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Biocatalyzed Production of Structured Olive Oil Triacylglycerols

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1. Introduction

Functional properties of fats and oils do not depend only on their fatty acid composition but also on the distribution of these fatty acids in the three positions of the glycerol backbone. This gives the fat or oil its commercial value. (Zhao,2005) There is a growing demand for lipids with desired characteristics ,thus researches have given way to these demands by the development of structured lipids with triacylglycerols that have predetermined composition and distribution of fatty acids. Structured lipids are now considered as alternatives to conventional fats not on the basis of saturate/polyunsaturate ratios but rather on their impact on cholesterol deposition. With the advances in the biotechnology and chemistry of fats and oils it is now possible to design fats and oils with properties that are desired. Recent years have seen great interest in the biotechnological modification and synthesis of structured triacylglycerols. Modification of fats and oil triacylglycerols to improve functionality have been carried out with various oils including olive oil. Olive oil enjoys a privileged position amongst edible oils and is still a buoyant commerce because of the large consumption of Mediterranean inhabitants (Oh et al,2009). It is one of the most expensive vegetable oils and of all the vegetable oils, olive oil is the best source of the monounsaturated fatty acid, oleic acid (72-83%) Risk factors for cardiovascular disease such as the level of homocysteine and total and low density lipoprotein (LDL) cholesterol in plasma have been reported to be reduced by oleic acid (Baro et al,2003). Olive oil is more than just oleic acid and because of its properties and qualities, it is used almost entirely in dietary consumption and even new markets have been created for this oil.

2. Triacylglycerol structure and characteristics

Triacylglycerols are by far the most abundant single lipid class and virtually all important fats and oils of plant or fat origin and most animal depot fats consist almost entirely of this lipid.

2.1 Triacylglycerol structure

Glycerol is a trihydric alcohol (containing three -OH hydroxyl groups) that can combine with up to three fatty acids to form monoacylglycerols, diacylglycerols, and triacylglycerols.

Fatty acids may combine with any of the three hydroxyl groups to create a wide diversity of compounds. A triacylglycerol (TAG)(Fig.1) consists of three fatty acids(R) to one glycerol molecule.

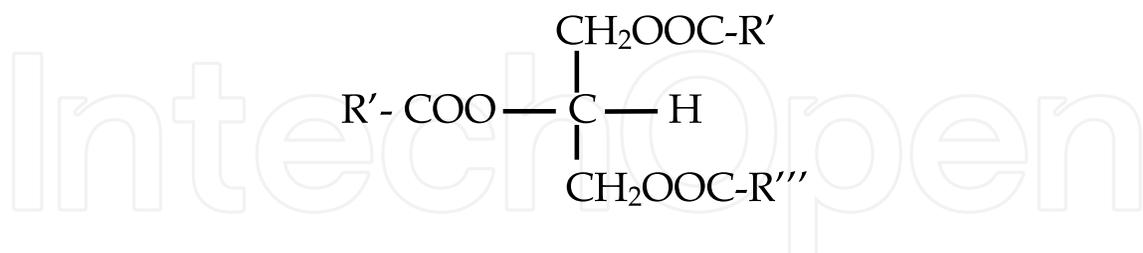


Fig. 1. Structure of a triacylglycerol

If all three fatty acids are identical, it is a simple triacylglycerol. The more common forms however are the “mixed” triacylglycerols in which two or three kinds of fatty acids are present in the molecule. The positions occupied by these fatty acids are numbered relative to their stereospecificity or stereospecific numbering (sn) as sn-1, sn-2 and sn-3. The orientation of the triacylglycerol structure specificity is as follows: if the fatty acid esterified to the middle carbon of the glycerol backbone is considered to the left (on the plane of the page), then the top carbon is sn-1, the bottom carbon is numbered sn-3 (below or behind the plane of the page) and the middle carbon is subsequently numbered as sn-2. The fatty acids in the triacylglycerol define the characteristics and properties of the triacylglycerol molecule. Both the physical and chemical characteristics of fats are influenced greatly by the kinds and proportions of the component fatty acids and the way in which these are positioned in the glycerol molecule (Breckenridge, W.C. 1978; Christie, W.W. 1982; Karupiah, T. & Sundram, K. 2007).

2.2 Triacylglycerol species of olive oil

The triacylglycerol composition is a relevant information for the restructuring of lipids. Most often this defines the properties being sought to make them more suitable for their end use. These are mainly nutritional or physical. Nutritional properties are important in structured lipids as there is a growing appreciation for this information because metabolism is intimately linked to triacylglycerol composition. Triacylglycerol composition by HPLC of olive oil as reported in literature is given in Table 1 (Christie, 1982; Uzzan, 1996; Aranda et al, 2004). Most prevalent triacylglycerols in olive oil is the oleic-oleic-oleic (OOO) triacylglycerol, followed, in order of incidence, by palmitic-oleic-oleic (POO), then oleic-oleic-linoleic (OOL), then palmitic-oleic-linoleic (POL), then by stearic-oleic-oleic (SOO). The triacylglycerol species show a small degree of asymmetry in the distribution of fatty acids among the three positions of the glycerol moiety.

However, a single symmetric triacylglycerol specie (OOO) represents almost half of the total triacylglycerols. New developments in analytical methodology have allowed the evaluation of the degree of asymmetry in other fractions. The information on the individual triacylglycerols would be very useful in the structured lipid production.

Triacylglycerol (TG)specie	% of Total TG (Range)
LLL	0 - 0.8
OLL	0.3 - 5.8
OLLn	0.9 - 0.6
OOLn	1.0 - 1.5
PLL	0.5 - 2.8
POLn	0.3 - 1.1
OOO	10.4 - 18.2
PoOO	0 - 1.1
POL	4.5 - 12.3
PPoO	0.4 - 1.2
PPL	0.7 - 2.1
OOO	21.8- 43.1
POO	20 - 23.1
PPO	2.9 - 5.3
PSPo	0 - 0.8
PPP	0 - 0.5
SOO	3.6 - 3.7
PSO	0.4 - 1.2
PPS	0 - 0.6

Table 1. Triacylglycerol Composition as Analyzed by HPLC

2.3 Fatty acid profile and distribution intriacylglycerols

The fatty acid composition of olive oil varies widely depending on the cultivar, maturity of the fruit, altitude, climate, and several other factors (Galtier et al, 2008). The major fatty acids in olive oil triacylglycerols are oleic acid ($C_{18:1}$), a monounsaturated omega-9 fatty acid which makes up 55 to 83% of olive oil. Another fatty acid is linoleic acid ($C_{18:2}$), a polyunsaturated omega-6 fatty acid that makes up about 3.5 to 21% of olive oil. Palmitic Acid ($C_{16:0}$), a saturated fatty acid that makes up 7.5 to 20% of olive oil, stearic Acid ($C_{18:0}$), a saturated fatty acid that makes up 0.5 to 5% of olive oil and linolenic acid ($C_{18:3}$) (specifically alpha-Linolenic Acid), a polyunsaturated omega-3 fatty acid that makes up 0 to 1.5% of olive oil. Olive oil contains more oleic acid and less linoleic and linolenic acids than other vegetable oils, that is, more monounsaturated than polyunsaturated fatty acids. This renders olive oil more resistant to oxidation. The different fatty acids have stereospecific distribution on the glycerol backbone rather than a completely random or "restricted random" distribution. In most vegetable oils either 18:1 or 18:2 are exclusively at the sn-2 position in the triacylglycerol species like OOO, LLL, POL and LLO. Linolenic acid ($C_{18:3}$) occurs less commonly, but when present, is at the sn-3 position as seen for OOLn in canola oil. Oleic acid is commonly at the sn-2 position of the olive oil triacylglycerols. (Karupiah & Sundram, 2007). Table 2 shows the fatty acid distribution in the three positions

of the glycerol molecule as reported by Uzzan (1996). In esterified olive oil, the content of saturated acids palmitic and stearic in position 2 is higher, with values of approximately 13-15% compared to the normal 1.5-2%.

Nature of FA	% total FA in 2	% total FA in 1+3
C14:0		
C16:	1.4	15.0
C18:0	-	3.4
C18:1	82.9	72.8
C18.2	14.0	7.4
C18:3	0.8	0.9

Table 2. Fatty Acid Distribution in the three positions of olive oil triacylglycerol

3. Structured triacylglycerols (sTAGS)

Structured lipids may be defined as triacylglycerols restructured or modified to change the fatty acid composition or their positional distribution in glycerol molecules by a chemical or enzymatic process. The term “structured triacylglycerol” was first introduced by Babayan (1987) to describe fats and oils that have been modified to change the fatty acid composition and the structure of triacylglycerols after the application of modification technologies. According to Hoy and Xu (2003) structured triacylglycerols (ST) generally are any fats that are modified or restructured from natural oils and fats, or fatty acids there from, having functionalities or nutritional properties for edible or pharmaceutical purposes. This definition covers any fats produced by either chemical or enzymatic methods for special functionality or nutritional use, including cocoa butter equivalents, breast milkfat substitutes, some low calorie fats, oils enriched in essential fatty acids γ -linolenic, arachidic, α -linolenic, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, margarines or plastic fats and structured triacylglycerols containing both long chain (essential) and medium/short chain fatty acids. Adamczak (2004) defined structured triacylglycerols as triacylglycerols with a precisely defined composition and position of fatty acids esterified with glycerol possible only with the use of lipases or enzymatic modification. Structured triacylglycerols are often referred to as a new generation of lipids that are considered as nutraceuticals or functional foods or functional lipids. Regardless of the definition restructuring of triacylglycerols can be designed for use as medical or functional food as well as nutraceuticals depending on the type of application.

3.1 Synthesis and production of sTAGS

Biotechnology has experienced considerable advances in the past years via the use of fats and oils. The enzymatic process of modification is one of the advantages of fats and oils biotechnology which gives additional levels of flexibility in controlling and designing structured triacylglycerols.

3.1.1 Lipases

In recent years, the use of lipases to modify the properties of triacylglycerols has received considerable interest and has been the subject of extensive research worldwide. Lipases catalyze three types of reactions and the catalytic action of lipases is reversible. They catalyze hydrolysis in an aqueous system, but also esterification (reverse reaction of hydrolysis) in a microaqueous system, where water content is very low. Transesterification is categorized into four subclasses according to the chemical species which react with the ester. Alcoholysis is the reaction with an ester and an alcohol, while acidolysis is the one with an ester and an acid. Interesterification is a reaction between two different esters, where alcohol and acid moiety is swapped. Lipases can be classified according to their positional specificity into two groups: 1,3-positional-specific and non-positional-specific. Usually, pancreatic and fungal lipases are 1,3-positional-specific, while yeast and bacterial ones are non-positional specific or weakly 1,3-positional-specific. It should however be noted that the positional specificity of lipases is not strictly divided into the two categories, but it varies widely in the range of very distinctly 1,3-positional-specific to very weakly specific or completely non-positional-specific. By exploitation of the specificity of lipases it is possible to produce acylglycerol mixtures which cannot be obtained by conventional chemical modification processes. Specificity of lipases can be utilized to produce products that cannot be produced otherwise which means that with 1,3 specific lipases, reactions involving triacylglycerol changes are confined to the sn-1 and sn-3 positions and the sn-2 acyl groups remain unaltered. There are several advantages connected to the use of lipases. The relatively mild reaction conditions for lipases reduce the amount of by products formed in a reaction. The use of lipases also renders it possible to process substances such as polyunsaturated fatty acids which cannot be processed by the conventional high temperature/high pressure processes. (Kennedy, 1991; Adamczak, 2004) With the application of new biotechnological techniques, companies are now able to produce lipases at lower costs. This will make the enzymatic processes far more competitive to the existing processes for the production of structured triacylglycerols.

3.1.2 Enzymatic processes of modification

Structured triacylglycerols may be prepared by hydrolysis of fatty acyl groups from a mixture of TAGs and random re-esterification follows onto the glycerol backbone. Depending on the desired metabolic effect, a variety of fatty acids are used in this process, including different classes of saturated, monounsaturated, and polyunsaturated fatty acids. Thus, a mixture of fatty acids is incorporated onto the same glycerol molecule. These manufactured lipids are structurally and different metabolically from the more simple, random physical mixtures of medium-chain triacylglycerols (MCTs) and long-chain triacylglycerol (LCT). Six possible fatty acid combinations could result for structured triacylglycerols prepared with an MCT and LCT and these are two MCFAs and one LCFA; one MCFA and two LCFAs; the two positional isomers; and small amounts of the starting MCT and LCT (Fig.2). Based on their high regioselectivity, lipases are effective biocatalysts for the manufacture of structured lipids that have a predetermined composition and distribution of fatty acids on the glycerol backbone. Structured lipids resembling TAGs of human milk have been produced by trans-esterification of tripalmitin, depending on the desired metabolic effect from plant oil, with oleic acid or PUFAs, obtained from plant oils,

using sn-1,3-specific lipases as biocatalysts. Such TAGs were found to closely mimic the fatty acid distribution of human milk and may be used in infant food formulations. Apart from imitating the human milk more closely, the occurrence of palmitic acid lipase catalyzed esterification has been used in fat modification to improve absorption properties and the nutritional value of lipids. The most commonly used method is acidolysis for the production of MLM type (M-medium chain fatty acid; L-long chain fatty acid using a regiospecific lipase to incorporate the medium chain fatty acids into the sn-1 and sn-3 positions of the triacylglycerol molecule. Currently interesterification is viewed as an alternate process to the partial hydrogenation of oils and fats. The process involves randomization among all three stereospecific positions of fatty acids in native edible oils and fats by enzymatic catalysis at low temperatures. The positional distribution of the fatty acids on the glycerol backbone is altered either through fatty acids switching positions within a triacylglycerol molecule or between triacylglycerols. If interesterification involves triacylglycerol species within the dietary fat, the fatty acid composition remains the same. There are many applications of interesterification. It is not only the management of the fatty acid mixtures which could lead to the improvement of physical properties such as in the case of cocoa butter equivalents or substitutes but it is used in the production of structured lipids which can provide specific metabolic effects for nutritive and therapeutic purposes (Kennedy,1991; Marangoni, 1993; Klemann,1994).

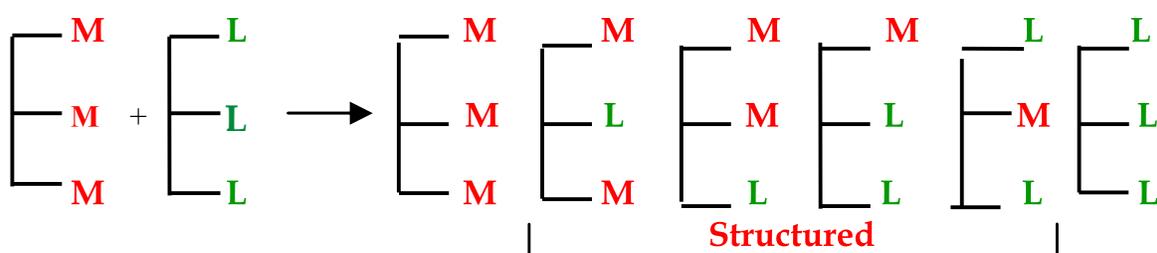


Fig. 2. Production of Structured Triacylglycerols

4. Structured olive oil triacylglycerols

Several researches have reported of the use of lipase catalyzed modification as the process for the production of structured olive oil triacylglycerols. In a study by Lee et al., 2006, olive oil triacylglycerols was used as a delivery medium for enrichment of conjugated linoleic acid in a dietary oil. Conjugated linoleic acid (CLA) are positional isomers of conjugated octadienoic acids two of which are cis-9,trans 11 and trans 10 cis-12 and are known to possess biological activity. The consumption of dietary CLA has decreased in recent years due to the replacement of animal lipids that contain little CLA. To consume more CLA and derive more health benefits, the enrichment of CLA in food has been attempted through modification of lipids in which synthesis of structured lipids is the most desirable method. Commercially produced CLA isomers were incorporated into extra virgin olive oil through a 1,3 specific lipase from *Rhizomucor miehii* that catalyzed acidolysis to produce the structured olive oil triacylglycerols. The olive oil synthesized structured olive oil contained reduced content of oleic acid which was 43.1 mol % from the original value of 75.7 mol% of the total fatty acids. The decrease was compensated by the increase of CLA content at 42.5 mol %. Major CLA isomers incorporated into the triacylglycerol molecules were cis-9,trans-

11 at 16.9 mol % and trans-10, cis-12 at 24.2%. The study suggests that restructuring olive oil may be a suitable way to incorporate or deliver CLA into human diets.

Structured triacylglycerols synthesized by the acidolysis of olive oil and capric acid was carried out with immobilized lipase derived from *Thermomyces lanuginosus* to produce olive oil triacylglycerols with medium chain fatty acids in its glycerol moiety. (Oh et al, 2009).

Medium chain triacylglycerols (MCTs) also offer numerous health benefits and have been widely studied for medical, nutritional and food applications. Structured lipids containing medium chain fatty acids at sn 1,3 positions and long chain fatty acids at the sn-2 position of triacylglycerols are more readily absorbed and oxidized for energy as compared to long chain triacylglycerols (LCT). Results of the study showed that the fatty acid composition of the olive oil triacylglycerols was significantly changed. The major fatty acid in the triacylglycerols was oleic acid originally, but after restructuring capric and oleic acids became the major fatty acids of the triacylglycerols. The study carried out by Fomuso and AKoh (2002) performed the lipase (1,3 specific lipase from *Rhizomucor miehiei*) catalyzed acidolysis of olive oil in a bench scale packed bed reactor. Findings showed olive oil to be characterized by four major clusters of triacylglycerol species with Equivalent carbon number (ECN), C_{44} , C_{46} , C_{48} , and C_{50} . Three monosubstituted products and two disubstituted products were detected after the reaction. Monosubstituted products have ECN of C_{36} , C_{38} , and C_{40} . And the disubstituted products had ECN of C_{30} , and C_{32} . Fatty acid distribution of the sn-2 position of olive oil was 74.8% oleic acid and 25.2% linoleic acid. The structured olive oil had 7.2% capryllic acid, 69.6% oleic acid, 21.7% linoleic acid and 1.5% palmitic acid at the sn-2 position. The results showed a structured olive oil that would have improved properties and nutritional value.

The production of cocoa butter equivalents is a promising application of the biotechnological production of structured lipids. Due to the high cost and fluctuations in supply and demand of cocoa butter, the industry has looked into the production of cocoa butter equivalents from other oil sources. Cocoa butter equivalents can be produced by the enzymatic acidolysis using sn 1,3 specific lipases that can catalyze the incorporation of palmitic acid (C_{16}) and stearic acid (C_{18}) to the sn-1,3 positions of a source containing oleic acid at sn-2 position until a similar composition of cocoa butter is obtained. The three main triacylglycerols are the 1,3 dipalmitoyl-2-oleoyl-glycerol (POP); 1(3)-palmitoyl-3(1)stearoyl-2-oleoyl glycerol (POS) and 1,3-distearoyl-2-oleoyl glycerol (SOS) with oleic acid at the sn-2 position of the glycerol backbone (Lipp, et al, 2001). Using olive pomace oil these three major triacylglycerols can be achieved to produce a cocoa butter like fat. Olive pomace oil's chemical composition does not differ from refined olive oil. It has the same triacylglycerol profile of olive oil because it is olive oil extracted via solvent. In a study by Ciftci and Fadiloglu (2009) utilizing the olive pomace oil for the production of a cocoa butter like fat, findings showed that the triacylglycerol composition of the prepared product was similar to that of the commercial cocoa butter which contained 18.9% POP, 33.1% POS, and 24.7% SOS. The triacylglycerol composition of refined olive pomace oils was redesigned so that properties such as the melting point, solid fat content and fat crystal network microstructures of the structured olive pomace oil and cocoa butter were very much similar.

In another study of Olive oil triacylglycerol restructuring, olive oil was blended with coconut oil to get a balanced proportion of saturated to unsaturated fatty acids and was subjected to lipase catalyzed interesterification to rearrange the fatty acids in the triacylglycerol molecule that would have both a short chain fatty acid and long chain fatty acid in one triacylglycerol molecule (Nagaraju & Lokesh, 2007). Results showed there were no significant differences between the blended and interesterified oils in terms of the fatty acid composition but HPLC analysis showed that there were new triacylglycerol molecular species formed. Studies have shown that structured lipids have a unique metabolism and exhibit better benefits when compared with the blended oils having similar fatty acid composition. The study of Nagaraju and Lokesh (2007) showed a reduction of serum cholesterol levels by 25% as compared to the oil blend. Cholesterol levels in rat liver was also reduced by 32% as compared with results using physical blending. The effect was certainly significant.

5. Conclusion

Through enzyme biotechnology, olive oil triacylglycerols can be structured to contain medium chain fatty acids or functional fatty acids like the conjugated linoleic acid for the production of a structured olive oil that would have improved biological and nutritional properties. Structured lipids provide attributes that consumers will find valuable whether for demands of healthier oils or for physical requirements to give appropriate properties. There is also a need for more researches with olive oil restructuring that will allow for better understanding and more control over the various interesterification processes and reduction in costs associated with large-scale production of structured olive oil triacylglycerols.

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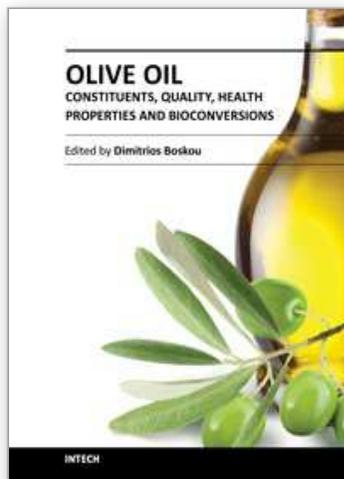
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The health-promoting effects attributed to olive oil, and the development of the olive oil industry have intensified the quest for new information, stimulating wide areas of research. This book is a source of recently accumulated information. It covers a broad range of topics from chemistry, technology, and quality assessment, to bioavailability and function of important molecules, recovery of bioactive compounds, preparation of olive oil-based functional products, and identification of novel pharmacological targets for the prevention and treatment of certain diseases.

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