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Candida antartica Lipase Assisted Enrichment of n-3 PUFA in Indian Sardine Oil

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Abstract

Indian oil sardine (Sardinella longiceps) are one of the richest and cheapest sources of n-3 polyunsaturated fatty acids (n-3 PUFA) such as Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA). Natural refined Indian Sardine oil generally contain about 25% (w/w) n-3 PUFA along with various unsaturated and saturated fatty acids in the form of mono, di, and triglycerides. Having high concentration of n-3 PUFA content in the glyceride form is most desirable to avail maximum health benefits. Thus, enhancing the n-3 PUFA content while retaining it in the glyceride form is the need of the hour. In this study, refined Indian Sardine oil was subjected to selective hydrolysis by Candida antartica lipase to enhance n-3 PUFA content. The enhancement of n-3 PUFA content was assessed before and after hydrolysis. Various reaction parameters were optimized by conducting trials with one parameter at a time approach. Incubating enzyme solution with refined sardine oil with a volume ratio of 1:1, at pH 7.0, for 60 minutes at 50 °C, with an enzyme load of 60 mg/ml was found to be optimum. After enzymatic treatment, the oil was refined to remove free fatty acids and moisture content using previously optimized refining technology. Enzymatic treatment at the optimal conditions resulted in the enhancement of Iodine number and n-3 PUFA content by 11.98 % and 82.27 % (w/w), respectively.

Keywords: Candida antartica, Lipase, n-3 Polyunsaturated fatty acids, Sardine oil.

Introduction

The n-3 polyunsaturated fatty acids (n-3 PUFA), like eicosapentaenoic acid (EPA) and docosahexanoic acid (DHA), has been the main focus of study because of their clinical significance. Both EPA and DHA have been found to influence several molecular pathways which contribute to various physiological functions such as lowering of plasma triglycerides, blood pressure and inflammation, hence reduce the risk of circulatory and inflammatory disorders (Mozaffarian and Wu, 2011). It is said that n-3 PUFA's are the main component in the phospholipid content of brain, also regulate neurotransmitters and helps in the development of visual system (Ruxton et al., 2004).

Humans cannot synthesize EPA and DHA in vivo. The n-3 PUFA, α-linolenic acid (ALA) is the precursor of EPA and DHA.ALA converts into EPA and DHA through elongation-desaturation pathway. The main sources of ALA include green leafy vegetables, rape-seed oil, soybean oil etc. (Calder, 2004). But studies show that the conversion rates of ALA to EPA and DHA in both men and women are limited (Williams and Burdge, 2006). Another main and direct source of it is found in seafood, especially oily fishes such as Sardines, Salmon, Mackerel and Anchovies. Although fish is a dietary source of n-3 PUFA, they do not synthesize it themselves and is obtained from the algae or phytoplanktons in their diets (Falk-Petersen et al., 1998). The Indian oil Sardine (*Sardinella longiceps*) is a species of ray-finned fish in the genus *Sardinella*. It is one of the richest and cheapest sources of n-3 PUFA.

The n-3 PUFAsare being sold in the form of nutritional supplements and functional food products, which requires concentration of n-3 PUFA into a chemical form which is easily digestible by humans. Many chemical methods are being used to concentrate n-3 PUFA from fish oil which include molecular distillation, urea complexation, chromatography, low-temperature crystallization and super critical fluid extraction (Rubio-Rodríguez et al., 2010). The traditional methods being used concentrate n-3 PUFA in the form of ethyl esters. Studies show that glyceride form of n-3 PUFA are absorbed easily than its ethyl ester form (Lawson and Hughes, 1988). Also, since these methods involve saponification process, EPA and DHA will easily get oxidized (Wijesundera et al., 2008).

Concentration of n-3 PUFA in fish oil is also possible by enzymatic treatments. Lipases act specifically by differentiating between fatty acids depending on their chain length and degree of unsaturation. Lipase hydrolyses saturated and mono-unsaturated fatty acids more efficiently than the n-3 PUFAs, because the 5 and 6 double bonds in EPA and DHA respectively which tend to offer steric hindrance at the active site of the lipases (Casas-Godoy et al., 2014). Therefore, enzymatic method is advantageous because lipases catalyze the reactions under milder conditions than the chemical methods and also the specificity of lipases enables the modification of oils and fats in more sophisticated way.

Several lipases have been used to enrich n-3 PUFAs in fish oil. Identifying more specific lipases for the enrichment of n-3 PUFA is still a major challenge. In this study, the potential of lipases from *Candida antarctica* (CAL A) has been investigated to develop n-3 PUFA concentrate in refined Indian Sardine oil by optimizing various reaction parameters.

Materials and Methods

Raw Materials

Crude Sardine oil was provided by Mukka Sea Food Industries Ltd (Mangaluru, India) which was refined using previously optimized refining technology (Charanyaa et al.,2017) and stored at 4˚C in the dark. *Candida antarctica* lipase A (CAL A) was procured from Kaypeeyes Biotech Pvt. Ltd., Mysore, India. Isopropanol, phenolphthalein indicator, potassium iodide, sodium thiosulphate, potassium hydroxide, wijs solution, chloroform, diethyl ether, hexanewas purchased from Lobachemei pvt ltd India. EPA and DHA methyl ester standards were purchased from Sigma Aldrich. All the reagents (analytical grade), solvents (chromatographic grade) were used without further purification.

Hydrolysis Reaction

The optimum reaction conditions for the hydrolysis of refined Indian Sardine oil was studied at different pH, temperature, enzyme load and oil to buffer ratio at different reaction times. The experimental trials were carried out in duplicates at different reaction parameters by varying only one reaction parameter at a time and keeping other parameters constant. The reaction system containing 2 g of fish oil and 2 g of 0.1 M potassium phosphate buffer were mixed in 25mL conical flask under constant stirring at 300 rpm to form a homogenous substrate to which 0.5 mL of Lipase preparations were added to carry out the hydrolysis. Reactions were carried out under vacuum conditions in order to prevent oxidation of oil. The reaction was stopped by adding 10 mL of absolute ethanol.

Determination of Degree of Hydrolysis (DOH)

The DOH was determined by measuring the acid value of both hydrolyzed and unhydrolyzed oils as well as the saponification value of the unhydrolyzed oil according to the equation (1)

$$DOH\ \% = \frac{AV\ (hydrolyzed\ oil\ -\ blank)}{SV\ (original\ unhydrolyzed\ oil)\ -\ AV\ (original\ oil)} X\ 100$$

Determination of Amount of Unsaturation in Oil

The amount of unsaturation was determined by measuring the Iodine number of both unhydrolysed oil and hydrolysed oil at different reaction times. It is expressed as the number of grams of iodine absorbed by 100g of fat sample. The higher the iodine number the more is the unsaturation in the oil. Iodine number was determined by Wijs method (Cd 1d-92) (AOCS 2009).

Determination of Concentration of n-3 PUFA (FAME Analysis)

The fatty acid methyl esters were obtained from glycerides present in the oil by transesterification (Ichihara and Fukubayashi, 2010) and were identified by gas chromatography. FAMEs were injected on a GC Agilent gas chromatograph connected to a DB-5 column with dimensions of 30 m*0.25 mm*0.2 µm using a flame ionization detector (FID) equipped with a split/ splitless injector. The analysis was performed at a starting temperature of 160˚C with a hold time for 1 minute at the rate of 5˚C/min. The temperature was increased to 185˚C with a hold time of 10 minutes at the rate of 8˚C/min. The third ramp was conditioned at a temperature of 240˚C with a hold time of 10 minutes. The right inlet and the detector temperatures were 280˚C and 300˚C respectively. FAMEs were identified by

comparing the retention time of the samples with FAME standards from Sigma Aldrich, expressed as percent of total fatty acids (%). Chromatograms were analysed using Chrom Cad software

Results and Discussion

Effect of Temperature

Hydrolysis was carried out at different temperatures ranging from 30˚C to 70˚C by keeping all other parameters constant. CAL A showed a maximum degree of hydrolysis of 2.43% at a temperature of 50˚C as shown in the Figure 1. However, a rapid decrease in the hydrolysis degree was observed at rest of the temperatures due to the denaturation of the enzyme structure at temperatures other than optimum temperature. Liu et al., (2012) reported that lipase from *Candida antarctica* ZJB09193 showed maximum activity at 52˚C and optimal temperature range was found to be 50-55˚C and activity dropped significantly at 60˚C and 70˚C. For further studies an optimum temperature of 50˚C was maintained.

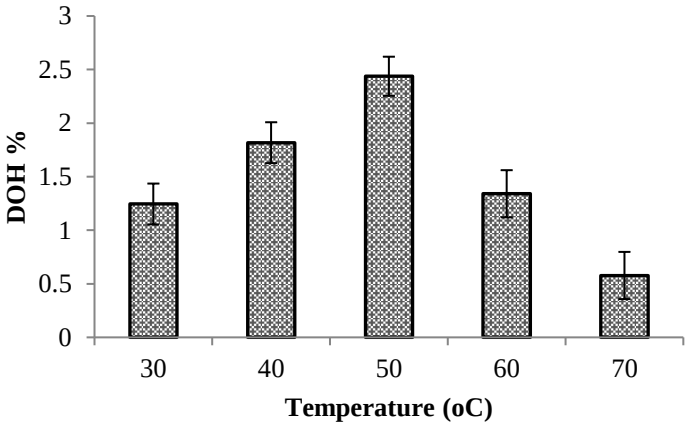

Figure 1: Effect of temperature on activity of CAL A [Reaction conditions: pH 7.0, enzyme load 10 mg/mL, oil to buffer ratio (1:1 w/w), reaction time 15 min].

Effect of pH

Hydrolysis reactions were carried out by varying pH from 6.0 to 8.5 (Figure 2). It shows maximum hydrolysis at neutral pH 7.0 because lipases normally contain residues of amino acids at the active site and result in maximum binding of the substrate at neutral pH. In alkaline solutions (pH>8.0), there could be partial damage of cysteine residues caused by β-elimination and in acidic solutions (pH<4.0) the labile peptide bonds sometimes found next to aspartic acid residues may be hydrolysed (Akova and Utsun, 2000). Also, at acidic pH, the enzyme may form high molecular weight aggregates or unspecific associations with other proteins due to its hydrophobic nature (Montero et al., 1993).

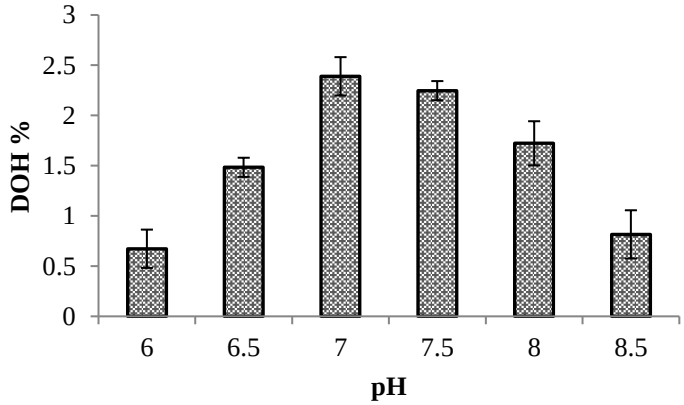

Figure 2: Effect of pH on activity of CAL A [Reaction conditions: Temperature 50˚C, enzyme load 10 mg/mL, oil to buffer ratio (1:1 w/w), reaction time 15 min].

The result of this study was similar to that of Pfefferet al., (2006) where the purified CAL A showed highest activity at pH 7.0. Therefore, for the rest of the study optimal pH of 7.0 was maintained.

Effect of Enzyme Load

The effect of enzyme concentration was studied by carrying out hydrolysis at different enzyme loads of 10 to 70 mg/mL, keeping other parameters a constant. The degree of hydrolysis increased linearly with the increase in CAL A concentration (Figure 3). A maximum degree of hydrolysis of 4.20% was seen at 60 mg/mL after which the hydrolysis degree remained constant. At low enzyme concentration and high oil content the rate of hydrolysis was low because of limited access of the active site at the oil water interface. At optimum enzyme concentrations a monolayer is formed around this interface resulting in high degree of hydrolysis and at higher concentration hydrolysis degree does not increase because of formation of multilayer of enzyme and accumulation of intermediates (Pongket et al., 2015). Hence, a further increase in CAL A concentration did not show much change in degree of hydrolysis. Therefore, further studies were carried out using 60 mg/mL CAL A.

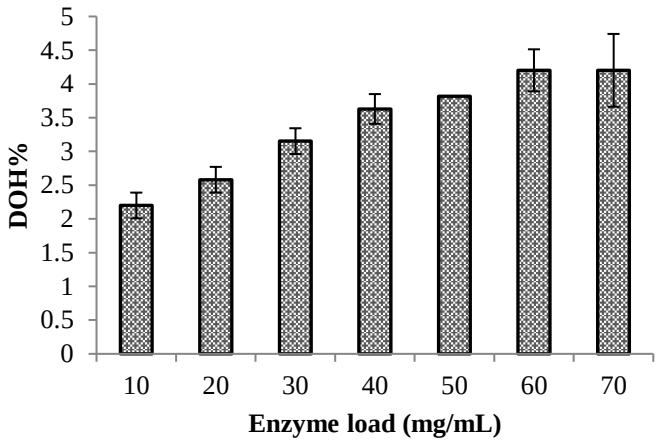

Figure 3: Effect of enzyme load on degree of hydrolysis [Reaction conditions: Temperature 50˚C, pH 7.0, oil to buffer ratio (1:1 w/w), reaction time 15 min].

Effect of Oil to Buffer Ratio

The degree of hydrolysis was highest at the oil to buffer ratio of 1:1 (w/w). The ratios above 1:1 (w/w) showed lesser activities (Figure 4) because high amount of water leads to a thicker water layer around the lipase leading to its denaturation (Sampath et al., 2016). Similar result was shown by Pongket et al., (2015) where the hydrolysis rate decreased with higher levels of buffer to oil ratio, which can be due to the alteration of ionization of the enzyme structure resulting in its denaturation. Lipase catalyses the cleavage of ester bonds of triglycerides with consumption of water molecules or in the presence of any aqueous environment. Hence it becomes important to study the amount of water/buffer required for the hydrolysis as it increases the functionality of the enzyme at the oil-water interface in the buffer system

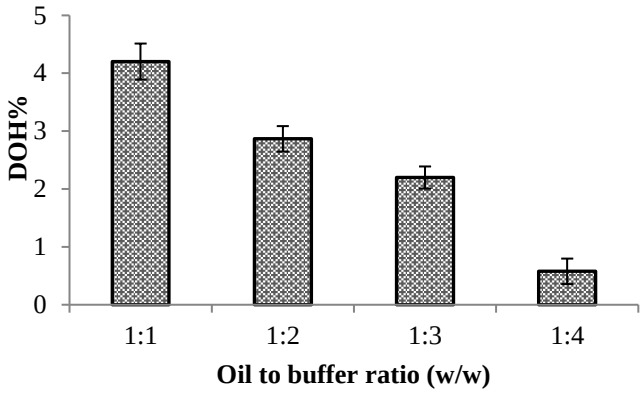

Figure 4: Effect of oil to buffer ratio on degree of hydrolysis [Reaction conditions: Temperature 50 ˚C, pH 7.0, enzyme load 60 mg/mL, reaction time 15 min].

Effect of Reaction Time

The time course of the hydrolysis reaction of CAL A shows maximum degree of hydrolysis of 12.11% at 60 minutes. It is clear from the trend observed in the Figure 5 that initially with increase in time the product formation was increased up to one hour. A further increase in the time did not lead to any improvement in the product formation. The progress of enzymatic reaction does not continue to increase linearly but it tends to slow down because of decrease in the substrate concentration or increase in the product concentration or inactivation of the enzyme (Gardossi et al., 2010; Sampath et al., 2016).

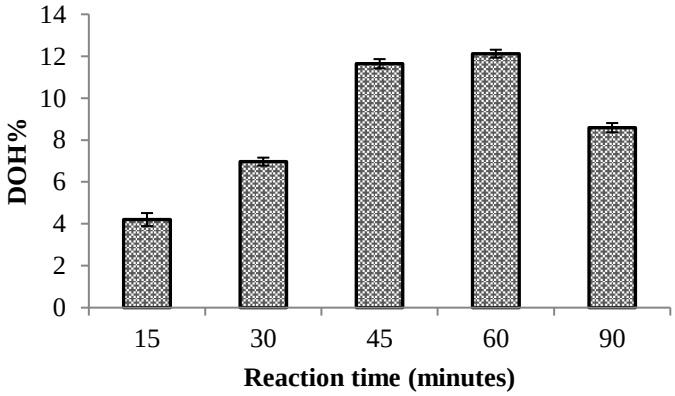

Figure 5: Effect of reaction time on degree of hydrolysis [Reaction conditions: Temperature 50˚C, pH 7.0, enzyme load 60 mg/mL, oil to buffer ratio (1:1 w/w)].

Amount of Unsaturation in Oil

The iodine number of unhydrolysed refined oil was determined to be 134.09 and the oil hydrolysed for1 hour was found to display an iodine value of 150.16. As shown in the Figure 6, there was an increase in the iodine number of the hydrolysed oil under optimized conditions of hydrolysis in comparison to the unhydrolysed refined oil. This signifies the increase in unsaturation and hence a possible enrichment of EPA and DHA, which was further confirmed by FAME analysis using gas chromatography

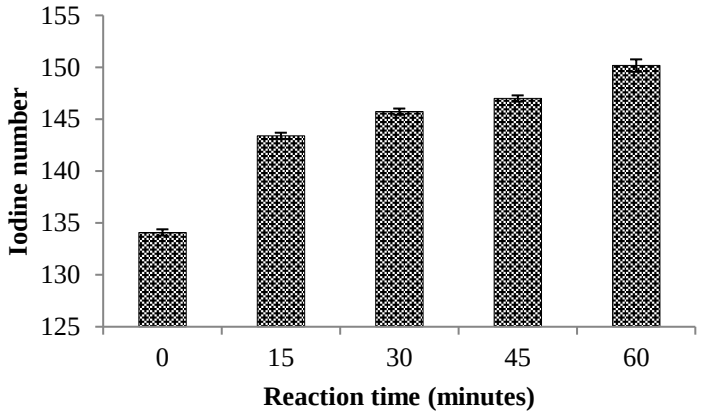

Figure 6: Amount of unsaturation in oil at different reaction times.

Concentration of n-3 PUFA in the Oil

The concentration of n-3 PUFA in the oil was determined by gas chromatography after removing the free fatty acids and moisture content using previously optimized refining method. The hydrolysis of refined Indian Sardine oil by CAL A at optimized conditions resulted in the enrichment of n-3 PUFA to 36.91% (30.51% EPA and 6.40% DHA) from 20.25% (17.86% EPA and 2.39% DHA) in the initial refined oil.

It is evident from above GC results that CAL A enhanced the nutritionally important n-3 PUFA like EPA and DHA in the sardine oil. This observation can be explained due to the fact of CAL A having fatty acid chain length specificity which shows an increased discrimination against the long chain PUFA like C18 to C22. CAL A hydrolyses the short chain fatty acids, saturated and mono unsaturated fatty acids because of the reduced steric hindrances when linked to the glycerol backbone, leading to the protection and enhancement of EPA and DHA (Okada and Morrissey, 2006). Usually lipases have sn-1,3 positional specificity, but CAL A has sn-2 preference (Domínguez De María et al., 2005) which might also be the reason for enrichment of EPA since it is preferentially located in the sn-1,3 position (Tengku-Rozaina and Birch, 2013). The hydrolyzed oil was found to contain enhanced quantities of DHA. Though theoretically DHA is mostly located in sn-2 position of the glycerol backbone (Charanyaa et al., 2017), there was enhancement in DHA content which can be due to the steric hindrance offered to the lipase and the fatty acid specificity of the lipase (Casas-Godoy et al., 2014).

Conclusions

The suitable reaction conditions for hydrolysis of refined Indian Sardine oil with *Candida antarctica* lipase A (CAL A) in buffer system have been determined. The lipase was observed to work optimally at pH 7.0, temperature 50°C with an enzyme load of 60mg/mL and 1:1(w/w) oil to buffer ratio for 1 hour. The hydrolysis of refined Indian Sardine by CAL A under optimal reaction conditions led to the increase in iodine number and hence considerable enhancement of n-3 PUFA. Hence, lipase catalysed hydrolysis can be considered as beneficial and feasible method for enhancement of n-3 PUFA in Indian Sardine oil for the use in nutraceuticals and functional food products.

References

Akova, A. Ustun, G., 2000. "Activity and adsorption of lipase from Nigella sativa seeds on celite at different pH values", Biotechnol.Lett., Vol. 22, pp. 355-359.

AOCS, 2009.Official methods and recommended practices of the American Oil Chemists Soc. Sampling anal. fats oils.

Calder, P. C. 2004. "n-3 Fatty acids and cardiovascular disease: evidence explained and mechanisms explored", Clin. Sci., vol. 107, no.1, pp. 1–11.

Casas-Godoy, L. et al. 2014. "*Yarrowia lipolytica* lipase Lip2: An efficient enzyme for the production of concentrates of docosahexaenoic acid ethyl ester", J. Biotechnol., vol. 180, pp. 30–36.

Charanyaa, S. Belur, P. D. and Regupathi, I. 2017. "A New Strategy to Refine Crude Indian Sardine Oil", J. Oleo Sci., vol. 66, no. 5, pp. 425–434.

Domínguez De María, P. et al. 2005. "Biotechnological applications of *Candida antarctica* lipase A: State-of-the-art", J. Mol. Catal. B Enzym., 37(1–6), pp. 36–46.

Falk-Petersen, S. et al. 1998. "Lipids and fatty acids in ice algae and phytoplankton from the Marginal Ice Zone in the Barents Sea", Polar Biol., vol. 20, no. 1, pp. 41–47.

Gardossi, L. et al. 2010. "Guidelines for reporting of biocatalytic reactions", Trends Biotechnol., vol. 28, no. 4, pp. 171–180.

Ichihara, K. and Fukubayashi, Y. 2010. "Preparation of fatty acid methyl esters for gas-liquid chromatography", J. Lipid Res., vol. 51, no. 3, pp. 635–640.

Lawson, L. D. and Hughes, B. G. 1988. "Human absorption of fish oil fatty acids as triacylglycerols, free acids, or ethyl esters", Biochem. Biophys. Res. Commun., vol. 152, no. 1, pp. 328–335.

Liu, Z. Q. et al. 2012. "Cloning, expression and characterization of a lipase gene from the *Candida antarctica* ZJB09193 and its application in biosynthesis of vitamin A esters", Microbiol. Res., vol. 167, no. 8, pp. 452–460.

Montero, S. et al. 1993. "Immobilization of *Candida rugosa* lipase and some properties of the immobilized enzyme", Enzyme Microb. Technol., vol. 15, no. 3, pp. 239–247.

Mozaffarian, D. and Wu, J. H. Y. 2011. "Omega-3 fatty acids and cardiovascular disease: Effects on risk factors, molecular pathways, and clinical events", J. Am. Coll. Cardiol., vol. 58, no. 20, pp. 2047–2067.

Okada, T. and Morrissey, M. T. (2007). "Production of n - 3 polyunsaturated fatty acid concentrate from sardine oil by lipase-catalyzed hydrolysis." Food Chem., vol. 103, no. 4, pp. 1411–1419.

Pfeffer, J. et al. 2006. "High yield expression of Lipase A from *Candida antarctica* in the methylotrophic yeast *Pichia pastoris* and its purification and characterisation", Appl. Microbiol. Biotechnol., vol. 72, no. 5, pp. 931–938.

Pongket, U. et al. 2015. "Enzymatic preparation of linoleic acid from sunflower oil: an experimental design approach", Biotechnol. Biotechnol. Equip., vol. 29, no. 5, pp. 926–934.

Rubio-Rodríguez, N. et al. 2010. "Production of omega-3 polyunsaturated fatty acid concentrates: A review", Innov. Food Sci. Emerg. Technol., vol. 11, no. 1, pp. 1–12.

Ruxton, C. H. S. et al. 2004. "The health benefits of omega-3 polyunsaturated fatty acids: A review of the evidence", J. Hum. Nutr. Diet., vol. 17, no. 5, pp. 449–459.

Sampath, C. Anita, N. Prasanna, B. D. Regupathi, I. 2016. Enzymatic Concentration of n−3 Polyunsaturated Fatty Acids from Indian Sardine Oil. In: Prasanna, B. D. Gummadi, S. Vadlani, P. (eds) Biotechnology and Biochemical Engineering. Springer.

Tengku-Rozaina, T. M. and Birch, E. J. 2013. "Enrichment of omega-3 fatty acids of refined hoki oil", J. Am. Oil Chem. Soc., vol. 90, no. 8, pp. 1111–1119.

Wijesundera, C. et al. 2008. "Docosahexaenoic acid is more stable to oxidation when located at the sn-2 position of triacylglycerol compared to sn-1(3)", J. Am. Oil Chem. Soc., vol. 85, no. 6, pp. 543–548.

Williams, C. M. and Burdge, G. 2006. "Long-chain n−3 PUFA: plant v. marine sources", Proc. Nutr. Soc., vol. 65, no. 1, pp. 42–50.