Enzymatic interesterification of heterotrophic microalgal oil with rapeseed oil to decrease the levels of tripalmitin

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Abbreviations:

CAD	Charged aerosol detector
DAG	Diacylglycerol
DHA	docosahexaenoic acid
FFA	Free fatty acids
FO	Fish oil
GC	Gas chromatograph(y)
HM	Heterotrophic microalgae
HPLC	High pressure liquid chromatography
LC-PUFA	Long-chained polyunsaturated fatty acids
MAG	Monoacylglycerol
QTOF MS	Quadrupole Time of Flight Mass Spectrometer
RO	Rapeseed oil
SMP	Slip melting point
TAG	Triacylglycerol

Abstract High lipid heterotrophic microalgae (HM) *Schizochytrium limacinum* is a good dietary source of long-chained polyunsaturated fatty acids. HM biomass have successfully been used in aquafeeds. However, the high saturated fatty acid content of the triacylglycerol (TAG) could limit the applications of HM as main feed lipid source. Enzymatic interesterification of HM oil with unsaturated oils may increase the utilization efficiency and remove the technical challenges in using such oils. In the present study, we mixed extracted oil from HM biomass (Alltech Inc.) with rapeseed oil and interesterified them enzymatically with either Lipozyme RM IM or Lipozyme 435 in reactions with no addition or 5 % addition of distilled water. The experimental oil mixes were formulated to target a total fatty acid profile similar to fish oil. No addition of water to the reaction mixture led to more efficient TAG recovery after interesterification. Overall, enzymatic interesterification of HM and rapeseed oils with

Lipozyme RM IM produced oils with lower levels of tri-saturated TAG isomers, higher content of TAG isomers with unsaturated fatty acids and lower slip melting point. Animal studies need to be performed to evaluate the biological effects of interesterified against unprocessed highly unsaturated oils.

Practical Applications: Heterotrophic microalgal oil as a pure product could have limited application in feeds and foods due to its structural content of triacylglycerol (TAG) with saturated fatty acids in all three positions due to both nutritional and practical reasons. Enzymatic interesterification of HM oil with rapeseed oil was in the present study shown to be an efficient technology to produce oils with more desirable TAG composition and reduced melting point for feed and food applications compared to the original raw material.

Introduction

The future growth of the aquaculture production is dependent of finding novel lipid sources with high content of essential omega-3 long-chained polyunsaturated fatty acids (n-3 LC-PUFA) [1]. The heterotrophic microalgae (HM) *Schizochytrium limacinum* provide such ingredients with high lipid content (55-77 % in dry matter) and high levels of docosahexaenoic acid (DHA) [2, 3]. Moreover, HM appears to be a good source of n-3 LC-PUFA for gilthead seabream *Sparus aurata* L [4] and Atlantic salmon *Salmo salar* L [5-8]. The high content of saturated fatty acids in HM, particularly palmitic acid (16:0), has however shown to lower the apparent digestibility of the saturated fatty acid fraction in diets for farmed salmon but not that of the mono and polyunsaturated ones, when HM is used to replace the supplemented dietary fish oil (FO) [6]. Increased dietary content of saturated fatty acids will in general increase the lipids' melting point and has also been shown to affect apparent lipid digestibility in fish [9]. However, lower apparent digestibility of saturated fatty acids is also observed in diets with HM compared to diets without, even when balanced to contain similar total levels of saturated fatty acids [7]. The high content of saturated fatty acids and their *sn*-position in the triacylglycerol (TAG) molecule, i.e. the potential presence of considerable amounts of fully saturated TAGs, is the most likely reason for the analyzed lower apparent digestibility of saturated fatty acids in HM lipids in salmon.

Enzymatic interesterification is a method that would modify the physicochemical properties of HM TAG by TAG hydrolysis and re-esterification of fatty acids to new positions, which could increase lipid digestibility of HM saturated lipids. In several food research areas,

enzymatic interesterification is used to remove and substitute position specific TAG fatty acids by others more desirable ones [10-11], creating products that could reach new markets. This method specifically targets ingredients with a high content of saturated fatty acids and high melting point, such as lard and tallow. Several studies have shown that enzymatic interesterification of solid fats in a blend with TAG lipids from sources with higher content of unsaturated fatty acids, increases the content of desirable TAG isomers. Palmitic acid at *sn*-2 position and unsaturated fatty acids at the *sn*-1,3 positions is facilitating digestion and absorption of nutrients, and is the most common TAG isomer in e.g. human milk fat [12]. These are often the targeted palmitic acid containing TAG isomers when lard or tallow are interesterified with highly unsaturated plant oils to reduce their content of fully saturated TAGs [11, 13-15].

Time and enzyme load are the main costs of most enzymatic processes, including interesterification, and higher enzyme load can compensate for longer reaction time. Zou et al. [11] showed that the tallow fat *sn*-2 16:0 content was reduced from 70 % to 62 % and 50 % after enzymatic interesterification with plant oils and Lipozyme RM IM load at 5 and 14 %, respectively, in a 6 hours' reaction. However, with only 3 hours incubation with an enzyme load of 14 %, the content of *sn*-2 16:0 was at the same level as after 6 hours with 5% enzyme. Temperature is another significant parameter in enzymatic reactions, which should be set at defined optimal levels. Moreover, water increases the speed of the interesterification process, mainly in terms of increased TAG hydrolysis rate. However, high reaction water level will increase the content of the resulting free fatty acids (FFA) that are not re-esterified to TAGs. This could affect the final product stability and nutritional value, as FFA can form calcium soaps in the intestine that negatively affect nutrient absorption [9] and may cause constipation [16].

Fish lipases hydrolyze *sn-2* fatty acids (with a bile salt-dependent lipase) that could increase the content of FFA 16:0 [17] when digesting 16:0 rich TAG containing ingredients compared to mammals that mainly secrete *sn*-1,3 position specific pancreatic lipase, which would produce *sn*-2 16:0 monoacylglycerols (MAG) that are more readily absorbed [18]. Hydrolysis of saturated fatty acids occurs slower compared to unsaturated fatty acids [9, 17]. Tripalmitin, i.e. TAG with 16:0 in all 3 positions, a common lipid in solid fat and most likely present in HM lipids, will therefore cause slower hydrolysis and absorption of these lipids compared to sources with a more varied content of TAG isomers.

In aquaculture context, and in order to be able to best utilize the HM lipids, rich in fully saturated TAG, enzymatic interesterification with other commonly used oils (e.g. rapeseed oil

[19]) can be considered as a pre-processing step to increase the bioavailability of these lipids. The present study exploits enzymatic interesterification of extracted HM oil and rapeseed oil (RO) using commercially immobilized *sn*-1,3-specific Lipozyme RM IM and *sn*-unspecific Lipozyme 435 as a method to decrease the saturation degree of TAG isomers in these oil blends.

Materials and Methods

Lipid sources

Rapeseed oil was purchased from Emmelev AS (Otterup, Denmark). Alltech Inc (Kentucky, USA) provided spay dried biomass of the heterotrophically produced *Schizochytrium limacinum*, a high-fat/high-DHA microalgae species.

Oil extraction

The HM biomass oil yield was studied using different lipid extraction methods for the selection of the most appropriate one to use in the large-scale oil extraction step of the present trial. In traditional laboratory scale oil extraction of 1-100 g samples it is common to use published methods [20-22]. However, large-scale oil extraction methods include often, simple one-solvent systems. In the present study, three solvents were tested separately (hexane, ethyl acetate and petroleum ether). In addition, we evaluated an up-scaled Bligh & Dyer [21] method and a super-critical-CO_2 extraction method.

Fatty acid analysis by GC

Fatty acid methyl esters (FAME) were prepared according to AOCS Official Method Ce 1b-89 [23] by transesterification of lipid sources or interesterified oil mixes with methanolic NaOH followed by methylation with boron trifluoride in methanol. C23:0 methyl ester was added as internal standard. The FAME solutions were extracted and diluted with isooctane to approximately 50 µg/mL. The GC analyses were conducted on a Trace GC gas chromatograph (Thermo Fisher Scientific) with flame ionization detector (GC–FID), with a 60 m x 0.25 mm BPX-70 cyanopropyl column with 0.25 µm film thickness (SGE, Ringwood, Victoria, Australia). Helium 4.6 was used as carrier gas at 1.2 mL/min constant flow. The injector temperature was 250 °C and the detector temperature was 260 °C. The oven was programmed as following: 60 °C for 4 min, 30 °C /min to 145 °C, then 1.2 °C /min to 217 °C, and 100 °C

/min to 250 °C, where the temperature was held for 7 min. The sample solutions (3.0 µl) were injected in splitless mode and the split was opened after 2 min. 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). Chromatographic peak areas were corrected by empirical response factors calculated from the areas of the GLC-793 mixture. Fatty acid compositions were 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.

Lipid class analysis by HPLC-CAD

Lipid classes were analyzed based on methods published by Homan and Anderson [24] and Moreau [25]. Approximately 50 mg oil were weighed into 50-mL volumetric flasks. The samples were dissolved in a total volume of 50 mL chloroform, and 20 µL of the solution were injected in a HPLC system (Perkin-Elmer, Waltham, MA) equipped with an ESA Corona® Plus charged aerosol detector (ESA Biosciences, Inc., Chelmsford, MA). The samples were separated on a LiChrosphere® 100, 5 µm diol column, 4 × 125 mm (Merck). A ternary gradient consisting of solvent A = isooctane, B = acetone/dichloromethane (1:2) and C = 2-popanol/methanol/acetic acid–ethanolamine–water (7.5 mM ethanolamine and 7.5 mM acetic acid) (85:7.5:7.5) was used with the following profile: at 0 min, 100:0:0 (% A:% B:% C); at 1 min, 90:10:0; at 8 min 70:30:0; at 11 min 40:50:10; at 13 min 39:0:61; at 26.3 min 40:0:60; at 28.4 min 0:100:0; at 30.9 min 100:0:0. The lipid components were identified by comparing their retention times with those of commercial standards and their content were quantified through external standard curves using a second order polynomial fit. The standards were obtained from Nu-Chek Prep, Inc., Elysian, MN (cholesterol ester, MAG, diacylglycerol (DAG), FFA) and Sigma–Aldrich, St Louis, MO (cholesterol, PE, PC, lyso-PC). Each sample analysis was performed in duplicate.

Enzymatic interesterification

Commercial Lipozyme RM IM with immobilized lipase from *Rhizomucor miehei* and Lipozyme 435 with immobilized lipase from *Candida antarctica* (Novozymes AS, Bagsvaerd, Denmark) were used as catalysts for enzymatic interesterification. We mixed 10 g pre-heated HM oil and 7 g RO in 100 mL glass bottles to achieve a calculated total distribution of fatty acids similar to fish oil (FO) (Table 1). The enzymatic reactions were performed in triplicate with 5 % enzyme concentration (w/w total reactants) during 8 hours with continuous shaking at either 60 or 80 ^{0}C, for Lipozyme RM IM or Lipozyme 435, respectively [13-14, 26]. Two

experiments were performed for each enzyme either with addition of distilled water to a total water content of 5% in the reaction (Trial 1) or no addition of water to the reaction (Trial 2), as previous results have shown that increased moisture content increase interesterification efficiency and reduce slip melting point (SMP) of the oil following interesterification [11, 13-14]. The reactions were terminated by heating the oils to 100 °C for 15 min.

Oil slip melting point determination

The slip melting point of the experimental oils were determined by the AOCS Official method Cc 3-25 [27], modified to facilitate analysis of samples with melting points below ambient temperature. Briefly, crystallized samples were melted at 50 °C and filtered through filter paper. Glass capillary tubes (i.d. 1 mm) were dipped in the melted fat to take up a 10 mm plug of sample in the tubes. The tubes were immediately placed horizontally in a freezer at -18 °C to allow crystallization of samples with low melting points and held at -18 °C overnight. The melting points of the samples were determined by monitoring the temperature at which the sample slips and rises inside the tubes when immersed in gradually heated water on a heating plate with magnetic stirring. Each analysis was performed in triplicate.

Oxipres

The induction periods (IP, hours) of the test oils were determined using an Oxipres apparatus (Mikrolab Aarhus, Denmark). We weighed 2.00 ± 0.02g oil samples into clean glass reaction containers and inserted them into pressure vessels at room temperature. The vessels were flushed with oxygen three times, then filled with oxygen (5.0, AGA AS, Norway) to 5 bar and then inserted into a preheated heat block held at 90 °C. Data sampling of pressure in the vessels initiated and repeated at 30 seconds' intervals. The induction period was determined graphically from the intersection of two tangents to the pressure curve using Paralog Software Version 3.10, build 422 (Mikrolab Aarhus, Denmark) (Fig. 1).

Lipidomic analysis

A chloroform/methanol (v/v 2:1) solution (400 µl) was added to 5 mg of accurately weighted oil samples. Samples were vortexed for 2 min, then allowed to stand for 30 min and centrifuged. Following, the samples were further diluted 1:10, 1:100 and 1:1000 with chloroform/methanol (v/v, 2:1) solutions and an internal standard mixture containing labeled lipids PC (16:0/0:0-D_3), PC (16:0/16:0-D_6) and TAG (16:0/16:0/16:0-$^{13}C_3$) was added. Stock solution of the standard compound TAG (17:0/17:0/17:0) was made in chloroform/methanol (v/v, 2:1) and further

diluted with the solvent containing the RO matrix in the same proportions as in the samples. The samples were analyzed on a Waters Q-Tof Premier mass spectrometer (Waters, USA) combined with an Acquity Ultra Performance LC™ (UPLC). The column was an Acquity UPLC™ BEH C18 2.1×100 mm with 1.7 µm particles. The solvent system included (A) ultrapure water (1% 1M NH_4Ac, 0.1% HCOOH) and (B) LC/MS grade acetonitrile/isopropanol (1:1, 1% 1M NH4Ac, 0.1% HCOOH). The gradient started from 65% A / 35% B, reached 80% B in 2 min, 100% B at 7 min, and remained there for 7 min. The flow rate was 0.400 ml/min and the injected amount was 1 µl (Acquity Sample Organizer, at 4°C). Reserpine was used as the lock spray reference compound. The lipid profiling was carried out using ESI in positive mode, and the data were collected at a mass range of m/z 300-1200 with scan duration of 0.2 sec. A set of standard samples (n=4) were analyzed together with the test samples in order to follow the instrument's performance. The QTOF MS instrument was calibrated according to the manufacturer's instructions and continuous Lock mass spray was used to assure mass accuracy.

The complete lipidomics data were processed using MZmine2 and Guineu softwares, and the samples were diluted 1:10 in order to detect as many compounds as possible as the detection limit is approximately 0.01 µmol/g and the abundant TAG were over the linear range of the method. The data processing included alignment of peaks, peak integration, normalization, and peak identification. TAGs were identified using an internal lipid library containing retentions times and *m/z* values of earlier identified lipids, Identification of certain TAGs were additionally confirmed by running MS/MS spectra. The data were normalized using the labeled internal standard TAG (16:0/16:0/16:0-$^{13}C_3$) to yield relative concentrations of all identified TAGs as µmol/g oil.

Statistics

Excel for Windows was used for general calculations of TAG hydrolysis (as the percent of each TAG content after enzymatic interesterification compared to the respective level prior to the reaction), content of TAG isomers with tri-saturated, tri-monounsaturated and a mixture of different fatty acids, and content of TAG isomers with 16:0 at *sn*-2. All statistical analyses were performed using Statistica 13 software for Windows (StatSoft Inc., Tulsa, USA). Data were subjected to General Linear model analysis by two-way ANOVA for detection of significance of differences ($P<0.05$), followed by Tukey post hoc test.

Results

HM-biomass oil extraction

The laboratory scale oil extraction of 20 g HM-biomass batches gave an oil yield of 63 and 65 % of the sample for Bligh & Dyer [21] and Folch et al [22] methods, respectively. Contrary, up-scaled one-solvent methods extracted only 10, 14 and 19 % oil of the samples, with hexane, ethyl acetate and Soxhlet methods, respectively. While, the super-critical CO_2 method extracted no more than 2 and 9 % oil from 1 and 5 g samples, respectively. An up-scaled Bligh & Dyer method was therefore used to extract the oil from 800-1200 g HM biomass samples. This method also showed variation in oil extraction efficiency, with higher oil yield at smaller sample sizes (Table 2).

Lipids and fatty acid composition of oil substrates

The composition of lipid classes was present in the following order of abundance in the HM extracted oil: TAG > FFA > cholesterol. One % phospholipids were detected in the laboratory but not identified in the HM oil extracted with the up-scaled method, while DAG were only detected in the HM oil extracted by the up-scaled method. Rapeseed oil had no other detected lipid classes than TAG (Table 3).

The characteristics of RO were like that of other plant oils, with high content of 18:1 (60%) followed by 18:2n-6 (20%), 18:3n-3 (10%) and some minor content of saturated fatty acids (Table 1). The HM extracted oil has in comparison only two major fatty acids, 16:0 (50%) and 22:6n-3 (22%). Fatty acids in FO, compared to RO and HM oil, have a high content of monounsaturated fatty acids (22:1/20:1/18:1) followed by saturated fatty acids (16:0>18:0/ 14:0) and several polyunsaturated fatty acids (22:6n-3>20:5n-3>18:4n-3>18:2n-6/18:3n-3). However, FO fatty acid profiles vary largely according to their origin from different fish species, harvest areas and seasons [28] (Table 1).

Analysis of fatty acid isomer composition of TAG molecules showed that the identified RO TAG are composed mainly of a mixture of saturated, monounsaturated and polyunsaturated fatty acids (82%) and only 18% tri-monounsaturated fatty acid TAG. In comparison, in the HM oil we identified 64% tri-saturated TAG, 36% mixed TAG and a negligible content of TAG with only monounsaturated fatty acids (Table 4).

Enzymatic interesterification

Oils from extracted HM and RO were mixed at a ratio 10:7, respectively, to achieve a total fatty acid composition comparable to that of a South American FO [28]. The total content of n-3 PUFA in the final mix was thus lower than in the HM oil, while the contribution of monounsaturated fatty acids from RO lowered the content of saturated fatty acids in the mixture compared to the respective levels in the HM oil (Table 1).

Higher moisture level in the reaction mix (Trial 1) resulted in significantly lower content of TAG and higher content of FFA when using Lipozyme RM IM compared to Lipozyme 435 (ANOVA, $P<0.01$). The initial HM/RO oil mix with 97% TAG was reduced by 63% to a TAG content of 35% after 8 hours reaction with Lipozyme RM IM, with the main reaction products being FFA followed by DAG, whereas no MAG were detected (Fig. 2). The TAG content of the oil mix after interesterification with Lipozyme 435 was not affected by the moisture content of the reaction. Lower moisture content (Trial 2) improved recovery of TAG after an 8 hour reaction with Lipozyme RM IM (82 %), but still significantly lower content of TAGs were analyzed compared to reactions with Lipozyme 435 (88 %) (ANOVA, $P<0.01$).

A total of 151 TAG isomers were separated in the analyzed oil samples. The extracted HM oil had high content of tri-saturated TAG isomers (261 µmol/g oil) that were not present in RO. On the other hand, the RO has higher content of tri-monounsaturated TAG isomers (97 µmol/g oil) and TAG isomers with a mixture of different fatty acids (432 µmol/g oil) that were present at lower levels in the HM oil (2 and 147 µmol/g oil, respectively). By mixing these oils, the high content of tri-saturated TAG isomers in HM oil was therefore diluted to 154µmol/g in the new oil mix and increased the content of TAG isomers with tri-monounsaturated and mixed fatty acid compared to the original HM oil (Fig. 3). Following interesterification, the total TAG isomer content reflected the interesterification efficiency of the performed experiments, in the following (descending) order: Trial 2 (low moisture) with Lipozyme 435 (TAG 1322±90 µmol/g oil) > Trial 2 (low moisture) with Lipozyme RM IM (TAG 1197±44 µmol/g oil) > Trial 1 (high moisture) with Lipozyme 435 (TAG 861±28 µmol/g oil) > Trial 1 (high moisture) with Lipozyme RM IM (TAG 820±268 µmol/g oil). Fatty acid *sn*-position was identified only in 43 out of the 151 separated TAG isomers. Those included 14 TAG isomers with tri-saturated fatty acids, 2 TAG isomers with tri-monounsaturated fatty acids and 27 TAG isomers with a mixture of different fatty acids (data not shown).

Enzymatic interesterification lowered the content of tri-saturated TAG isomers in the HM/RO mixes (Fig. 3). The resulting tri-saturated TAG of the interesterified oils were significantly lower with Lipozyme RM IM compared to Lipozyme 435 (ANOVA, $P<0.05$) for both reaction moisture levels. The final content of tri-monounsaturated TAG was significantly

higher in Trial 1 (high moisture) with use of Lipozyme 435 compared to Lipozyme RM IM and compared to both enzymatic reactions in Trial 2 (ANOVA, $P<0.05$). The content of TAG with mixed fatty acids after interesterification significantly varied with moisture level, with significantly higher final content in at low (Trial 2) compared to high (Trial 1) reaction moisture levels (ANOVA, $P<0.05$). The particularly higher resulting TAG hydrolysis level with the use of Lipozyme RM IM compared to Lipozyme 435 in Trial 1 was only reflected in the resulting lower content of tri-saturated and tri-monounsaturated TAG but not in the content of mixed TAG. At lower reaction moisture levels, the resulting content of the targeted mixed TAG increased, from 147 µmol/g in the HM oil and 264 µmol/g in the oil mix to 544 and 511 µmol/g in the oils after interesterification in Trial 2 with Lipozyme 435 and Lipozyme RM IM, respectively. This was also reflected in the slip melting point, which in Trial 2 (low moisture) was reduced from 46 °C in HM oil (Table 2) to 35 and 26 °C in the interesterified oils using Lipozyme 435 and Lipozyme RM IM, respectively (Fig. 4). In comparison, the slip melting points of the interesterified oils from Trial 1 (high moisture) were higher, 41 and 33 °C, with Lipozyme 435 and Lipozyme RM IM, respectively. Considering both trials, interesterification with Lipozyme RM IM resulted in oils with significantly lower slip melting point compared to Lipozyme 435 (ANOVA, $P<0.05$). Interesterified oil mix with Lipozyme RM IM from Trial 2 (low moisture), with a lower slip melting point and higher content of TAG with mixed fatty acids, had a shorter oxipres induction period (8 hours) compared to the oil mix from Trial 1 (35 hours) interesterified with Lipozyme RM IM (Fig. 5).

Total fatty acid profile analysis showed that 16:0 is the most abundant fatty acid in HM oil (Table 1). This was reflected in the TAG isomers, where 26 of the 43 identified TAG isomers had one or more 16:0 esterified to the molecule (data not shown). TAG isomers with 2 or 3 16:0 were present at higher content in the HM oil and were significantly reduced following interesterification (Table 4). Lipozyme RM IM with either high or low reaction moisture level (Trial 1 and 2, respectively) reduced the content of tri-saturated TAG isomers in the interesterified oils, significantly more compared to Lipozyme 435 (ANOVA, $P<0.05$). On the other hand, higher interesterification reaction moisture level (Trial 1) resulted in significantly lower levels of most identified tri-saturated TAG isomers compared to low reaction moisture levels (Trial 2) (Two-way ANOVA, $P<0.05$). There was however a significantly higher content of TAG isomers with 16:0 at *sn-2* in Trial 2 compared to Trial 1 (ANOVA, $P<0.05$; Fig. 6). This was mainly due to the presence of significantly higher levels of TAG isomers with mixed fatty acid at *sn*-1,3 positions in Trial 2 compared to Trial 1. The high contribution of oleic acid (18:1n-9) in RO was also highly reflected in the resulting TAG isomers, where 22 out of 29

identified TAG isomers containing unsaturated fatty acids were esterified with one or more 18:1n-9 molecules. In general, most identified mixed TAG isomers were present at significantly higher content following interesterification with lower (Trial 2) compared to higher (Trial 1) reaction moisture levels. In addition, the type of enzyme used had significant effect on the resulting levels of the different mixed TAG identified (Two-way ANOVA, $P<0.05$; Table 4).

The second most abundant fatty acid in HM oil 22:6n-3 (DHA), was identified in 3 TAG isomers (14:0/14:0/22:6; 14:0/16:0/22:6; 22:6/18:1/18:1) in the analyzed interesterification product oils. While the content of the TAG isomer 14:0/14:0/22:6 decreased and 14:0/16:0/22:6 remained unchanged at a low level, 22:6/18:1/18:1 isomers increased to a significantly higher content after interesterification. The content of these isomers were significantly higher after interesterification with low (Trial 2) compared to high (Trial 1) reaction moisture levels and by the use of Lipozyme RM IM (Two-way ANOVA, $P<0.05$), with a resulting particularly lower content of 22:6/18:1/18:1 after interesterification with Lipozyme 435 in Trial 1 (Table 4). TAG isomers with 18:2n-6 (linoleic acid) identified in RO were hydrolyzed to a larger extent compared to other unsaturated fatty acids, except when using Lipozyme 435 where we analyzed significantly higher content of these isomers compared to when using Lipozyme RM IM in Trial 1 (ANOVA, $P<0.05$) (Table 4).

Discussion

Heterotrophic microalgae biomass (HM) is a promising high lipid DHA-rich ingredient that can substitute FO in aquafeeds [4-8] or be used as DHA supplement in other feeds and foods. However, as we showed in the present study, 64% of the identified TAG isomers in extracted HM oil have saturated fatty acids at all 3 positions of the TAG molecule. This is most likely the reason for the reduced apparent digestibility of the saturated fatty acid fraction in HM diets in Atlantic salmon reared at low water temperatures (ca. 10 °C) [7]. Reduced emulsification and enzymatic hydrolysis due to the high melting point of these lipids and calcium soap formation of free saturated fatty acids [9] are probably the main reasons for this effect. Moreover, should HM oil be used in feed production and not whole HM biomass [6-7], this would be in solid state at ambient conditions posing technical challenges that feed manufacturers would need to overcome. Enzymatic interesterification of HM oil with unsaturated oils can increase the utilization efficiency and remove the technical challenges in using such oils. In the present study, we achieved significant reduction of the fully saturated TAG isomers in HM and a

significantly increase in TAG isomers with unsaturated fatty acids following interesterification of HM oil with RO using Lipozyme RM IM and Lipozyme 435.

In the present study, the extracted HM oil was mixed with RO resulting in an oil mix with similar fatty acid profile to that of FO. The use of RO in our study is also relevant in terms of the current commercial practices in Atlantic salmon farming [19]. A mix of oils from HM and rapeseed will in general dilute the content of tri-saturated TAG isomers and increase the total digestibility of the oils mix in the feed but will not increase the digestibility of the fully saturated TAG isomers. In the present study TAG isomers abundant in RO increased 1-10-fold in a mix with HM oil interesterified with Lipozymes. In addition, one TAG isomer with DHA, present at low levels in HM oil, increased 10-fold following interesterification. Enzymatic interesterification therefore opens new possibilities for making customized functional n-3 LC PUFA oils for the aquafeed industry and other food or feed applications.

Water is essential for performing lipid hydrolysis. However, moisture content in an interesterification process over a certain level will favor hydrolysis and reduce re-esterification of fatty acids to TAG [11, 13-14]. This was confirmed in the present study, where higher hydrolysis degree was achieved by adjusting the moisture content of the reaction mix to 5% (Trial 1) compared to reactions with no water addition (Trial 2) and thus only contribution of moisture by the enzyme products used (2% in Lipozyme 435 and 4% in Lipozyme RM IM). Higher reaction moisture content resulted in significant increased hydrolysis of TAG to FFA only in the case of Lipozyme RM IM and not Lipozyme 435, as also shown in a study with interesterification between beef tallow and RO by Gruczynska et al [26]. Contrary, Kowalski et al [13-14] showed that increasing reaction moisture content from 2 % to 10 % increased TAG hydrolysis to a larger degree for beef tallow interesterified with RO using Lipozyme 435 compared to Lipozyme RM IM. The reason for this discrepancy is unknown but could be due substrate specificity for the enzymes. In the present study, the relative efficiency in hydrolysis and re-esterification of FFA to form new TAG species of the two Lipozymes products used was significantly different also with no addition of water. This strengthens the theory that endogenous water content of the enzyme product may affect the enzyme's function and be part of the reason for the differences between the enzymes in Trial 2.

Our experiments showed that the main products of the hydrolysis were FFA and DAG, whereas no MAG were detected. This agrees with other studies in enzymatic interesterification of oils where it has been reported that the resulting MAG content was in general low (~1%) due to fast esterification to DAG in the presence of FFA [29]. In our study, this was not true at higher moisture levels using Lipozyme RM IM, where more FFA and less TAG and DAGs

were present following the reaction. Nevertheless, re-esterification of FFA to new TAG was efficient at lower reaction moisture levels with both enzymes tested, and at higher moisture level using Lipozyme 435.

The slip melting point (SMP) as a consequence of saturated fatty acids in lipid sources is known to affect lipid digestibility [9]. Kowalski et al [13-14] showed that reaction time, temperature and catalyst load affected SMP in interesterification products after reactions of beef tallow and RO with the same enzymes used in the present study. The authors measured SMP in a range between 19 and 28 °C in separated TAG products by column chromatography, while SMP in the crude interesterified product was 2-3 °C lower. HM oil SMP was 46 °C, while SMP in RO in known to be below 0 °C. SMP for beef tallow was not reported in the reviewed studies, where the authors concluded that the reduced SMP following interesterification was a result of altered TAG structure due to exchange of fatty acids within and between TAG molecules. Enzymatic interesterification with Lipozyme RM IM resulted in significantly lower SMP in the products of the present study, probably reflecting the lower content of tri-saturated TAG isomers, compared to when Lipozyme 435 was used.

Apparently, Lipozyme 435 has the ability to hydrolyze all fatty acids on all three positions of TAG and resulted in the highest total TAG levels following interesterification in the present study. Nevertheless, a significantly higher content of TAG isomers with unsaturated fatty acids was observed after interesterification with Lipozyme RM IM. TAG-hydrolysis in Atlantic salmon favors formation of 1,2/2,3-DAG prior to 1,3-DAG [17]. Hydrolysis is thus more likely to start at *sn*-1/3, with faster hydrolysis of unsaturated compared to saturated fatty acids in fish [9, 17]. The use of Lipozyme 435 for exchanging fatty acids at *sn-2* does not give any advantage compared to Lipozyme RM IM that is mainly *sn*-1/3-specific. Contrary to the present study that showed an increase of TAG isomers with 16:0 in *sn-2* after interesterification at lower reaction moisture levels, Gruczynska et al [26] showed that Lipozyme 435 reduced the content of this TAG fraction. This will depend on the substrates used and have different implications depending on the scope of each experiment. In the case of our study where we aimed at reducing the total content of fully saturated TAG isomers, the positioning of the esterified unsaturated fatty acids after interesterification is not expected to have a practical importance other than that 1,3-positioned unsaturated fatty acids are hydrolyzed and absorbed faster compared to saturated fatty acids in fish. In the present study we observed a reduction in the content of tri-saturated TAG isomers in the processed HM/RO mixes compared to the original product. This was a result of *sn*-1/3 hydrolysis of 16:0 that made available a large amount of MAG and DAG with

16:0 at *sn*-2 that were then esterified to mixed TAG isomers with unsaturated fatty acids in *sn*-1/3, resulting thus, in a transformed oil expected to be more digestible than the original one.

However, the TAG products with lower SMP and higher content of unsaturated fatty acids oxidized faster (8 hours) compared TAG with higher SMP and higher content of fully saturated TAG (35 hours). Kowalski et al [13-14] showed that interesterified lipids were faster oxidized (4-5 hours) compared to the initial blend (15 hours). The author also isolated TAG from these interesterified samples and showed that these were oxidized even faster (< 2 hours) than the crude products containing all hydrolyzed fractions and concluded that the lower oxidative stability was a result of change in TAG structure. However, this could also be a result of small scaled experiment with a low amount of oil exposed to air compared to industrial process with considerable larger ratio of oil:air in a closed system. Lipid hydrolysis products, especially containing unsaturated fatty acids, would however in general increase the speed of oxidation [30] in agreement with the present results, and in practice they would need to be used more cautiously in terms of antioxidant protection during further processing and storage.

In summary, the present study showed that enzymatic interesterification of a mixture of oils from HM and rapeseed significantly reduces tri-saturated TAG isomers, increasing the content of TAG isomers with a mixture of saturated and unsaturated fatty acids and decreasing the slip melting point of the oils. Lipozyme 435 was more efficient in the production of TAG compared to Lipozyme RM IM under the conditions of our study, given that the latter contained higher moisture levels (4%) compared to the former (2%), which may have affected the results. Overall, enzymatic interesterification with Lipozyme RM IM produced a final oil with lower levels of tri-saturated TAG isomers, higher content of TAG isomers with unsaturated fatty acids and lower slip melting point. Lipozyme 435 which is not position specific in its hydrolytic activity did not reduce the content of *sn-2* 16:0 TAG, which gave the *sn*-1,3 specific Lipozyme RM IM an advantage in the fast exchange of palmitic acid molecules at these positions forming a higher number of TAG isomers with reduced content of palmitic acid.

Enzymatic interesterification of HM oil is thus an efficient technology to produce customized oils with desirable TAG isomer composition. Larger scale studies need to be performed to evaluate the biological importance of using interesterified against unprocessed oils.

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Compliance with Ethical Standards

Conflict of interest There are no conflicts of interest to report.

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Figure captions

Fig. 1 Oxipres induction period (IP) of oils, determined by the intersection of two tangents.

Fig. 2 Triacylglycerol (TAG), diacylglycerol (DAG) and free fatty acids (FFA) in initial heterotrophic microalgae (HM) oil / rapeseed oil (RO) mix and following enzymatic interesterification in reaction with: A) 5% water (Trial 1), and B) with no addition of water (Trial 2) by the use of Lipozyme 435 (L 435) and Lipozyme RM IM (L RM IM). Values are mean ± standard deviation; n=3. Data were subjected to two-way ANOVA for the variable moisture level, enzyme and the interaction of these, followed by Tukey post hoc test for single lipid classes (P<0.0.5).

Fig. 3 TAG isomer composition of only saturated fatty acid (Saturated TAG isomers), only monounsaturated fatty acids (Monounsaturated TAG isomers) or a mixture of different fatty acids (Mixed TAG isomers) of heterotrophic microalgae (HM) oil, rapeseed oil (RO), a mixture of 10g HM oil and 7 g RO, and after enzymatic interesterification in reaction with 5% water (Trial 1) and with no addition of water (Trial 2) by the use of Lipozyme 435 (L 435) and Lipozyme RM IM (L RM IM). Values are mean ± standard deviation; n=3. Data were subjected to two-way ANOVA for the variable moisture level, enzyme and the interaction of these, followed by Tukey post hoc test for single TAG isomers (P<0.0.5).

Fig. 4 Slip melting point of heterotrophic microalgae (HM) oil, and after enzymatic interesterification in reaction with 5% water (Trial 1) and with no addition of water (Trial 2) using Lipozyme 435 (L 435) and Lipozyme RM IM (L RM IM). Values are mean ± standard deviation; n=3. Data were subjected to two-way ANOVA for the variable moisture level, enzyme and the interaction of these, followed by Tukey post hoc test (P<0.0.5).

Fig. 5 Oxipres induction period (IP, hours) of a mixture of 10g HM oil and 7 g RO after enzymatic interesterification with Lipozyme RM IM in reactions with 5% water (Trial 1; n=2) and with no addition of water (Trial 2, n=3).

Fig 6 Content of TAG isomers with 16:0 at *sn*-2 in heterotrophic microalgae (HM) oil, rapeseed oil (RO), a mixture of 10g HM oil and 7 g RO (Oil mix), and after enzymatic interesterification in reaction with 5% water (Trial 1) and with no addition of water (Trial 2) by the use of Lipozyme 435 (L 435) and Lipozyme RM IM (L RM IM), respectively. Values are mean n=3 ± standard deviation. Data were subjected to two-way ANOVA, for variables moisture level, enzyme type and the interaction, followed by Tukey post hoc test (P<0.0.5).

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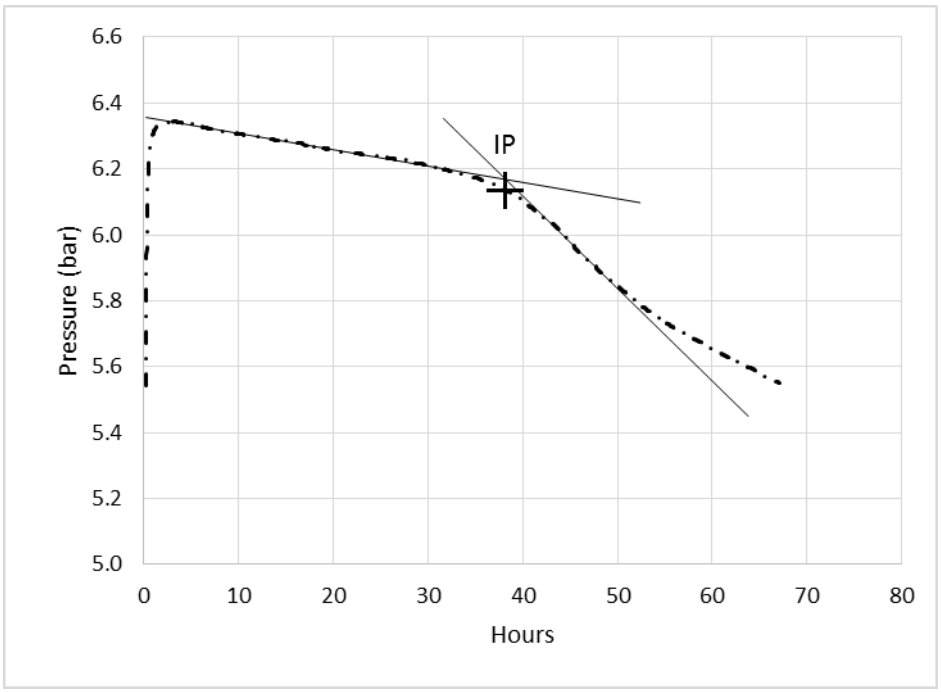

585

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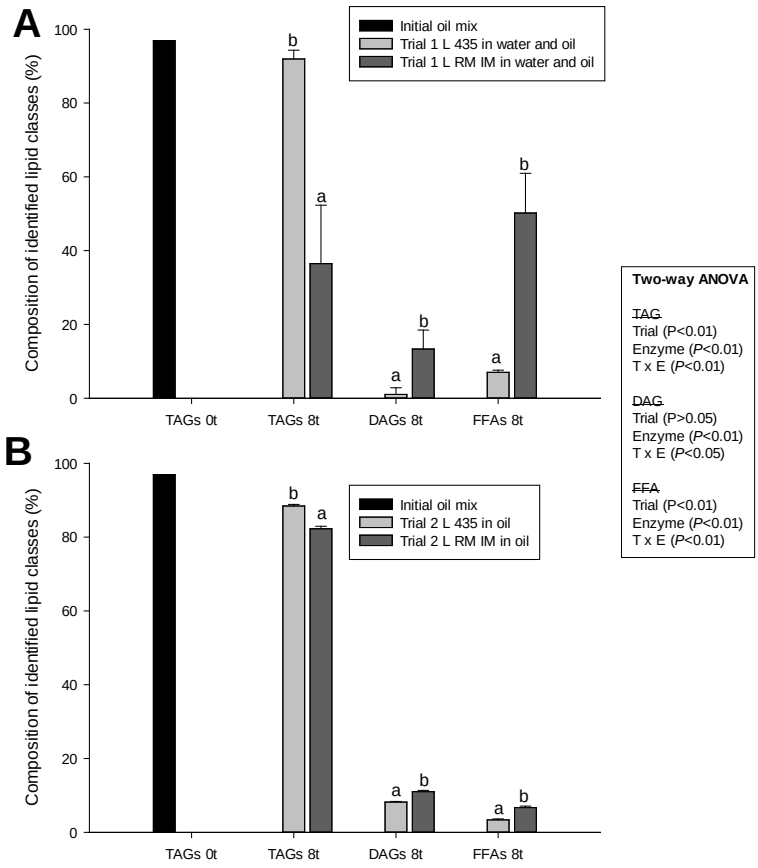

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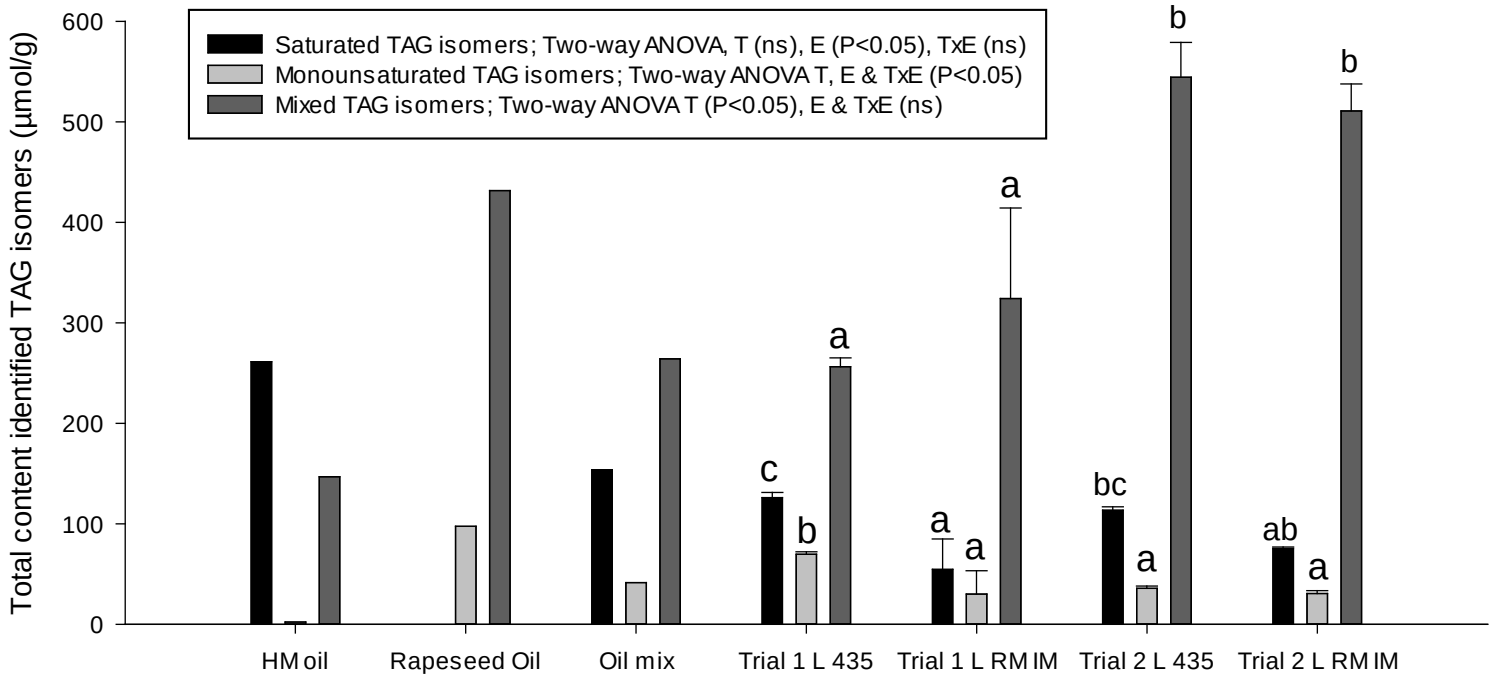

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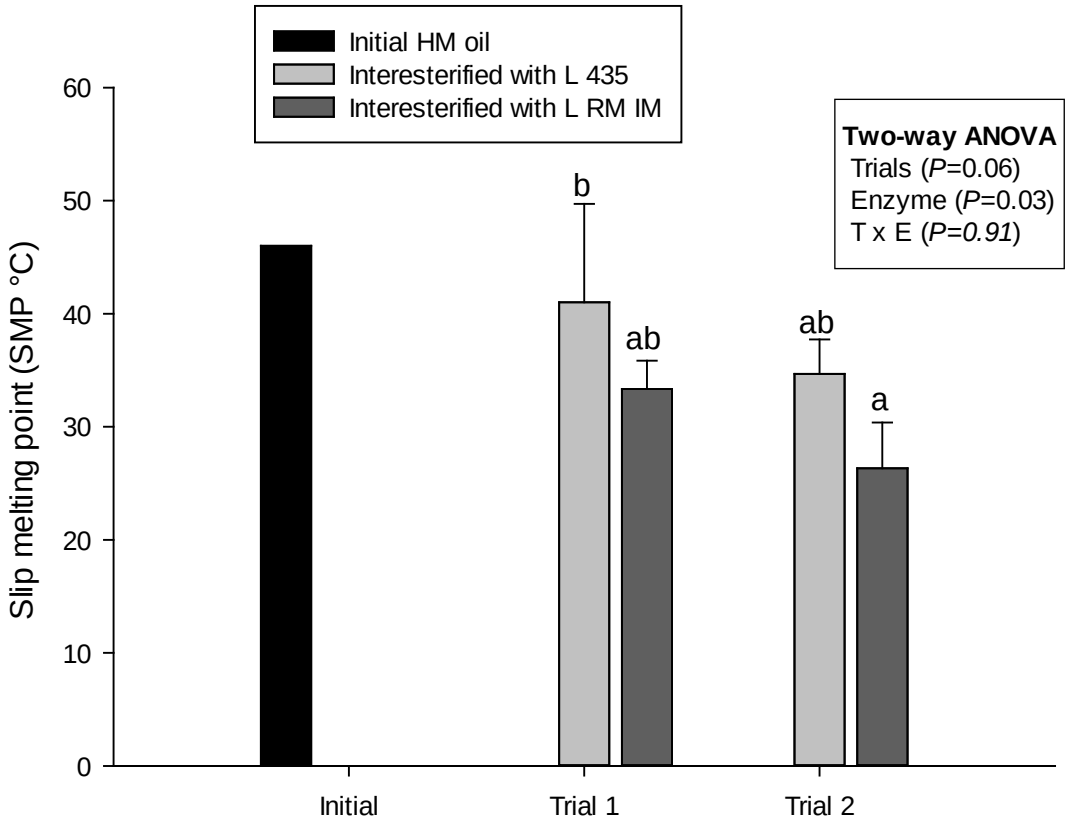

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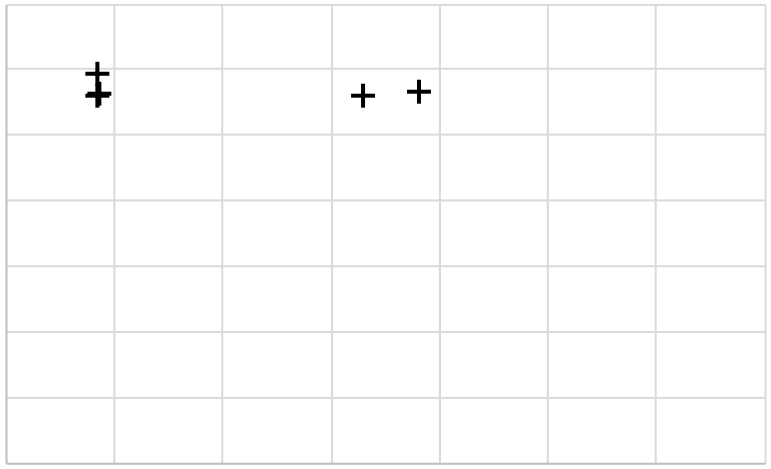

607

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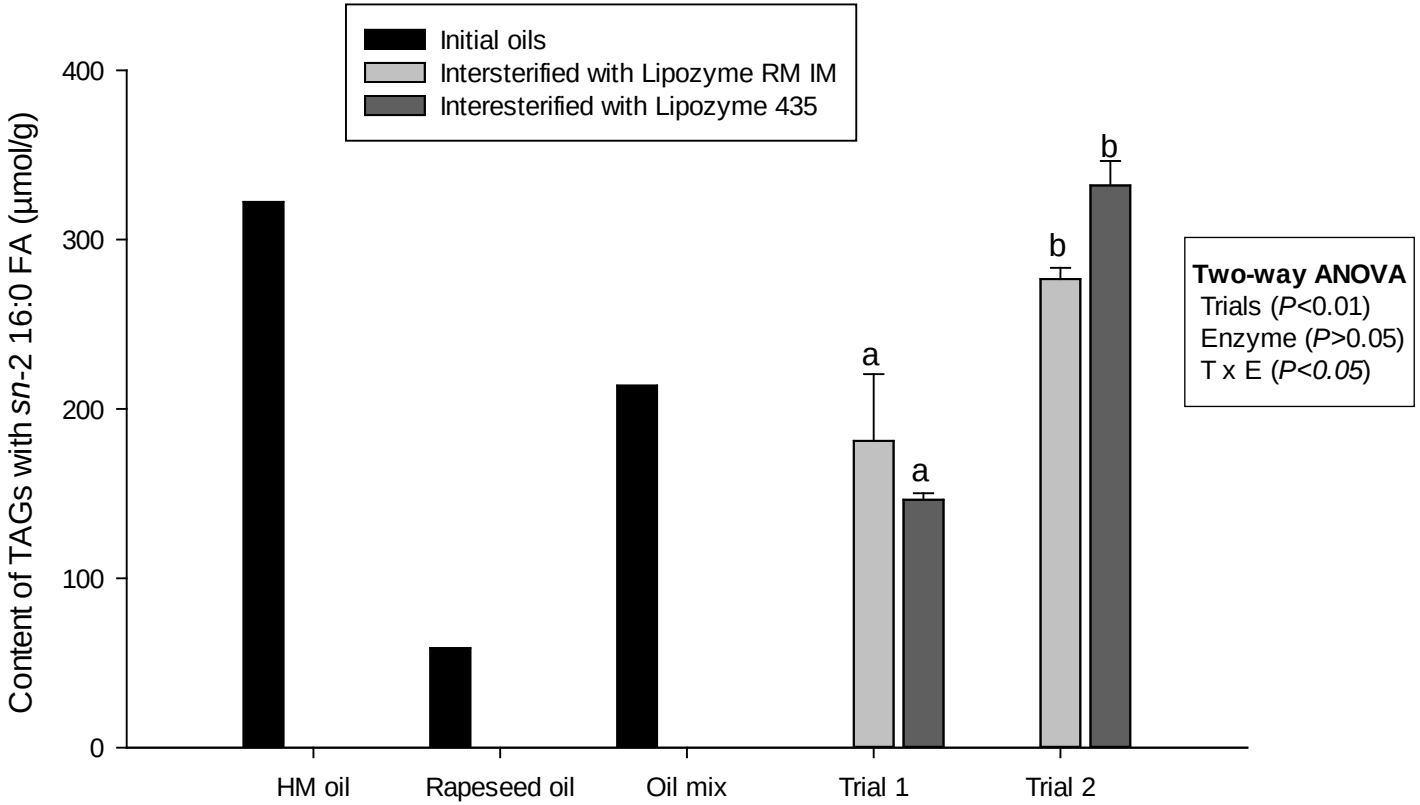

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611 **Fig 6** Content of TAG isomers with 16:0 at *sn*-2 in heterotrophic microalgae (HM) oil, rapeseed oil (RO), a mixture of 10g HM oil and 7 g RO (Oil mix), and
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615 **Table captions**

616 Table 1. Total fatty acid composition of commercial oils and the experimental RO/HM (7/10) oil mix.

617 Table 2. Efficiency of oil extraction methods used for HM.

618 Table 3. Lipid class composition (percent of identified lipid classes) of rapeseed oil (RO) and HM oil
619 extracted in small and larger scale.

620 Table 4. Content of selected TAG isomers in rapeseed oil (RO), extracted HM oil, and oil mix
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622 water (Trial 2) by the use of Lipozyme 435 (L 435) or Lipozyme RM IM (L RM IM).

623 Table 1. Total fatty acid composition (g/100g) of commercial oils and the experimental RO/HM (7/10)
624 oil mix.

Fatty acids	**HM oil**	**RO**	**Oil mix***	**South American FO (Gutierrez & da Silva, 1993)**
14:0	4.4		2.6	0.8-11.5
16:0	50.0	4.6	31.3	14.1-27.4
18:0	1.2	1.5	1.5	1.3-11.1
20:0	0.3	0.5	0.4	0.0-0.6
22:0	0.1	0.3	0.2	0.0-0.8
Sum saturated fatty acids	**56.3**	**6.9**	**36.0**	**23.6-40.8**
16:1	0.1	0.2	0.1	1.3-20.6
18:1	0.3	60.3	25.0	9.8-27.9
20:1 + 18:3		1.1	0.5	0.3-12.3
22:1 + 22:3			0.0	1.8-16.9
Sum monoenoic fatty acids	**0.4**	**61.6**	**25.6**	**19.7-54.4**
18:2 n-6	0.4	19.9	8.4	0.2-7.5
20:2 n-6		0.1		0.0-4.2
20:4 n-6				0.0-1.5
22:4 n-6				0.0-3.5
Sum n-6 PUFA	**0.4**	**20.0**	**8.4**	**1.8-9.4**
18:3 n-3	0.1	10.1	4.2	1.1
18:4 n-3	0.1		0.1	2.6
20:4 n-3	0.3		0.2	0.5
20:5 n-3	0.2		0.1	1.5-24.2
22:5 n-3			0.0	0.4-5.9
22:6 n-3	21.7		12.8	1.4-34.3
Sum n-3 PUFA	**22.4**	**10.1**	**17.3**	**4.6-48.9**
Total content fatty acids	**79.6**	**98.7**	**87.5**	**88.3-100**

625 *Estimated FA composition of oil mix with 7g RO and 10g HM oil.

626

627 Table 2. Efficiency of oil extraction methods used for HM.

Method	Sample size HM powder (g)	Oil extracted (g/100g)
Bligh & Dyer (1963)	20	63
Folch (1967)	20	65
Hexan-extraction	270	10
Ethylacetate-extraction	270	14
Soxhlet	270	19
Bligh & Dyer (1963)	800-1200	23-31
Super-critical CO_2	1-5	2-9

628

629 Table 3. Lipid class composition (percent of identified lipid classes) of rapeseed oil (RO) and HM oil
630 extracted in small and larger scale.

Lipid classer	HM oil lab	HM oil extracted	RO
Triacylglycerol	95	95	100
Diacylglycerol	-	1	-
Monoacylglycerol	-	-	-
Free fatty acids	3	3	
Cholesterol	1	1	-
Phospholipids	1	-	-
Other properties			
Water content	<1	<1	<1
Melting point (°C)	46	46	<5

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Table 4. Content of selected TAG isomers in rapeseed oil (RO), extracted HM oil, and oil mix following enzymatic interesterification in reaction with 5% water (Trial 1) and with no addition of water (Trial 2) by the use of Lipozyme 435 (L 435) or Lipozyme RM IM (L RM IM).

Qualitative content (µmol/g oil)	Initial oils			Interesterified oils				ANOVA		
	RO	HM oil	Oil mix*	Trial 1 L 435	Trial 1 L RM IM	Trial 2 L 435	Trial 2 L RM IM	T	E	T*E
TAG(14:0/16:0/16:0)	-	67	40	36 ± 1	14 ± 10	24 ± 1	18 ± 1	ns	*	*
TAG(16:0/14:0/14:0)+TAG(16:0/12:0/16:0)	-	17	10	8 ± 1	2 ± 2	3 ± 0	2 ± 0	**	**	**
TAG(16:0/16:0/16:0)+TG(14:0/16:0/18:0)	0.2	101	59	54 ± 3	29 ± 13	54 ± 3	35 ± 0	ns	**	ns
TAG(16:0/16:0/15:0)	-	27	16	4 ± 0	2 ± 1	3 ± 0	2 ± 0	ns	**	*
TAG(16:0/16:0/18:0)	-	30	18	14 ± 0	6 ± 4	19 ± 0	12 ± 0	**	**	ns
TAG(18:1/18:1/18:1)	89	2	38	66 ± 2	29 ± 22	34 ± 2	29 ± 3	*	*	*
TAG(14:0/16:0/22:6)	10	62	40	4 ± 1	4 ± 1	9 ± 1	11 ± 0	**	ns	ns
TAG(16:0/14:0/18:3)	0.1	2	1	1 ± 0	5 ± 1	10 ± 1	10 ± 0	**	**	*
TAG(16:0/16:0/18:1)	5	2	3	3 ± 1	46 ± 2	73 ± 6	63 ± 3	**	**	**
TAG(16:0/16:0/18:2)+TAG(16:0/16:1/18:1)	6	1	3	4 ± 0	26 ± 5	45 ± 2	42 ± 1	**	**	**
TAG(16:0/16:0/18:3)+TAG(16:0/16:1/18:2)	3	2	2	2 ± 0	17 ± 2	31 ± 2	29 ± 1	**	**	**
TAG(16:0/18:0/18:1)	5	0.2	2	2 ± 0	11 ± 1	24 ± 1	19 ± 1	**	**	**
TAG(16:0/18:1/18:1)	40	1	17	24 ± 1	37 ± 8	65 ± 5	60 ± 3	**	ns	*
TAG(16:0/18:1/18:2)	42	4	20	23 ± 0	35 ± 8	56 ± 3	61 ± 4	**	*	ns
TAG(18:0/18:1/18:1)	26	0.2	11	15 ± 1	9 ± 5	20 ± 2	14 ± 1	**	ns	*
TAG(18:1/16:0/18:3)	33	9	19	14 ± 0	22 ± 6	41 ± 1	38 ± 4	**	ns	*
TAG(18:1/18:2/18:1)+TAG(18:0/18:1/18:3)	87	16	46	59 ± 1	28 ± 20	32 ± 2	30 ± 2	ns	*	*
TAG(18:2/18:2/18:2)+TAG(18:1/18:2/18:3)	82	1	35	40 ± 2	15 ± 11	16 ± 1	19 ± 2	*	**	**
TAG(18:2/18:2/18:3)+TAG(18:1/18:3/18:3)	48	3	22	24 ± 1	8 ± 7	7 ± 0	7 ± 1	**	**	**
TAG(22:6/18:1/18:1)	0	1	0.4	0.4 ± 0.2	19 ± 4	36 ± 2	36 ± 3	**	**	**

*Calculated TAG isomer composition of oil mix with 7g RO and 10g HM oil.

Values are mean ± standard deviation; n=3. Data were subjected to two-way ANOVA for the variable Trial (T), enzyme (E) and the interaction of these (P<0.0.5).