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Enrichment of Olive Oil with Alpha Linolenic Acid Catalyzed by Lipase Mediated Trans-Esterification

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Abstract: Consumption of Polyunsaturated fatty acid omega-3 is the most recommended fatty acids which have a health benefits for brain, kidney and eye. The conversion of plant-derived omega-3 (n-3) α-linolenic acid (ALA, 18:3n-3) to long-chain eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) can enhance ALA sufficient diets in compare to ALA deficient diets. Alpha-linolenic acid (ALA) was extracted and enriched from flax seed oil. Commercial lipases *Candida rugosa*, *Pseudomonas cepacea and P. fluorescens* were used for transesterification of ALA enriched from flax seed oil into olive oil TAG back bone. Gas chromatography of olive oil showed it contained high amounts of oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6) and palmitic acid (C18:0) with 0% of ALA. Among the commercial lipase *C. rugosa* has more preference to ALA and 27% of ALA was incorporated to TAG back bone of olive oil. In 24 hours reaction time, the ALA concentration in TAG of olive has increased to 26% while oleic acid decreased to 60% and palmitic acid decreased from 25 to 7%. The highest incorporation of ALA into olive oil (29%) occurred for ALA: olive oil mole ratio (1:2). There was a decline in incorporation of ALA in olive oil backbone with an increase in the amount of water. ALA incorporation of 25% occurred with 100 µl water while without water it was 27%; it decreased to 15% with 1 ml of water.

Key words: Lipase · α-linolenic acid · Enriched olive oil · Polyunsaturated fatty acid · Transesterification

INTRODUCTION

Alpha-linolenic acid (ALA) is a series of n-3 polyunsaturated fatty acid (PUFA), which was found mainly in vegetable oil such as flaxseed oil, walnut oil, rapeseed oil and perilla oil [1]. Omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and ALA are associated with health benefits [2, 3]. Consumption of appropriate amounts and proportions of these fatty acids are essential. Long -chain n-3 PUFAs such as DHA can be converted from the precursor α-linolenic acid (LNA, 18:3n-3), via delta-6 desaturase (D6D), delta-5 desaturase (D5D) and elongases, although the conversion is limited. Therefore, mammals obtain DHA [4] either as DHA itself or the precursor LNA. Also obtained from intermediates between LNA and DHA, like eicosapentaenoic acid (EPA, 20:5n-3) [5]. It has been reported that adequate intake of only LNA is sufficient to maintain normal brain DHA concentration [4].

Flax seed oil contains large amount of ω-3polyunsaturated fatty acid which can delay PUFA oxidation. ALA is partitioned to chylomicron TAG and very little to phospholipids after absorption [6]. It has recently been reported that ALA can be metabolized with emphasis on inter conversion as well as the oxidation and carbon recycling [4]. The conversion of the plant-derived omega-3 (n-3) α-linolenic acid (ALA, 18:3n-3) to the long-chain eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) can increase ALA sufficient diets in compare to ALA deficient diets [7].

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Enzymatic enrichment of PUFA has shown great potential in production of high quality nutrients in mild process conditions (e.g. neutral pH and low temperatures).

Olive oil is enriched in mono-unsaturated fatty acids (MUFA), specially oleic acid (C:18 n:1). It is a desirable substrate for transesterification because a diet rich in olive oil has been shown to reduce plasma low-density lipoprotein cholesterol (LDL), while leaving high-density lipoprotein cholesterol (HDL) unchanged [8]. Since high concentration of LDL causes high risk for coronary heart disease, it is recommended to reduce intake of dietary saturated fatty acids (SFAs) and increase the consumption of PUFA and MUFA [9]. According to Indian Council of Medical Research, the desirable proportions of saturated acid, oleic acid and PUFA in the dietary fats should be in proportion of about 1:1:1 [10]. Recently, many efforts have been carried out in transesterification or inter-esterification reactions to modify and enrich the content of n-3 PUFA in vegetable oil, melonseed oil, borage oil and evening primrose oil with implementation of biocatalysts such as lipases [11, 12].

The use of lipases for olive oil enrichment in presence of ALA enriched from flax seed oil can be a novel idea. In this study, preparation of ω-3 PUFA enriched acyl-glycerol from olive oil via enzymatic transesterification was conducted using three commercial lipases. The purpose of present work was to evaluate effects of several parameters such as temperature, reaction time, ratio of ALA with olive oil and water content on ω-3 PUFA enrichment of olive oil with specific lipase.

MATERIALS AND METHODS

Chemicals: Lipases of *Candida rugosa* (lipase 1, Cat EU029c), *Pseudomonas cepacea* (lipase 4, Cat EU088C) *and P. fluorescens* (lipase 21, Cat EU084) were purchased from Euoropa Bioproducts (Cambridge, UK). Fatty acid methyl ester mixture (Supelco 37 FAME Cat 47885-U), methyl linoleniate, triolein, 1, 2 di-olein, 1,3 di-olein, 1 mono-olein, 2 mono-olein and para-nitrophenyl palmitate (pNPP) were purchased from Sigma. All the solvents of HPLC grade or AR grade and thin layer chromatography plates (TLC, Silica gel_{60}, aluminum support) were purchased from Merck, Mumbai, India. Flax seeds and olive oil were locally purchased.

Preparation of Olive Oil Fatty Acid Methyl Ester: Fatty acid methyl esters derived from olive oil were prepared by mixing one gram of purified TAG with 3N methanolic KOH at 60°C for 3h. Unreacted TAG was extracted by hexane, while adding 3N methanolic HCl acidify the mixture and extracted fatty acids in hexane. The recovered fatty acids were subjected to esterification reaction by addition of 3N methanolic HCl and hexane at 60°C, left over night. Then FAME were extracted 3 times in hexane and dried over Na_2SO_4 and filtered through normal Whatman filter paper and evaporated under vacuum, FAMEs were analyzed by Gas chromatography (Chemito-1000). The GC was equipped with FID detector, capillary column (30m length, 0.25mm ID, 0.25µm film thickness, Supelco Company). Nitrogen gas was used as carrier gas with flow rate of 0.8ml/min. The detail of GC settings are stated in our previous published work [13].

Transesterification Reaction: A small scale transesterification reaction was conducted in 50 ml round bottom capped flask. According to author previous work [13], α-linolenic acid (ALA) which is used in transesterification reaction was enriched up to 80% by urea complexation method. The reaction mixture contained olive oil (0.4g) with enriched α-linolenic acid (ALA) (0.1g) from flax seed oil were incubated at 35°C, in stirring condition with individual free lipase sources (300U) for 24h, in 5ml hexane. The reaction was terminated by adding anhydrous sodium sulfate to the reaction mixture and filtration was carried out was through filter paper to remove the enzyme. Hexane was removed under vacuum, the reaction mixture was loaded on 10 cm Alumina column and individual components of TAG and FA were eluted with 150 ml hexane: diethyl ether as mobile phase to separate the TAG from fatty acids. Five ml fractions were collected and analyzed by TLC for absence of fatty acids. The pure TAG fractions were collected, the solvent was evaporated and purified TAGs were collected. To 10 µl of the concentrated TAG sample 1 ml methanolic HCl and 1 ml hexane were added, the mixture was transferred to reaction tubes and subjected to esterification reaction for FAME preparation with stirring condition at 60°C. The FAME produced was analyzed for determination of fatty acid components of TAG by GC as described earlier.

Effect of Different Ratio of Olive Oil: Ala on Transesterification Reaction: Transesterification reactions were performed in the same condition as

described above using different ratios of ALA: olive oil as 1:2, 1:4, 1:8 to optimize the effect of the ratio of oil and α-linolenic acid on transesterification.

Effect of Incubation Time on Transesterification Reaction: Transesterification reactions were performed for different time intervals like 1, 3, 6, 12 and 24 h in the same condition as described above and the product were analyzed for FAME content in TAG by GC.

Effect of Water Content on Transesterification Reaction: The transesterification reactions were performed with different quantities of water for instance 100, 250, 500 and 1000 µl in a reaction volume of 6ml, before addition of the enzyme to determine the effect of water activity on transesterification reaction. The fatty acid compositions of the TAGs purified from the reaction mixture by column chromatography were determined by GC analysis of FAME as described earlier.

RESULT AND DISCUSSION

Some vegetable oils have only limited applications in food products when they are used in their native form. They are often chemically or physically modified in order to enhance their quality. The quality of fat is determined by its value and chemistry of fat and also human requirements encourages modifying the fat to counter changing functional and nutritional challenges. Enzymatic interesterification is an efficient way of controlling the melting characteristics of edible oils and fats. This is performed by controlling the degree of conversion and reaction. No aggressive chemicals are used in the process and "trans" fats are not formed as in other production methods. Lipase mediated hydrolysis and synthesis are preferred techniques because of their well-known specificity and milder reaction conditions. Table 1 shows α-linolenic acid contents of some vegetable sources [14, 15]. Low amount (1.2%) of long chain fatty acids (C20-C22) was found in safflower oil. Linoleic acid content of poppy and safflower oil has 74% of it [16]. α-linolenic acid content of Perilla, linseed and flaxseed oil was more than 50%.

Table 1: α-Linolenic acid content of vegetable oils

Oil	α-Linolenic acid content (g/100 g of oil)
Perilla	54-65
Linseed	50-54
Flaxseed	53
Cohni	5.9-14.5
Canola	9-11
Wheat germ	6-9
Soybean	6.8
Modified canola	22–44

Enrichment of ALA by Urea Complexation: As discussed in our early stage of the work [13] urea complexation was found to be a suitable technique for enrichment of ALA from the fatty acid mixture. The GC analysis of the free fatty acids after urea complexation showed that the ratio of ALA enhanced to 82 %. It was also found that the ratio of 4:1 (urea: fatty acids) is the best for the enrichment of ALA [13]. This was presumably because of the removal of fatty acids like linoleic and palmitic acids by complexation and crystallization of the urea-fatty acid crystals. The technique of urea crystallization is specific and used for enrichment of ALA from the mixture of other fatty acids [17]. Also urea being highly water-soluble it was possible to separate ALA from urea by simple extraction, subsequently.

Fatty Acid Composition of Olive Oil: Olive oil is used as a substrate for enrichment with ALA. It contained high amounts of oleic acid (C18:1, n-9), linoleic acid (C18:2, n-6) and palmitic acid (C18:0) as shown in Fig. 1. It is found that it contains 70% monounsaturated fatty acid, 9% n-6 polyunsaturated fatty acid and it is a suitable source to provide a glycerol backbone and enriched with n-3 Polyunsaturated fatty acids.

Effect of Different Enzyme on Incorporation of ALA in to Olive Oil: Transesterification of olive oil with ALA was performed with enrichment of ALA in TAG backbone of olive oil was up to 27% by lipase from *C. rugosa* (Table 2) and GC chromatogram has confirmed the incorporation of ALA in to TAG back bone (Fig. 2). According to the results the degree of incorporation of ALA by commercial enzymes were *C. rugosa*> *P. fluorescens* > *P. cepacea.*

Table 2: Incorporation of ALA into olive oil back bone by different enzyme sources

	Palmitic acid %	Oleic acid %	Linoleic acid %	α-linolenic acid %
Olive oil	18.28	71.81	9.90	-00.01
Candida rugosa	10.79	52.29	9.79	27.13
Pseudomonas cepacea	22.87	57.27	6.79	13.07
Pseudomonas fluorescens	14.24	57.08	7.19	21.49

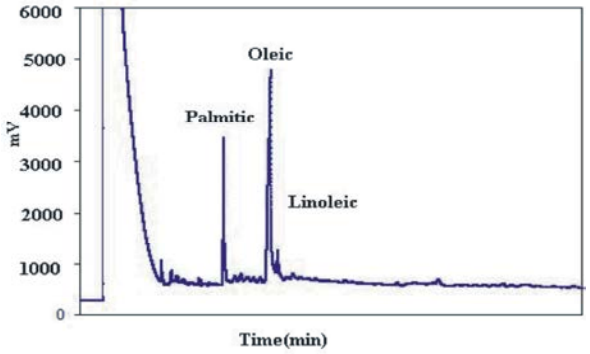

Fig. 1: GC chromatogram of olive oil fatty acids

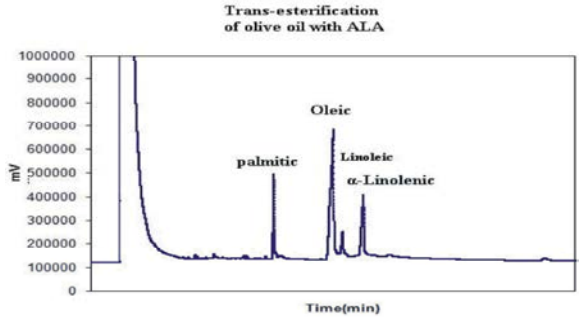

Fig. 2: GC chromatogram of Trans-esterified olive oil with ALA

It showed that free lipases can distinguish between PUFA and other fatty acids. Also, lipase shows less hydrolytic activity towards ester bonds of PUFAs that makes it more suitable for concentrating ALA. It was reported that lipase has been utilized for the recovery of EPA and DHA from marine oils and γ-linolenic acid from borage seed oil [8]. DHA-rich triglycerides were prepared from fish oil with lipases obtained from *C. rugosa* and *Chromo-bacterium viscosum* [18]. Lipase purified from *P. fluorescens* HU380 was used to concentrate EPA and DHA from oils [19]. About 19.6% incorporation of EPA and DHA in to hazelnut oil was reported by Can and Ozcelik [5]. All of these reports show that our free lipase from *C. rugosa* has a better effect on transesterification reaction which has incorporated 27% ALA into olive oil back bone.

Structured lipids containing EPA and DHA acids were synthesized in a batch reactor by lipase-catalyzed acidolysis of fish oil with caprylic acid. The following free lipases (Lipase AP, *A. niger* ; Lipase P, *Pseudomonas sp*.; Lipase A Y, *C. rugosa*; Lipase AK, *P. fluorescens*; Lipase F, *Rhizopus oryzae*; Lipase D, *R. delemar*) were used for esterification reaction. [20]. According to Tsao-Jen Lin and ShinWan Chen [21] after 6h reaction time, the degree of n-3 PUFA incorporation attained with various lipases

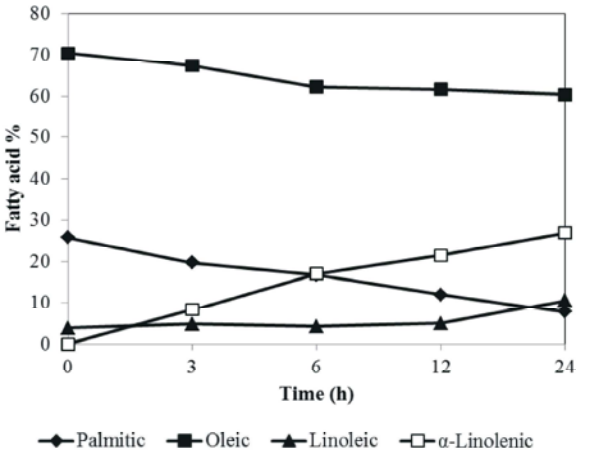

Fig. 3: Effect of time on incorporation of ALA in to TAG of olive oil

was in order of *M. miehei* > *C. rugosa* > *C. Antarctica* > *Pseudomonas sp* a nd the 1,3-specific lipase from *M. miehei* resulted highest degree of incorporation 56% of PUFA. Senanyake and Shahidi [22] have reported the degree of DHA incorporation into borage oil in hexane at ambient temperature was in order of *C. antarctica* >*Pseudomonas sp*.> *M. miehei* > *C. rugosa*.

Effect of Time on ALA Incorporation in to Olive Oil: The dependence of reaction rate of transesterification with respect to time was investigated at 35°C in presence of 300U lipase. When the reaction time increased the ALA incorporation has increased and oleic acid concentration decreased from TAG back bone. The fatty acid compositions of TAG before and after transesterification are shown in Fig. 3. After transesterification for 6h ALA increased from zero to 17% and the content of oleic acid decreased from 70 to 62%, as the esterification progressed more ALA was consumed and incorporated in TAG back bone. In 24 hours of reaction time, the ALA concentration in TAG of olive has increased up to 26% while oleic acid decreased to 60% and palmitic acid decreased from 25 to 7%. In similar studies the optimal reaction time changed depending on enzyme and substrate used [22]. Incorporation of DHA into borage oil by *C. antarctica* lipase was increased as the incubation time increased to 24 h [22, 23], whereas Akoh and Moussata [24] have reported 40 h as the optimal reaction time for the incorporation of EPA and capric acid into borage oil. In addition, Can and Ozcelik [5] have reported 30-40h for incorporation of EPA and DHA in hazelnut oil,

Table 3: Effect of incorporation of ALA in TAG back bone by ratio of ALA: olive oil

Ratio of ALA: olive oil	Incorporated ALA in TAG back bone %
1:2	29
1:4	27
1:8	25

the C18-C20 acyl chain lengthened n-3 PUFAs (EPA and LA) in the triglyceride mixture were found to increase proportionally with the progress of hydrolysis up to 6 h [25].

In a similar study, Rao *et al.* [26] have used RSM to optimize reaction conditions in the modification of coconut oil TAG by lipase-catalyzed acidolysis in hexane to incorporate n-3 or n-6 PUFA. It was found that maximal incorporation of n-3 PUFA occurred at a 1:4 molar ratio of TAG/FFA when incubation carried out for 34 h at 54°C.

Effect of Ratio of ALA: Olive Oil for Enrichment of Olive Oil with ALA: Effect of ratio of ALA: olive oil on the level of incorporation of ALA in olive oil was examined at 35°C for 24 h. When the concentration of olive oil was increased from 1:4 to 1:8 (ALA: olive oil), the incorporation of ALA did not change significantly. The highest incorporation of ALA into olive oil (29%) occurred when a mole ratio of ALA: olive oil (1:2) was employed while the incorporation rate at 1:4 and 1:8 ratio were rather similar, 27 and 25%, respectively (Table 3).

This indicated that the incorporation of a foreign fatty acid into triacylglycerol is limited to replacement of one fatty acid molecule on TAG by polyunsaturated fatty acid irrespective of the concentrations and time of reaction. This is an interesting finding that requires deeper and further studies. In enzymatic enrichment of PUFA in oil, ratios can be adjusted according to the desired amount of fatty acid in product. In present study, the incorporation of ALA into olive oil increased by increasing the molar ratio of ALA: olive oil. Other investigators have also reported that the incorporation of n-3 fatty acid into seed oil raises by increasing the substrate molar ratio [11, 22]. Also, it has been stated that incorporation rate can be improved by choosing high substrate molar ratios while reducing the reaction time, but Jennings and Akoh [27] have pointed out a significant modification in large scale, using excess amounts of n-3 fatty acid had resulted in lipase inhibition and decreased in product yield while removing excess fatty acids. Fajardo *et al.* [28] have reported the incorporation of EPA into palm oil relatively enhanced (27.6%) in small volume of reaction (21.5%) at the mole ratio of 1:3.

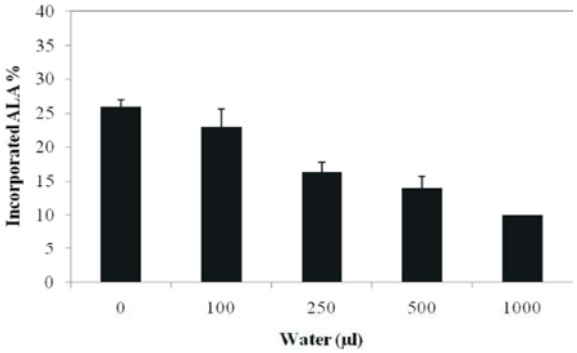

Fig. 4: Effect of water on ALA incorporation

However, there were no significant changes in terms of percentage of incorporation of DHA. Increasing the mole ratio of EPAX EE (fish oil concentrate high in n-3 PUFA) from 3 to 4 has increased the incorporation of both EPA and DHA by 11.3 and 6.5%, respectively; compared to when small quantities of the reactants used. When the mole ratio was further increased from 4 to 5, the percentage of incorporation slightly decreased. This may be due to the effect of mass transfer limitations on product yield, or the reaction may have reached to an equilibrium state. As the reactant quantities increased it has reflected on mass transfer of the reactants to the enzymes. Other researchers have shown that as the high mole ratio reflected on decrease of the reaction time; that has improved the reaction rate and resulted in less acyl migration.

Effect of Water Content on Incorporation of ALA in to Olive Oil Back Bone: The ability of lipase to catalyze reactions in organic solvents has been investigated. It is of common knowledge that the rate of enzyme catalyzed reactions in such systems strongly depend both on the amount of water present in the system and on the nature of the organic solvent used [29]. Catalytic activity and stability of enzymes are markedly influenced by hydration levels [30]. Often higher hydration levels are known to result in collection of enzyme leading to reduce yields [31]. Each enzyme displays different levels of water content profile for maximal activity [32]. A small variation in water activity can have a significant influence on the catalytic activity and selectivity of lipase. It is known that some water is necessary for catalytic activity and the precise level of water is important for equilibrium of a reaction. The transesterification reaction was performed in presence of 0 to 1000 µl water. There was a decline in incorporation of ALA in olive oil backbone with an increase in the amount of water. Fig 4 shows ALA

incorporation of 25% occurred at 100 µl water while without water it was 27% and it decreased to 15% with 1 ml of water. That is indicating the change in ALA incorporation rate is because of the shifting of the transesterification reaction to hydrolysis rather than synthesis, in presence of excess water.

It was reported that the incorporation of C10:0 into rice bran oil with increasing amounts of water (0-12%) for the solvent-free reactions indicates that the water content of the unmodified rice bran oil was 0.07% [27]. The influence of water content on the synthesis of butyl butyrate by transesterification reaction (alcoholysis) using lipases from different sources was reported [33], which indicated a direct relationship between water and reaction rate. Maximum esterification yield (92%) was obtained with *C. rugosa* lipase when compared to the yields from other lipases (*R. oryzae*, 45%; *Mucor javanicus*, 84%; *A. niger*, 56% and *Penicillium roqueforti*, 67%). It indicates that at higher water content the reaction equilibrium in favor of the hydrolysis and equilibrium shifts to synthetic mode when operated at low water levels.

CONCLUSION

The lipase mediated transesterification of ALA to olive oil TAG back bone was demonstrated as a suitable technique for enrichment of n-3 PUFA into olive oil which can be modified for other edible oils as well. Free lipase from *C. rugosa* identified as a good source of lipase for transestrification of ALA with olive oil and enriched olive oil with 27% of ALA. Use of the enriched olive oil with omega-3 can give a new kind of edible oil which prevents high cholesterol and heart problems. With the above promising results, it is recommended that in future work, the use of transesterification may require more investigation with different sources of seed oil which contain PUFA to prepare nutritional supplements.

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Persian Abstract

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چکیده

استفاده از اسیدهای چرب حاوی امگا ۳ برای سلامت مغز، کلیه و چشم توصیه شده است. تبدیل امگا ۳، اسید آلفا لینولنیک گیاهی (ALA) به اسید چرب زنجیر بلند ۲۰ کربنی غیر اشباع ایکوسپنتانوئیک و اسید دکوساهگزانوئیک صورت می گیرد و کیفیت رژیمی روغن با کمبود ALA را بهبود بخشد. لیپاز تجاری کاندیدا روگوسا، سودوموناس کپاکا و سودوموناس فلوروسانس موجب ترانس استریفیکاسیون روغن پنبه دانه غنی از ALA به روغن پایه TAG زیتونی می گردد. نتایج کروماتوگرافی نشان می دهد که روغن زیتون حاوی اسید اولئیک (C18:1, n-9)، لینولنیک (C18:2, n-6) و پالمتیک (C18:0) و ۰٪ ALA می باشد. در میان لیپازهای تجاری کاندیدا روگوسا برتری بیشتری برای تبدیل ALA دارد که موجب تبدیل ۲۷٪ ALA به پایه TAG روغن زیتون شده است. در زمان واکنش ۲۴ ساعت غلظت ALA روغن پایه TAG زیتون به میزان ۲۶٪ افزایش داده است و میزان اسید اولئیک به ۶۰٪ و پالمتیک از ۲۵ به ۵٪ کاهش داده است. بیشترین تبدیل ALA به روغن زیتون ۲۹٪ بوده که نسبت کسر مولی ALA به روغن زیتون (۱:۲) بوده است. با افزایش میزان آب در روغن زیتون میزان به کارگیری ALA کاهش یافته است. تبدیل ALA با ۱۰۰ µl ۲۵٪ بوده در حالیکه با افزایش آب به میزان ۱ میلی لیتر میزان بکارگیری ALA به ۱۵٪ کاهش یافته است.