribution statement

Ruben Smets: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Visualization, Writing – Original Draft

645 **Peter Goos:** Methodology, Formal analysis, Writing – Review & Editing

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647 **Mik Van Der Borcht:** Conceptualization, Writing – Review & Editing, Supervision, Project
648 administration

649 **Declaration of Competing Interest**

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

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