SHORT COMMUNICATION

European Journal of Lipid Science and Technology

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Fractionation of Oil from Black Soldier Fly Larvae (*Hermetia illucens*)

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Black soldier fly larvae (BSFL; *Hermetia illucens*) are subjected to a conventional fishmeal process, or room-temperature formic acid hydrolysis, and lipid yield and composition between the two processes compared. Acid hydrolysis of BSFL results in higher protein yield in the meal and higher oil yield. Oils separated after acid hydrolysis have a lower trilaurin content (triacyglycerol with lauric acid (12:0) in all *sn*-positions) and a lower melting point (23 °C) compared to oils separated after conventional (fishmeal) processing (26 °C). Further reduction of trilaurin content and melting point (20 °C) are achieved by dry-fractionation (winterization) of the oil.

***Practical Applications*: Fractionation of black soldier fly larvae oil could yield products with targeted levels of trilaurin and melting points adapted to different applications in feeds, foods, and cosmetics.**

1. Introduction

BSFL are a rich source of protein and minerals that to a large extent can replace fish meal or soy products in feeds for farmed animals.[1–3] In addition, products developed from side-streams can be used in food and cosmetics.[4,5] Insects will be an important resource in the circular economy and can be co-located with producers or users of primary products (e.g., grain producers, breweries) to utilize nutrient substrate side-streams and heat.[6] The substrate can furthermore be selected to target desired protein and fat composition, but also content of specific amino acids, fatty acids and minerals.[7–9] Substrate selection and optimal harvest timing are important for efficient growth and to achieve the targeted nutritional composition of the larvae.

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DOI: 10.1002/ejlt.202100252

BSFL has a fat content in the range of 12–34%[10] so efficient defatting is essential to produce a meal with high level of protein. Reduced fat content has previously been reported in BSF larvae,[11] and several substrates are suggested to grow high protein/low fat larvae.[12] BSFL fat is also an important resource in many applications. However, BSFL oil is dominated by a few fatty acids (mainly 12:0, lauric acid)[13] which limit its inclusion levels in cold-water aquaculture feeds. These feeds normally include fish oil and rapeseed oil with a large distribution of fatty acids including polyunsaturated acids.

Replacement of corn oil with BSFL oil at 0, 4, 6 and 8% showed a linear increase of growth in nursery pigs.[14] In addition, lauric acid from BSFL demonstrated antimicrobial properties on gastrointestinal bacteria. While 5% inclusion of BSFL oil to a basal broiler chicken diet had no effect on growth,[15] 50 and 100% replacement of soybean oil with BSFL oil reduced growth in broiler chickens.[16] In a diet to rainbow trout (*Oncorhynchus mykiss*), replacement of fish oil with BSFL oil had no effect on growth. This was performed in freshwater RAS on trout growing from 50 to 350g. Atlantic salmon (*Salmo salar*) given three diets where 100% of the fish meal in the control was replaced with two BSFL meal with a total lipid content 17 or 26% respectively, increased the lauric acid content from 0.2 mg/g in the control diet to 11 and 16 mg/g in the respective BSFL diets. The replacement reduced growth, but appeared not to affect digestibility of lauric acid.[2] However, the percentage of lauric acid in the whole fish was unchanged between the dietary groups, contrary to Belghit[17] who observed a linear increase in whole fish lauric acid content in salmon fed 1/3, 2/3 and 100% replacement of fish meal with defatted BSFL meal (18% lipid). While Lock[2] performed their study with post-smolt salmon (250 g) in tanks, Belghit[17] used salmon in sea cages (1.4–3.7 kg), which could indicate that utilization of lauric acid is different at different sizes of fish.

Although digestibility in salmon appears not to be affected by BSFL lipid inclusion, reduced lipid digestibility has previously been observed in Atlantic salmon given feed with a high content of saturated fatty acids. Examples include diets with palm oil as the only coated oil,[18] lard from bovines,[18] or high inclusion of *Schizochytrium sp*. microalgae-biomass.[20] Common for these is a high inclusion of palmitic acid (16:0), which is known to be digested slower than lauric acid. Furthermore, the high melting point due to a large fraction of triacylglycerols (TAG)

with saturated fatty acids at all three *sn*-positions,[21] makes these structures less soluble in bile. This affects content viscosity and digestibility in the gut,[22] and may thus pose technical challenges in feed and food applications. Increased inclusion of BSFL meal or oils in diets has generally caused increased saturated fatty acid content in the edible meat products.[17,23] The content of saturated fatty acids in meat is closely related to the texture. Thus an increased ratio of polyunsaturated fatty acids is more desirable both for meat quality and human health.[24] Nevertheless, the high saturated fatty acid content of the BSFL could be beneficial in terms of energy and antimicrobial activity.[25–27] Various methods are available for altering the lipid composition and properties. Hydrolysis and/or fractionation of lipids with low digestibility can increase availability of lipid structures for digestibility and improve technical properties. The hydrolysis of lipids could be controlled by lipases[28] or acid hydrolysed with endogenous enzymes.[29] Dry fractionation/winterization is a partial crystallization of a fat followed by separation of the liquid and solid phases (olein and stearin) which have lower and higher melting points, respectively, than the starting lipid. As is known from the palm oil industry, a variety of BSFL oil fractions with targeted melting points and technofunctional properties could be produced by iterative.[30] In the present study lipid classes, fatty acids and TAG isomers are analyzed in BSFL oils after two different down-stream processes and winterization.

2. Experimental Section

BSFL, received on dry ice from Bestico B.V. (www.bestico.nl), were stored frozen (−20°) until use. The larvae were heated to 90 °C in a microwave oven (10 min) to inactivate endogenous enzymes (specifically lipases). To 2000 g BSFL was added 5.0 g Grindox 1032 Danisco/Dupont (Copenhagen, Denmark) antioxidant blend (20% tocopherol-rich extract of natural origin (E306)). The mixture was homogenized and divided into two equal portions.

2.1. Down-stream processing

2.1.1. Acid hydrolysis

A glass vessel was charged with 1000 g BFSL and 25 g 99% formic acid (VWR, Leuven, Belgium). The thick mass was stirred at room temperature for 66 hours. The viscosity of the mass was unchanged after the process, and therefore 500 g tap water was added and stirring continued for 3.5 hours. The mixture was heated to 90 °C and kept at that temperature for 10 min. After centrifugation (Sorvall LYNX 6000, Thermo scientific, Waltham, MA, USA) at 20 000 *g* for 10 min, the liquid phase was decanted from the solids (45.4%) into a separatory funnel, and oil (7%) and stickwater (47.5%) were separated. C*onventional down-stream process (fishmeal process)*: The other half of the BSFL mass was mixed with 500 g tap water and heated to 90 °C and kept at that temperature for 10 min with continuous overhead mechanical stirring of the mass. Centrifugation and separation of the phases was performed as described above, yielding solids (44.3%), oil (5.4%) and stickwater (50.3%).

2.2. Winterization

Oil separated after acid hydrolysis (18.84 g) was stored at 20 °C overnight, resulting in partial crystallization. After centrifugation at 20 °C, 20 000 *g*, 30 min, the olein (liquid phase, 8.66 g, 46% yield) was decanted from the stearin (the solid phase, 11 g, 54% yield). Both fractions were analyzed for lipid classes, fatty acids, TAG isomers and slip melting point.

2.3. Slip Melting Point Analysis

Melting point of the oils were determined by a modified AOCS method Cc3-25[31] to facilitate melting points below ambient temperature. The samples were melted at 50 °C and filtered through filter paper. A 10 mm plug of the melted fat were taken up in a glass capillary tubes (i.d 1 mm). The tubes were immediately placed horizontally in a freezer at −18 °C overnight. The tubes were the next day gradually heated in water and melting point temperature determined when the sample slips and rises inside the tubes. The analyses were performed in triplicate.

2.4. Fatty Acid Analysis by GC

Fatty acid methyl esters (FAME) were prepared according to AOCS method Ce1b-89[32] by transesterification of oils with methanolic NaOH followed by methylation with boron trifluoride in methanol. The FAME solution was extracted and diluted with isooctane to approximately 50 µg/mL. The GC analyses was conducted on a Trace GC gas chromatograph (Thermo Fisher Scientific) with flame ionization detector (GC–FID). The FAMEs were identified by comparing the elution pattern and relative retention time with the reference FAME mixture (GLC-793, Nu-Chek Prep Inc., Elysian, MN, USA). Fatty acid composition was calculated by using 23:0 fatty acid methyl ester as an internal standard and reported on a sample basis as g/100 g fatty acid methyl esters.

2.5. Lipid Class Analysis by HPLC-CAD

Lipid classes were analyzed based on methods published by Homan and Anderson[33] and Moreau.[34] Approximately 50 mg oil were weighed into a 50-mL volumetric flask. The sample was dissolved in chloroform to a final volume of 50 mL, and 20 µL of the solution were injected on an HPLC system (Perkin-Elmer, Waltham, MA, USA) equipped with an ESA Corona® Plus charged aerosol detector (ESA Biosciences, Inc., Chelmsford, MA, USA). The samples were separated on a LiChrosphere® 100, 5 µm diol column, 4 × 125 mm (Merck, Darmstadt, Germany). The lipid components were identified by comparing their retention times with those of commercial standards and their concentrations were quantified through external standard curves using a second order polynomial fit.

2.6. Lipidomics

TAG subclasses were quantified at VTT (Espoo, Finland). Analyses were performed on a Waters Q-Tof Premier mass

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spectrometer combined with Acquity ultra-performance liquid chromatography (UPLC). The identification of TAGs was based on VTT´s lipid MS/MS-library and on literature data. Trilaurin contents were quantified by using a calibration curve of trilaurin reference compound within a concentration range from 1.25 to 62.5 mg/mL.

2.7. Statistics

Excel for Windows was used for general calculations. All statistical analyses were performed using Statistica 13 software for Windows (StatSoft Inc., Tulsa, USA). Trilaurin content in the different oils were subjected to General Linear model analysis by one-way ANOVA for detection of significance of differences ($P<0.05$), followed by Tukey post hoc test. Replicates for other TAG isomers were not available, and these were therefore subjected to linear regression against the trilaurin content between the different oils.

3. Results and Discussion

Black soldier fly larvae's high tolerance to a range of substrate makes it a potentially important species for the future circular economy world-wide.[10,35] Substrate composition and harvest time make it possible to engineer nutrient composition to a certain extent, though high levels of lauric acid appear to be inherent in the larvae.[36,37] Processing to a final ingredient also affects its properties and the qualities of feeds incorporating the ingredient. The BSFL is a high protein/high fat raw material, analyzed in the present study to have a dry matter content of 37%, of which 38% are fat, 40% protein and 5% ash on dry matter basis. A defatting process that preserves the quality of the protein is of major interest for the feed markets.[23] Heat and pressure are the main process parameter used to remove fat from a protein source. Too high temperature will affect protein quality and digestibility.[38] In the present study we used conventional down-stream process, as used for fish meal, with temperature < 100 °C and fractionation by centrifugation, which is known to produce high quality ingredients. However, the yield of BSFL oil could be improved by, for example, addition of protease enzymes. Acid hydrolysis with formic acid, is commonly used to preserve raw materials (e.g., fish silage;[29]), and takes advantage of endogenous enzyme for hydrolysis. In the present study endogenous enzymes were inactivated to prevent total hydrolysis to FFA.[39] High levels of FFA may affect both oxidative stability and digestibility negatively.[40] Acid hydrolysis prior to oil separation reduced losses of protein to the stickwater and increased separation of fat to the oil phase (73.6% of the fat) in the present study. Thus, producing a high protein solid phase (meal), compared to conventional down-stream process where only 55.4% of the fat was separated in the oil phase (**Table 1**).

Ingredients that are partly hydrolyzed are known to increase feed intake as long as the gut is not overloaded with products that are not absorbed thus affecting gut filling and ingredient digestibility.[40,41] As expected, oil of BSFL after acid hydrolysis contained a lower TAG content, but also a higher content of FFA (8.1% after acid hydrolysis compared to 3.9% after conventional process) and unidentified lipid classes (most likely partially hydrolyzed lipid classed). How these products will affect feed intake or digestion of these lipids is unknown. However, feed intake and digestion are to a large extent determined by the fatty acid composition in the lipid structures.

Table 1. Composition BSFL raw material and after process, and yields to different phases (DM = Dry matter; N.A. = Not analyzed).

	Raw material	Conventional process			Acid hydrolysis process		
		Stickwater	Solids	Oil	Stickwater	Solids	Oil
Dry matter (%)	37.0	9.3	34.5		8.6	31.6	
Fat (Bl& D ; %DM)	37.8	0.0	27.0		0.0	16.5	
Protein (Nx6,25; %DM)	39.5	44.1	50.4		34.9	57.6	
Ash (%DM)	5.4	10.8	5.5		17.4	4.1	
Protein yield (%)	100	20.3	75.8	N.A.	14.3	83.0	N.A.
Fat yield (%)	100	N.A.	42.3	55.4	<0.1	24.7	73.6

A total of 97% of the fatty acids in the oils were identified, mainly being lauric acid (36–37%), followed by 16:0 (15%), 18:1 (13–14%) and 18:2 (15–16%) (**Table 2**). Short chain saturated fatty acids (< C14:0) are known to be readily absorbed, while medium chain saturated fatty acids (C16:0 and C18:0) could lower digestibility – particularly if these appear in all positions of the triglyceride (TAG) structured as tripalmitin and tristearin, respectively. Whereas, trilaurin and trimyristin (TAG with only C12:0 or C14:0) are readily digested and absorbed by mammalians,[42] low temperature can affect digestibility of saturated fatty acids in poikilothermic animals as fish, where chain length and melting point determine digestibility.[22] In the present study acid hydrolysis prior to oil separation lowered the melting point of the oil (23 °C) compared to oil separated from conventional process (26 °C) (Table 2). The lower melting point may be a result of the higher level of FFAs. High degree of fat separation, partly hydrolyzed lipids and lower melting point appear to be advantages of acid hydrolysis prior to oil separation of BSFL. However, further reduction in the content of lauric acid and/or lower melting point may make BSFL oil available for new food applications. Thus, fractionation for targeted markets might be a solution, especially winterization, which is a cost-effective method to separate oils with different melting points.

Oil separated after acid hydrolysis was assumed to be the main interest for winterization as this allowed us to target a low melting point. The process lowered the portion of identified lipid classes, giving slightly lower content in olein (66%) compared to stearin (73%). Olein had lower content of TAG (54%), and higher level of FFA (9%) compared to stearin (65 and 7%, respectively). The content of lauric acid was lower in olein (31%) compared to the stearin fraction (41%), and the olein contained more monounsaturated fatty acids (16:1, 18:1 and 20:1), *n*-3 and *n*-6 polyunsaturated fatty acids. Olein had also, as expected, a lower melting point (20°) compared to stearin (26°) (Table 2).

Lipid structures of TAG with saturated fatty acids in all three *sn*-positions are often associated with lower lipid digestion.[42]

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Table 2. Lipid classes and fatty acids in BSFL oil after process.

BSFL oils	Conventional process	Acid hydrolysis	Winterization	
	BSFL-oil	BSFLE-oil	BSFLE olein	BSFLE stearin
Triacylglycerol	86.0	70.0	56	65
Diacylglycerol	0.5	0.5	1.0	0.6
Monoacylglycerol	<1.0	<1.0	<1.0	<1.0
Free fatty acids	3.9	8.1	8.9	6.9
Cholesterol esters	<0.5	<0.5	<0,5	<0,5
Cholesterol	<0.5	<0.5	<0,5	<0,5
Phosphatidylethanolamine	<0.5	<0.5	<1.0	<1.0
Phosphatidylinositol	<1.0	<1.0	<1.0	<1.0
Phosphatidylserine	<1.0	<1.0	<1.0	<1.0
Phosphatidylcholine	<1.0	<1.0	<1.0	<1.0
Lyso-Phosphatidylcholine	<0.5	<0.5	<1.0	<1.0
Total polar lipids	<1.0	<1.0	<1.0	<1.0
Total neutral lipids	90.4	78.6	65.9	65.9
Total sum lipids	90.4	78.6	65.9	72.9
12:0	36.7	36.3	30.9	40.6
14:0	8.1	7.9	6.3	9.2
16:0	14.7	14.7	14.4	15.0
18:0	2.7	2.8	2.9	2.6
20:0	1.8	2.0	1.9	1.5
Sum SFA	63.7	64.0	56.5	69.0
16:1*n*-7	1.7	1.8	2.0	1.6
18:1(*n*-9)+(*n*-7)+(*n*-5)	12.6	13.5	15.2	12.0
20:1(*n*-9)+(*n*-7)	1.1	1.2	1.4	1.1
Sum MUFA	15.4	16.5	18.6	14.7
18:2n-6	14.6	15.8	17.7	14.0
Sum *n*-6 PUFA	14.6	15.8	17.7	14.0
18:3n-3	0.5	0.6	0.7	0.5
18:4n-3	0.3	0.3	0.4	0.3
Sum *n*-3 PUFA	0.8	0.9	1.1	0.8
Sum identified FA	97.3	96.9	93.9	98.5
Sum unidentified FA	2.7	3.1	2.9	2.4
Melting point (°C)	26.0	23.0	20.0	26.0

Although, lauric acid from BSFL is a highly digestible fatty acid – high portion of trilaurin may also challenge optimal digestibility if included at high levels in diets to animals (especially in fish at low temperature). Lipidomics of BSFL oil in the present study confirms that the oils melting point is correlated to the quantified trilaurin content (**Figure 1**). Oil separated after acid hydrolysis had a significantly lower trilaurin content (198mg/g) compared to BSFL oil separated from conventional process (216mg/g). Concluding from the results above, a higher content of FFA and lower melting point after acid hydrolysis, confirms that trilaurin are determining for the melting point. Furthermore, olein fraction after winterization showed the lowest content of trilaurin (168mg/g), while melting point and trilaurin content in the stearin (216mg/g) were comparable to levels found in oil separated from conventional down-stream process of BSFL raw material (Figure 1).

Only minor changes in the content of other TAG isomers were observed between oils from different processes, and after winterization. In total 25 isomers of TAG were identified, although, the method did not distinguish between position isomers *sn*-1/3 and *sn*-2 for most of them, and shown only summarized chain length for the fatty acids in TAG. Example as TAG(38:0), with only saturated fatty acids and according to fragmentation of MS contains only lauric acid and myristic acid in a 2:1 ratio –, e.g., TAG(12:0/12:0/14:0), but *sn*-position of myristic acid is unknown. For TAG(40:0) mainly lauric acid and palmitic acid, but may also have myristic acid. From MS-results majors isomers being TAG (12:0/12:0/16:0) and a minority being TAG (14:0/14:0/12:0). Analysis of the isomers confirms the high portion of saturated fatty acid in BSFL oils with 22 – 25% of the identified TAG isomers having 12:0, 14:0 and/or 16:0. In addition, there were several TAG isomers with 18:1 and 18:2. Except trilaurin (Figure 1), no large changes in other TAG isomers were observed as an effect of process. Differences between oil separated after acid hydrolysis compared to conventional down-stream process are also reflected in differences between olein and stearin phase after winterization. With overall, olein having the lowest content if three-saturated TAG (22%) and stearin having the highest content (25%), but otherwise small changes in isomers, only indications of some isomers with monosaturated fatty acid to be more dominant in olein. The TAG isomer composition of the olein fraction is comparable to a 3:2-ratio mix between BSFL oil from conventional down-stream processing and fish oil (FO), but with a contribution of more unsaturated fatty acids from FO (**Table 3**).

Producers of BSFL are today mainly targeting production of high-quality protein meal for the highest paying feed and food markets. Customized processes for development of products to different market segments, species and different life stages of animals may be the future for these resources. High-protein meals are of high interest for several aquaculture species, including Atlantic salmon. Poultry and swine production may utilize ingredients with lower protein content. Similarly, BSFL oil could be processed to target markets. Today, BSFL oil is used in feeds to pig nurseries and appear to be beneficial for their development and health.[15] If further studies are shown that trilaurin is an important nutrient for this effect, fractionation of the oil could target some of the oil to more exclusive markets (compare with palm oil).

4. Conclusions

Oil extraction efficiency could be improved by acid hydrolysis prior to down-stream processing of BSFL. The separated oil from conventional down-stream process has a high content of trilaurin giving a melting point at 26 °C that may cause technical issues for some feed and food applications. Lower trilaurin content and melting point can be achieved by acid hydrolysis of BSFL and/or winterization of the oils. This will allow producers of BSFL to tailor oil properties to various markets.

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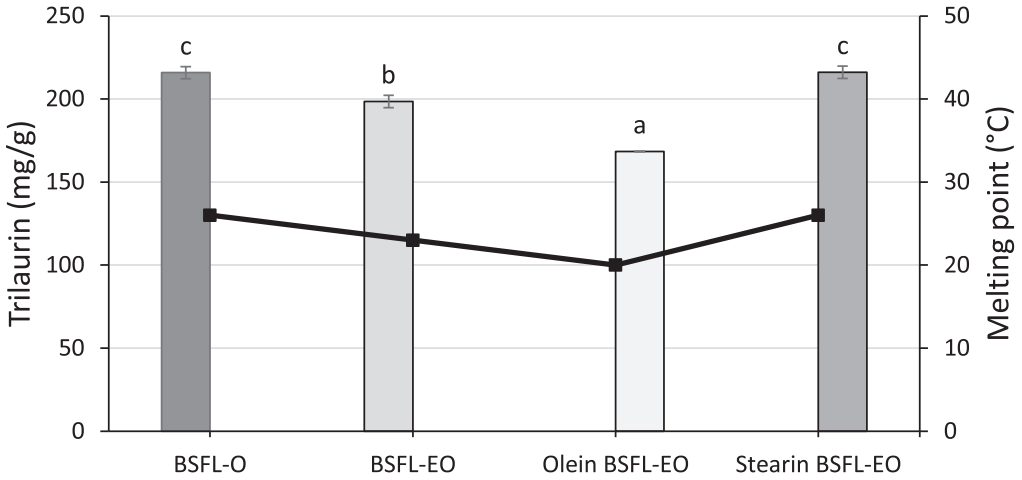

Figure 1. Trilaurin content in processed oil from BSFL after conventional down-stream process (BSFL-O), acid hydrolysis (BSFL-EO), olein- and stearin phases after winterization. Average ± standard deviation; $n = 2$). ANOVA, followed by Tukey post hoc test ($P<0.05$). Black line indicates melting point of the oils.

Table 3. Other identified TAG-isomers in processed oil from BSFL after conventional down-stream process (BSFL-O), acid hydrolysis (BSFL-EO), olein- and stearin phases after winterization, and in a BSFL-fish oil (FO) mix.

TAG isomers (% of sum identified)	BSFL-O	BSFL-EO	Olein BSFL-EO	Stearin BSFL-EO	Regression		BSFL-O : FO (3:2)
					R^2	*P*	
TAG(36:0)	6.84	8.09	7.73	8.10	0.48	0.71	7.90
TAG(38:0)	7.51	8.00	6.82	7.92	0.58	0.22	7.77
TAG(40:0)	7.40	7.63	7.40	8.81	0.50	0.32	8.04
TAG(42:1)	2.97	3.23	3.32	2.64	**0.66**	**0.04**	2.73
TAG(42:0)	5.61	5.61	3.65	6.66	0.86	0.23	3.31
TAG(44:2)	2.93	2.55	2.85	2.64	0.02	0.12	2.70
TAG(44:1)	2.31	2.23	2.47	2.13	**0.65**	**0.03**	2.05
TAG(46:2)	6.99	7.97	6.51	6.62	0.03	0.28	7.68
TAG(46:1)	6.72	5.57	6.49	7.00	0.10	0.34	4.50
TAG(48:3)	3.99	4.42	4.54	3.73	**0.76**	**0.03**	3.95
TAG(48:2)	5.60	5.84	5.89	4.54	0.39	0.10	4.66
TAG(14:0/16:0/18:1)	3.51	3.47	3.93	3.31	**0.85**	**0.01**	2.83
TAG(48:0)	0.62	0.56	0.33	0.88	0.78	0.22	0.47
TAG(50:3)	2.20	1.91	2.17	1.85	0.12	0.13	1.95
TAG(14:0/18:1/18:1)+TG(16:0/16:1/18:1)	4.27	4.36	4.80	3.78	**0.77**	**0.03**	3.59
TAG(50:1)	2.92	2.64	2.74	2.44	0.18	0.14	2.80
TAG(18:1/16:1/18:2)+TG(18:2/18:2/16:0)	5.21	5.36	5.89	6.06	0.08	0.12	4.85
TAG(16:0/18:2/18:1)+TG(16:1/18:1/18:1)	7.43	6.98	6.96	8.51	0.45	0.46	5.42
TAG(16:0/18:1/18:1)	4.14	3.96	4.21	3.60	0.35	0.06	4.12
TAG(54:5)	3.71	3.34	3.92	2.95	0.41	0.10	2.53
TAG(18:1/18:2/18:1)	3.36	2.92	3.55	2.78	0.38	0.11	2.53
TAG(18:1/18:1/18:1)	2.63	2.37	2.64	2.13	0.25	0.12	2.39
TAG(16:0/18:1/20:1)+TG(18:0/18:1/18:1)	1.04	0.93	1.12	0.85	0.45	0.09	3.67
TAG(56:2)	0.06	0.05	0.07	0.04	0.36	0.17	4.09
TAG(58:2)	0.01	0.02	0.01	0.01	0.84	0.48	3.47

TAG isomer regression to trilaurin content between different BSFL processed oils

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Acknowledgements

This study was supported by the Research Council of Norway (RCN) through the Nofima basic grant (194 050/F40). The authors also want to thank the skillful laboratory personnel at Nofima BioLab.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

A.S.B. Conceptualization; Formal analysis; Investigation; Methodology; Project administration; Resources; Validation; Visualization; Writing – original draft; Writing – review & editing.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

acid-hydrolysis, black soldier fly larvae, lipidomics, oil fractionation, oil separation efficiency, trilaurin, winterization

Received: December 21, 2021
Revised: February 4, 2022
Published online: February 25, 2022

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