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Lipase-produced omega-3 acylglycerols for the fortification and stabilization of extra virgin olive oil using hydroxytyrosyl palmitate

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ABSTRACT

Pure concentrates of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids were used to produce monoacylglycerol and diacylglycerol (MDG) oils via enzymatic glycerolysis in a solvent-free system. Immobilized lipase from *Candida antarctica* B (Novozym 435) was employed for the glycerolysis reaction with up to 90% product formation in one hour. This lipase also favoured diacylglycerol (DAG) over monoacylglycerol (MAG) production. Products were purified using silica gel chromatography and characterised by capillary chromatography with flame ionization detection (Iatroscan-FID). Purified products were added to extra virgin olive oil (EVOO) at a concentration of 20% w/w. The fortified olive oil was stabilized using hydroxytyrosyl palmitate, an antioxidant prepared by conjugating hydroxytyrosol with palmitic acid using Novozym 435 lipase. *In vitro* hydrolysis of the fortified oil was also carried out using porcine pancreatic lipase to investigate the mechanism of action of this enzyme on the oil mixtures.

1. Introduction

Monoacylglycerol (MAG) and diacylglycerol (DAG) are partial acylglycerols, naturally occurring in small quantities in plants and animals. DAGs are mainly present in vegetable oils such as cottonseed and olive oils at a concentration of about 2 - 10% (Takase, 2007). MAG and DAG are non-ionic molecules and account for about 75% of the world's emulsifier production (approximately 250,000 t/year) (Damstrup et al., 2005; Ferreira-Dias., 2001). These emulsifiers are widely applied in the food, cosmetic and pharmaceutical industries. Unlike non-ionic surfactants such as tensoactives, MAG and DAG showed no side effects or skin irritation after ingestion and have the "Generally Recognized as Safe (GRAS)" status from the Food and Drugs Administration, USA (Freitas et al., 2008).

Omega-3 fatty acids such as EPA and DHA have been widely studied for their health benefits. The most common forms of omega-3 concentrates are triacylglycerol (TAG) and ethyl ester (EE) (Destaillats et al., 2018). EEs are even more common than TAGs in the market. For instance, Lovaza is an EE omega-3 drug with 84% EPA+DHA (900 mg/capsule) approved in USA and Europe (Rupp, 2009). Epadel in Japan and Vascepa in USA are EPA-EE (96.5%) drugs approved by the EU and the American authorities (Harris, 2009). A number of studies have shown that DAG and MAG show better digestion and absorption compared to TAG and EE, due to differences in absorption, digestion and stereo specificity (Banno et al., 2002; Cruz-Hernandez et al., 2012; Destaillats et al., 2018; Valenzuela et al., 2005). MAG and DAG require minimal enzyme digestion before entering the enterocytes (Destaillats et al., 2018). Moreover, small amounts of ethanol presence in the EE form of omega-3 s have nutritional or/and religious consequences (Cowan, 2010) and concentration of omega-3 by selectively removing non-omega-3 fatty acids results in oils with EPA and DHA remaining at their natural positions on the glycerol backbone. Therefore, there is market interest in developing omega-3 MAG and DAG concentrates as an alternative to TAG and EE forms.

Since studies have shown that lipid mixtures with high levels of MAG may be oxidatively unstable (Mistry and Min, 1988; Xia et al., 2017), production and stability of high DAG omega-3 concentrates should be investigated. Commercial production of DAG involves the use of extreme processing conditions such as high temperature (220 °C-260 °C) (Feltes et al., 2010). This process is not suitable for the heat sensitive and oxidatively unstable omega-3 fatty acids. Reactions where lipases are involved can be carried out under mild conditions and so are preferable to high temperature, chemical production methods for omega-3 lipids (Akanbi et al., 2019). Currently, there are no published studies on the production of high-DAG-low-MAG omega-3 concentrates.

In this study, the abilities of lipases to produce omega-3-enriched monoacylglycerol and diacylglycerol (MDG) oils using EPA/DHA ethyl esters were screened. Reactions were carried out under solvent-free conditions, and the effects of enzyme amount, substrate molar ratio, and

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time on product yield were studied and optimized. Scale-up and purification of products was carried out. The purified products were used to fortify extra virgin olive oil (EVOO) and stability studies were performed. *In vitro* hydrolysis of the oil after fortification was also studied.

2. Materials and methods

2.1. Materials

Two omega-3 ethyl esters (EE) samples; high EPA (h-EPA) and high DHA (h-DHA) were provided by BASF Australia Ltd (Melbourne VIC., Australia). These were converted to free fatty acids (FFA) according to a previously reported method (Akanbi and Barrow, 2015). Immobilized lipases from *Rhizomucor miehei* (Lipozyme RMIM), *Thermomyces lanuginosus* (Lipozyme TLIM) and *Candida antarctica* B (CLAB, Novozym® 435) were obtained from Novozymes Australia Pty. Ltd. Anhydrous glycerol (99.9%), molecular sieves (3 Å, 4 – 8 mesh) and porcine pancreatic lipase were purchased from Sigma–Aldrich (Castle Hill, Australia). Thin layer chromatography and gas chromatography standards were purchased from Nu-Chek Prep (Elysian, MN, USA) and Sigma Aldrich (Castle Hill, Australia) respectively. Extra virgin olive oil (Cobram Estate) was purchased from a local supermarket and the declared antioxidants on the label were vitamin E (230 mg/kg), polyphenol (330 mg/kg) and squalene (4130 mg/kg). All other reagents were of analytical grade.

2.2. Lipid class analysis by Iatroscan-FID

Lipid class analysis was carried out using Iatroscan-with flame ionisation detector (FID) Capillary (Iatroscan MK5, Iatron Laboratories Inc., Tokyo, Japan) as previously reported (Akanbi et al., 2013).

2.3. Analysis of fatty acid compositions by gas chromatography

Before analysis by gas chromatography, fatty acids in the oil samples were converted to methyl esters according to a previously described method (Akanbi et al., 2013). Samples were analysed using an Agilent 6890 gas chromatograph with flame ionisation detector (FID), equipped with a BPX70 SGE column (30 m, 0.25 mm i.d., 0.25 μm film thickness). The oven was programmed from 140 °C (5 min hold) to 240 °C (5 min hold) at a rate of 4 °C/min for a total run time of 30 min. A volume of 1 μL of solution was injected with a split ratio of 50:1 (injector temperature, 250 °C). Helium was used as the carrier gas (1.5 mL/min, constant flow). Detector gases were 30 mL/min hydrogen, 300 mL/min air and 30 mL/min nitrogen. Peak areas were integrated by ChemStation software and corrected using theoretical relative FID response factors (Craske and Bannon, 1987). The GC fatty acid standards used were a mixture of saturated, monounsaturated and polyunsaturated fatty acids ranging from carbon 4 to 24 (C4 - 24).

2.4. Enzymatic glycerolysis

Enzymatic glycerolysis was performed in a round bottom flask at 50 °C under vacuum with magnetic stirring at 360 rpm. The reaction mixture contained 2 g substrate (3:1 glycerol:omega-3 FFA), lipase (0.12 g, 6% w/w of total substrates) and molecular sieves (0.2 g, 10% w/w of total substrates). Immobilized lipases from *Rhizomucor miehei* (Lipozyme RMIM), *Thermomyces lanuginosus* (Lipozyme TLIM) and *Candida antarctica* B (CLAB, Novozym 435) were screened. Aliquots of sample (0.5 g) were periodically withdrawn from the reaction mixture for analysis.

2.5. Optimizing glycerolysis conditions for scaled-up synthesis

The effects of process parameters such as reaction times (1–24 h), enzyme amount (4–15% w/w of reaction mixture) and substrate molar ratios (glycerol:FFA) on MDG oil formation were investigated. Each of the factors was varied while others were kept constant. To provide sufficient samples for further experiments, the optimised conditions were combined to scale-up production of the MDG oil.

2.6. Extraction, purification and characterisation of MDG oils

Upon completion of glycerolysis, the reaction mixtures were suspended in *n*-heptane and the unused glycerol and immobilised lipase were separated by centrifugation (6800 rpm, 6 min). Then, *n*-heptane was evaporated off *in vacuo* and the unreacted FFA was separated away from the product using a silica gel column (solvent system was 60:17:0.2 – *n*-heptane:diethyl ether:acetic acid). Using the same solvent system, the lipid classes of the MDG oil (MAG and DAG) were separated on a silica gel plate (60 F_{254}, Merck KGaA, Darmstadt, Germany) and their fatty acid compositions were analysed by GC-FID.

2.7. Fortification of extra virgin olive oil (EVOO) with MDG oils

To achieve higher omega-3 levels in the EVOO, 20% w/w of purified MDG oils were added and the fatty acid analysis of the fortified oil was carried out using GC-FID.

2.8. Stabilization of fortified oil

Hydroxytyrosyl palmitate was added to the oil at a concentration of 1180 mg/kg oil (Akanbi and Barrow, 2018) and the accelerated oxidation technique involving Rancimat (Metrohm Ltd. Switzerland) was used to measure the oil stability index (OSI). The Rancimat method is widely used in the food industry for determining the stability of edible fats and oils and fat-containing foods because of its ease of use and reproducibility (Farhoosh, 2007). Using Rancimat, oil samples (4 mL) were heated at 90 °C under a purified air with a flow rate of 20 L/h. These conditions were selected because they were found to be suitable for testing the oxidative stability of omega-3 rich oils (Wang et al., 2014).

2.9. In vitro *hydrolysis with porcine pancreatic lipase*

Porcine pancreatic lipase (PPL) was used to hydrolyse the fortified EVOO was performed using PPL (porcine pancreatic lipase) as previously reported (Akanbi et al., 2014) with some modifications. Three hundred milligram (300 mg) sample mixture (EVOO with 20% h-EPA MDG or h-EPA EE oils) was combined with 22% calcium chloride solution ($CaCl_2$, 100 µl), 0.1% bile salt solution (320 µl) and 1 M tris (hydroxymethyl) aminomethane, 200 µl at pH 7.7 in a 5 mL flask. Then the mixture was incubated at 37 °C under nitrogen with 200 rpm agitation for 3.5 h. Percent FFA released was determined using Iatroscan-FID and purified on a TLC plate. The FA compositions of these were tested using GC-FID as described in Section 2.3.

2.10. Statistical analysis

Experiments were carried out in triplicates and results were presented as mean values±standard deviations (SD). Data analysis was done by one-way analysis of variance (ANOVA) using IBM SPSS Statistics 25 software. Significant differences between means were determined by Tukey's HSD (Honestly Significant Difference) multiple comparison. All test procedures were made at 5% significant level. The graphical representations of data were performed by Excel Microsoft Office 365 ProPlus.

3. Results and discussion

3.1. Enzymatic synthesis of omega-3 MDG oils in a solvent-free system

Enzymatic synthesis of omega-3 MDG oil was carried out in a solvent-free system by reacting h-EPA FFA with glycerol in the presence of

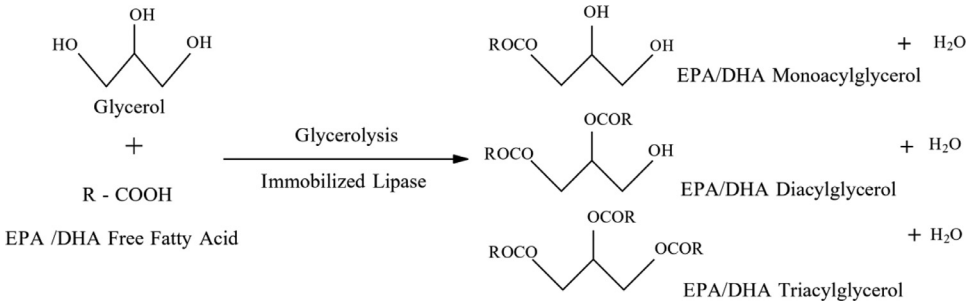

Fig. 1. Schematic diagram of enzymatic glycerolysis for conversion of EPA/DHA free fatty acids to EPA/DHA acylglycerols using immobilized lipase. *EPA* eicosapentaenoic acid, *DHA* docosahexaenoic acid.

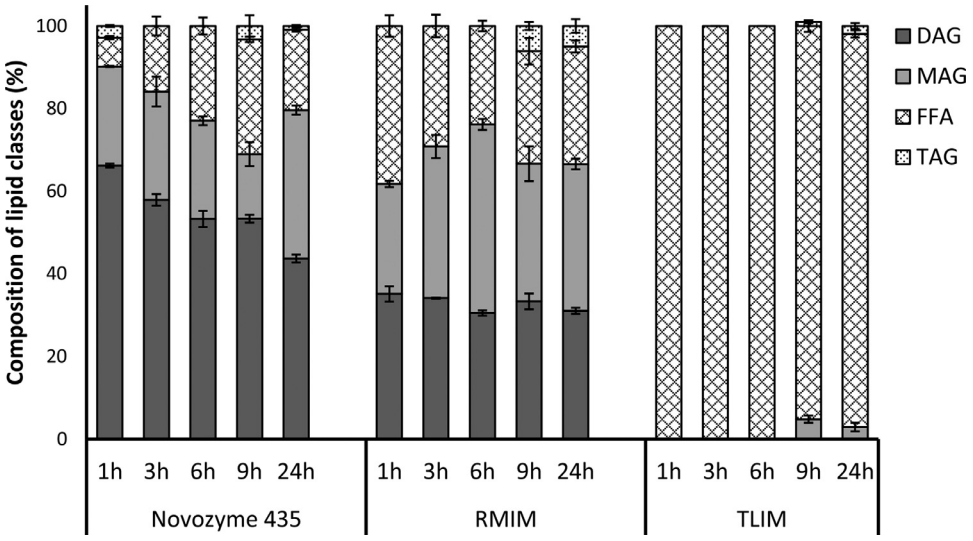

Fig. 2. Lipase screening for the production of DAG and MAG. The reaction mixture contained 2 g substrate (3:1 glycerol-to-FFA molar ratio), lipase (0.12 g, 6% w/w of total substrates) and molecular sieves (0.2 g, 10% w/w of total substrates). The mixture was incubated at 50 °C with magnetic stirring at 360 rpm under vacuum. Results are means ± SD of three replicates. *DAG* diacylglycerol, *MAG* monoacylglycerol, *TAG* triacylglycerol, *FFA* free fatty acids.

immobilized lipase (Fig. 1). Progression of the reaction was monitored using capillary chromatography with flame ionization detection (Iatroscan-FID) after removing the unreacted glycerol (as explained in Section 2.6). Thin layer chromatography standards (Nu-Chek Prep, Elysian, MN, USA) were used to identify the peaks corresponding to MAG, DAG, TAG or FFA. Percent product formation was determined using SIC-480 II software for multiple chromatogram processing, by comparing the percentage peak areas of the unreacted FFA and the acylglycerols (MAG, DAG, TAG) formed.

3.1.1. Screening of enzymes for glycerolysis reaction

Immobilized lipases from *Candida antarctica* lipase B-(CLAB/Novozym 435), *Rhizomucor miehei* (RMIM) and Lipozyme *Thermomyces lanuginosus* (TLIM) were screened for the production of MDG (MAG and DAG) oils. The enzymes' selections were based on their commercial availability and broad usage in esterification and transesterification reactions. As shown (Fig. 2), both Novozym 435 and Lipozyme RMIM enabled the formation of significant amount of MAG and DAG, while Lipozyme TLIM gave poor results. The inability of Lipozyme TLIM to produce MAG and DAG under the reaction condition may be because non-emulsified lipids could not effectively access the enzyme active site. This enzyme has been primarily used for the hydrolysis of emulsified lipids releasing MAG, DAG and FFA (Akanbi et al., 2013). The initial reaction rate of Novozym 435 was very rapid with maximum DAG (66%) and MAG (24%) production within 1 h, compared to RMIM which had maximum DAG (31%) and MAG (46%) production in 6 h. Since MAGs may be more oxidatively unstable than DAG, Novozym 435 was used for subsequent experiments because it produced higher DAG than did Lipozyme RMIM.

3.1.2. Effect of enzyme concentration

The effect of enzyme concentration on the production of omega-3 MDG oils using Novozym 435 was investigated, with results presented in Fig. 3(a). As shown, the highest DAG production occurred with 6% w/w Novozym 435 within 1 h. Higher enzyme load did not affect product yield. Whereas with a low enzyme load (4% w/w), the reaction took a longer time to reach maximal DAG production.

In a previous study on the glycerolysis of oleic acid, 50% DAG production was obtained with 5% Novozym 435. Authors (Liu et al., 2016) found that higher enzyme load (7.5%) led to a decrease in DAG production. Excessive use of enzyme may lead to protein aggregation, thus reducing product yield (Ghamgui et al., 2006; Liu et al., 2016). This may also lead to poor mixing, limiting mass transfer and reaction rate (Valério et al., 2010). Therefore, 6% w/w Novozym 435 was used for subsequent experiments.

3.1.3. Effects of molar ratio

The effect of changing the molar ratios of substrate (glycerol and omega-3 fatty acids) was investigated by varying the level of glycerol while keeping the concentration of the omega-3 fatty acids constant. As shown in Fig. 3(b), the mole ratio of substrate was varied from 3:1 to 6:1 (glycerol: omega-3 fatty acids) and higher DAG yields were obtained within 1 h for all samples. The highest DAG formation occurred with a 6:1 molar ratio (67%), followed by 4:1 (64%) and 3:1 (61%). Excess stoichiometric glycerol amount (6:1 glycerol:oleic acid) has been reported to favour high DAG production (>60%) during the enzymatic glycerolysis of oleic acid (Zhao et al., 2011). However, excess glycerol tends to create a very viscous mixture that can be difficult to mix, leading to loss of homogeneity and entrapment of the immobilized enzyme's particles, impacting results. (Yang et al., 2005b). We found that 4:1 (glycerol: omega-3 fatty acids) substrate ratio was optimum for subsequent experiments as this was a less viscous mixture than when higher amounts of glycerol were used.

In summary, the optimized conditions for the enzymatic synthesis of omega-3 MDG were lipase (Novozyme 435; 6% w/w of reaction mixture), molar ratio of substrates (4:1; glycerol:omega-3 FFA) and reaction

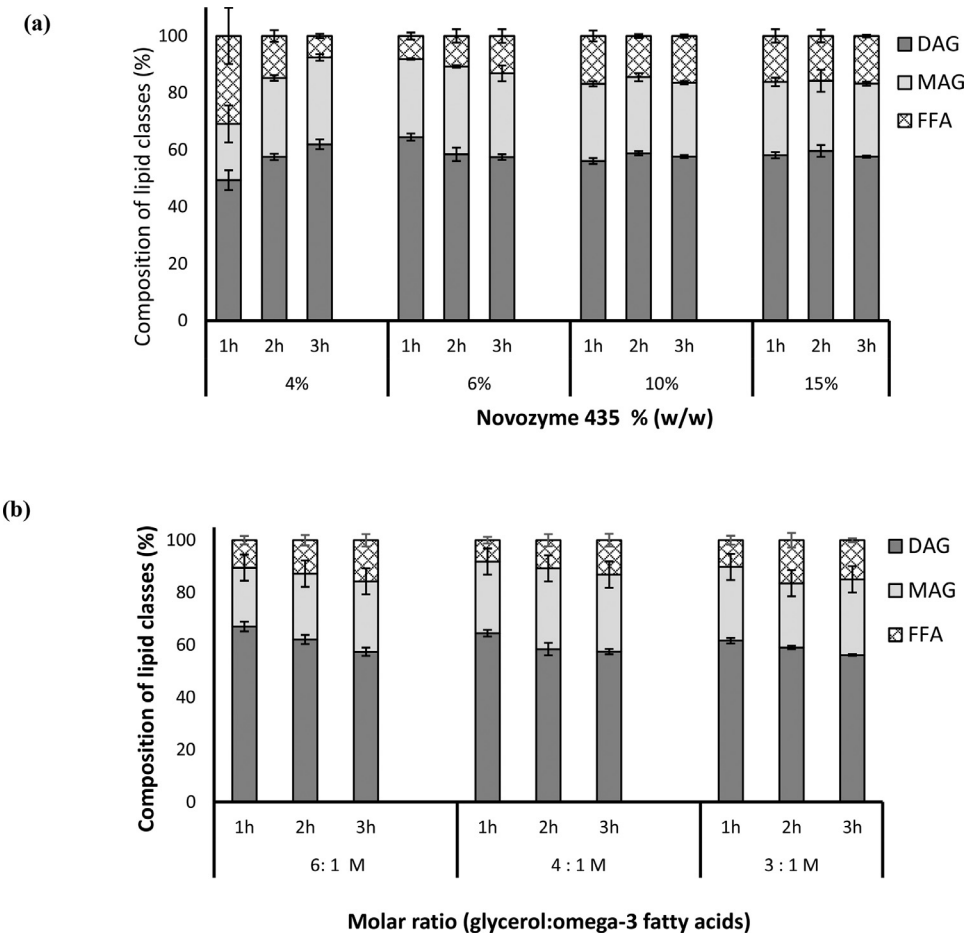

Fig. 3. Effect of (a) enzyme concentration and (b) substrate molar ratio on the production of DAG and MAG. Values are mean ± SD for three replications.

Table 1
Lipid class profile of purified products.

Lipid classes (%)	h-EPA product	h-DHA product
DAG	71.45 ± 1.16[a]	73.13 ± 1.39[a]
MAG	25.34 ± 0.73[b]	23.45 ± 0.94[b]
TAG	3.22 ± 1.03[c]	3.42 ± 1.04[c]

Values are mean ± SD of three replications. Mean values in the same row with different letters (a-c) differ significantly ($p<0.05$). Lipid class profiles (%) were determined using capillary chromatography-FID (Iatroscan MK5).
DAG diacylglycerol, *MAG* monoacylglycerol, *TAG* triacylglycerol, *h-EPA* high eicosapentaenoic acid, *h-DHA,* high docosahexaenoic acid.

time (1 h). This solvent-free enzymatic method can be used for the large-scale production of omega-3 acylglycerols. It is an efficient and rapid method that utilises highly reusable immobilised lipase to produce acylglycerol in a short time. Therefore, this enzyme technology can be used to produce omega-3 rich functional ingredients such as emulsifiers and stabilisers that can be used in the food, pharmaceutical, and cosmetic industries.

3.2. Characterisation of synthesised MDG oils using Iatroscan-FID and GC-FID

The synthesised MDG oils were purified by silica gel column chromatography and quantified using Iatroscan-FID with results presented in Table 1. Results show that the total glycerolysed products (MAG+DAG+TAG) in both samples are not significantly different ($p<0.05$). These similarities indicate that under solvent-free conditions and with FFA as substrate, Novozym 435 showed preference for DAG synthesis over MAG. A number of studies have shown that MAG and DAG production by Novozym 435 are highly influenced by the type of fatty acid (FA) donor. For instance, a previous glycerolysis study where TAG (rapeseed oil) was used as FA donor under solvent-free conditions, Novozym 435 produced 45% DAG and less than 5% MAG after 6 h (Weber and Mukherjee, 2004). Meanwhile, during the glycerolysis of sunflower oil (TAG), Novozym 435 produced 70% MAG in the presence of *tert*-butanol as solvent (Yang et al., 2005a). These results show the versatility of Novozym 435 for producing a broad range of structured lipids.

Since some lipases show preferences for fatty acid based on chain length or degree of unsaturation (Akanbi et al., 2013), we determined if Novozym 435 showed a preference for either EPA or DHA in the glycerolized products by analysing the fatty acid compositions of the MAG and DAG portions using GC-FID. MAG and DAG were first extracted using TLC plate before being converted to FAMEs for GC analysis. The results presented in Fig. 4 show that EPA and DHA distribution in the DAG and MAG portions of both h-EPA and h-DHA products are similar to what was in the original samples before glycerolysis. These results show that under the glycerolysis conditions employed in this study, Novozym 435 was not selective towards either EPA or DHA. A previous study found that Novozym 435 shows less discrimination towards omega-3 fatty acids (Halldorsson et al., 2003).

3.3. Fortification of EVOO with omega-3 MDG oils

Clinical benefits of EPA and DHA have been widely reported and foods rich in these fatty acids are highly recommended for their health benefits. Thus, addition of EPA and DHA to commonly consumed foods

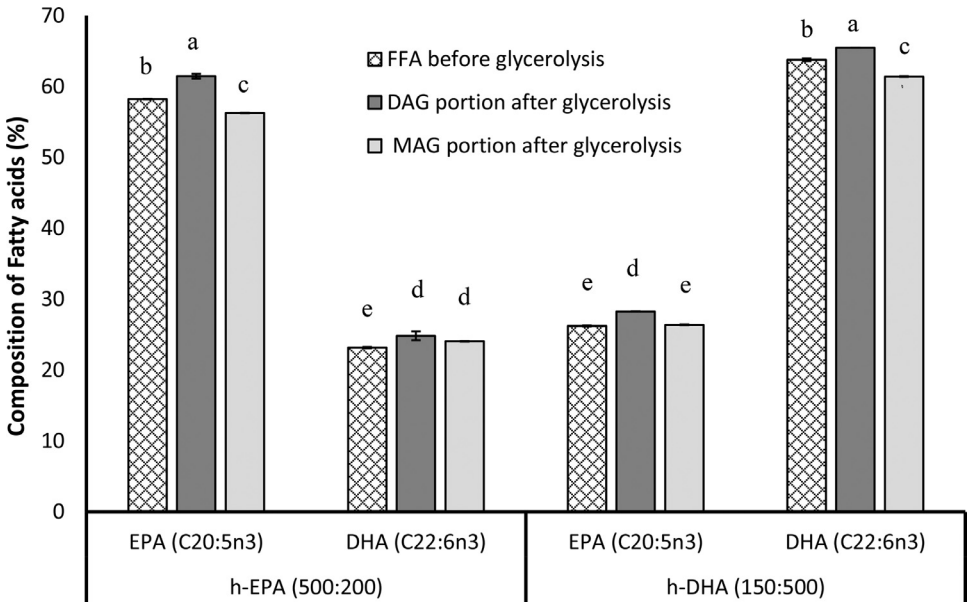

Fig. 4. EPA and DHA composition before and after glycerolysis. Values are mean ± SD for three replications. Bars with different letters (a-d) indicate significant difference ($p<0.05$) between mean values in each sample (h-EPA and h-DHA). *h-EPA* high eicosapentaenoic acid, *h-DHA* high docosahexaenoic acid.

is of interest. Today, vegetable oils such as olive oil forms a major part of the western diet. This oil is rich in natural polyphenols that act as antioxidant, however, it has less than 3% omega-3 fatty acids. From the nutritional point of view, fortifying olive oil with EPA and DHA will reduce the consumption of other saturated and monounsaturated fatty acids. Besides, a diet containing this functional olive oil (omega-3 enriched olive oil) would have a favourable lipid profile compared to a diet containing only olive oil. It has been shown that dietary olive oil supplemented with omega-3 EPA and DHA significantly reduced the levels of colonic tumour necrosis factor α (TNFα) and leukotriene B_4 (LTB_4) and may be beneficial in the management of inflammatory bowel disease (IBD) (Camuesco et al., 2005). With these in mind, 20% w/w of the purified omega-3 MDG oils were added to EVOO to deliver EPA and DHA.

3.3.1. *Fatty acid composition of fortified EVOO*

The fatty acid compositions of fortified olive oil samples were determined by GC-FID. Results show that oleic C18:1n9c (72%), palmitic C16:0 (13%) and linoleic acids C18:2n6c (9%) were the major fatty acids present in EVOO. The total SFA, MUFA and PUFA in the oil before fortification were 16%, 75% and 9.5%, respectively. After fortification, the total omega-3 PUFA in the oil rose from 0.75% to 13% in the h-EPA product and 16% in the h-DHA product. With the increase in omega-3 FA contents in the fortified samples, the total SFA decreased while the total PUFA increased.

3.3.2. *Oxidative stability of fortified EVOO*

EPA and DHA are known to be oxidatively unstable because they have multiple double bonds. In this study, we investigated the oxidative stability of the fortified extra virgin olive oil with and without added antioxidant. The antioxidant used in this study was hydroxytyrosyl palmitate, an antioxidant enzymatically synthesised by conjugating palmitic acid with hydroxytyrosol (Akanbi and Barrow, 2018). Hydroxytyrosyl palmitate has been found to be more effective than alpha-tocopherol in stabilizing omega-3 oils (Akanbi and Barrow, 2018). Oxidative stability tests of oil samples were carried out using Rancimat as described in Section 2.8 and results are presented in Fig. 5. As shown, the oil stability Index (OSI) values of fortified oils without antioxidant were much lower compared to those with added antioxidant. The mean OSI value of the olive oil fortified with the h-EPA product (24 h) was more than the one fortified with h-DHA product (16 h). The higher stability of the h-EPA product may be due to its lower DHA content. DHA is known to be less stable than most PUFAs including EPA because of its high degree of unsaturation (Wijesundera et al., 2008). Although the OSI value of olive oil without added antioxidant was 56 h (data not shown), the significant decrease observed after fortification with the h-DHA (16 h) and h-EPA (24 h) products necessitated the addition of antioxidant. The added hydroxytyrosyl palmitate doubled the OSI values of the fortified oils, increasing them to 32 h (h-DHA product) and 44 h (h-EPA product), respectively. These findings show that lipophilic esters of olive-derived polyphenols such as hydroxytyrosol are effective antioxidants for stabilizing omega-3 containing oils.

3.3.3. In vitro *hydrolysis with pancreatic lipase*

During digestion, lipolysis of dietary fat by pancreatic lipase releases FFAs and MAGs, followed by the formation of micelles which facilitate bioavailability (Arterburn, 2006; Cuenoud et al., 2020). However, unlike TAGs, the hydrolysis of EEs does not result in MAGs formation because they lack a glycerol backbone. Thus, to understand the lipolysis of complex lipid mixtures, we used porcine pancreatic lipase (PPL) to hydrolyse EVOO fortified with h-EPA MDG and h-EPA EE for 3.5 h. We quantified the FFAs released using Iatroscan-FID, extracted them using TLC plate and analysed their FA composition by GC-FID. We used PPL for hydrolysis because it is similar to human pancreatic lipase (Wishart et al., 1993). Iatroscan-FID results showed that 91% FFA was released from the EVOO fortified with h-EPA MDG, while 80% FFA was released from the EVOO fortified with h-EPA EE. The high FFA contents were anticipated because all the major fatty acids in the EVOO have been previously reported as suitable substrates for this enzyme (Akanbi et al., 2014). Meanwhile the GC-FID results (Fig. 6) showed that more EPA and DHA are present in the FFA portions of the EVOO fortified with h-EPA MDG than that fortified with h-EPA EE. As shown in Fig. 6, up to 2-fold EPA (10.2% *versus* 5.4%) and 4-fold DHA (3.86% *versus* 0.86%) were found in the h-EPA MDG sample than in the h-EPA EE sample. These results suggest that omega-3 s in the MAG and DAG forms are better substrates for PPL than those in the EE forms. These results indicate that in a complex lipid mixture, acylglycerol forms of omega-3 PUFAs can be more readily digested and absorbed in humans than can the ethyl ester form.

A recent study found that MAG forms of omega-3 had a significantly greater absorption than omega-3 EEs in humans (Cuenoud et al., 2020). They found that in obese individuals, intake of nutritional doses of omega-3 MAG supplement resulted in an increase in EPA absorption (Cuenoud et al., 2020). Another study on diets containing 10% TAG or 10% DAG oils found that the amount of FFA in the stomach in the DAG diet group was higher than that in the TAG diet group (Osaki et al.,

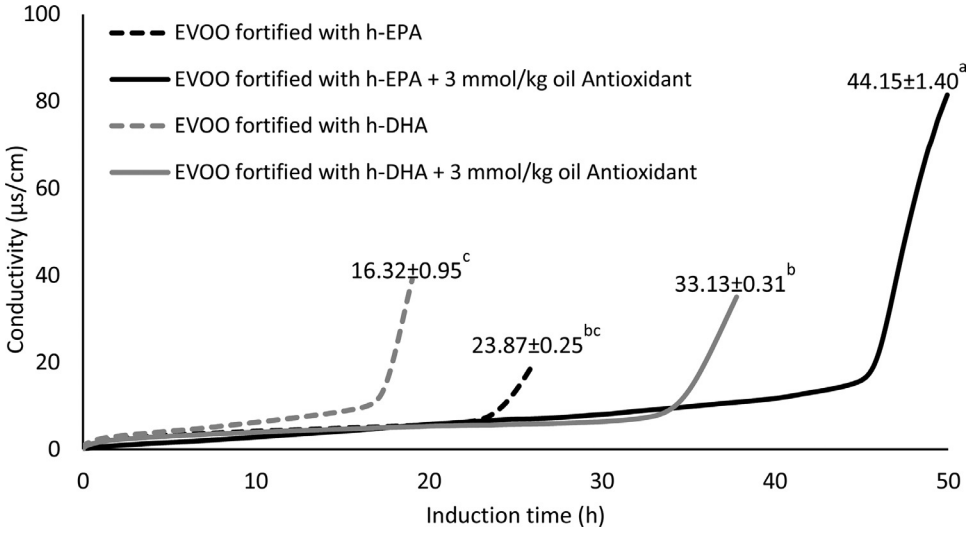

Fig. 5. Oxidative stability index (OSI) of extra virgin olive oil (EVOO) fortified with MDG oil (h-EPA and h-DHA) with and without antioxidant (3 mmol/kg oil) at 90 °C. Values are mean ± SD of three replicates. Mean values with different letters (a-c) indicate significant difference ($p<0.05$).

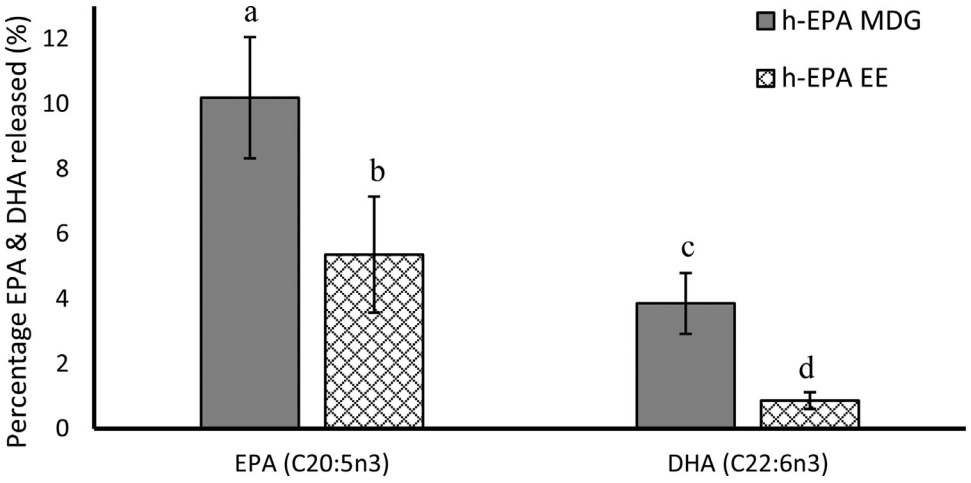

Fig. 6. EPA and DHA released during the hydrolysis of fortified extra virgin olive oil (EVOO) by h-EPA MDG and h-EPA EE. Mean values with different letters (a-d) indicate significant difference ($p<0.05$). *h-EPA MDG* high eicosapentaenoic monoacylglycerol and diacylglycerol, *h-EPA EE* high eicosapentaenoic ethyl ester.

2005). This implies that even more EPA and DHA would be absorbed in humans when these fatty acids are in the MAG or DAG form, because these lipid classes are the "pre-digested" forms of TAG. Omega-3 TAGs have also been found to show more rapid absorption than those in the EE forms. Up to 68% EPA and DHA was absorbed in the TAG form in human system compared to only 21% in ethyl ester form (Lawson and Hughes, 1988).

Conclusion

In the present study, omega-3 MDG oils were produced in a solvent free system by reacting h-EPA FFA and h-DHA FFA with glycerol in the presence of immobilized lipase. Product formation was rapid when Novozym 435 lipase was used. The purified omega-3 MDG oils were added to EVOO and stabilised using hydroxytyrosyl palmitate. The added antioxidant (hydroxytyrosyl palmitate) was produced enzymatically using ingredients (palmitic acid and hydroxytyrosol) that are naturally present in extra virgin olive oil. This oil blend may be referred to as "functional olive oil or omega-3 enriched olive oil" and not as extra virgin olive oil for regulatory purposes. *In vitro* hydrolysis of the oil blend was carried out using porcine pancreatic lipase (PPL). Results indicate that omega-3 MDG oils in a complex lipid mixture can be more readily digested and absorbed in humans than the omega-3 EE forms.

Declaration of Competing Interest

The authors whose names are listed immediately below certify that they have **NO** affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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