Food Chemistry 250 (2018) 60–66

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

# Synthesis and concentration of 2-monoacylglycerols rich in polyunsaturated fatty acids

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ARTICLE INFO

*Keywords:*
2-Monoacylglycerols
Polyunsaturated fatty acid
Enzymatic ethanolysis
Low temperature crystallization
Molecular distillation

ABSTRACT

Polyunsaturated fatty acids (PUFA) in 2-monoacylglycerols form exhibit various biological activities and have potential applications in food and pharmaceuticals. Preparation of 2-monoacylglycerols was conducted by enzymatic enthanolysis. The effects of lipase type, substrate weight ratio, reaction time and lipase load on the 2-monoacylglycerols content in the crude product were investigated. Lipozyme 435 behaved as 1,3-specific and high-catalytic-activity lipase in this reaction. Under the optimal conditions (ethanol:oil = 3:1 (w/w), 8% Lipozyme 435, 3 h), 27% 2-monoacylglycerols were obtained. After solvent extraction of 2-monoacylglycerols, the abilities of low temperature crystallization and molecular distillation to concentrate 2-PUFA-monoacylglycerols were compared. Low temperature crystallization concentrated 81.13% and 74.29% PUFA by acetonitrile and hexane, respectively, with over 90% in 2-monoacylglycerol forms. Conversely, molecular distillation yielded a PUFA concentration of 72% but decreased the 2-monoacylglycerols content to 69.81%. Thus, the method including enzymatic ethanolysis and low temperature crystallization is suitable for preparation of 2-monoacylglycerols rich in PUFA.

## 1. Introduction

Monoacylglycerols (MAGs), possessing both hydrophilic and hydrophobic portions, are widely used as nonionic surfactants and emulsifiers in the food and pharmaceutical industries due to their excellent emulsifying, stabilizing and plasticizing properties (Damstrup et al., 2005; Wang, Li, Wang, Jin, & Wang, 2014). Currently, novel functional MAGs of nutritional interest, primarily those enriched in n-3 polyunsaturated fatty acids (n-3 PUFA), have received considerable attention. It is well-known that PUFA, such as eicosapentaenoic acid (20:5n-3, EPA), docosapentaenoic acid (22:5n-3 & 22:5n-6, DPA) and docosahexaenoic acid (22:6n-3, DHA), have health-promoting functions, such as prevention of cardiovascular disease (Calder & Yaqoob, 2012; Investigators et al., 2008; Kris-Etherton, Harris, & Appel, 2002), anti-inflammation (Dasilva et al., 2015; Oh et al., 2010), inhibition of cancer growth (Fetterman & Zdanowicz, 2009; Murphy, Mourtzakis, & Mazurak, 2012), anti-hyperlipidemia (Gotoh et al., 2009), anti-depression (Peet, Brind, Ramchand, Shah, & Vankar, 2001) and promotion of brain and nerve development in infants (Horrocks & Yeo, 1999).

MAGs rich in n-3 PUFA also have some beneficial effects on the prevention of hypertension and the improvement of immune and other physiological functions (Morin, Blier, & Fortin, 2015; Morin, Rousseau, Blier, & Fortin, 2015). According to the digestive characteristic of dietary lipid, fatty acids can be absorbed when released from the triacylglycerols (TAGs) structures as free fatty acids or as 2-MAGs after digestive lipolysis (Michalski et al., 2013). The 2-MAGs are most readily absorbed through the intestinal mucosa and are used directly for the resynthesis of TAGs (Michalski et al., 2013). Furthermore, 2-MAGs are involved in the synthesis and degradation of endocannabinoids, which can regulate appetite, pain sensation, inflammation and lipid metabolism (Blankman, Simon, & Cravatt, 2007; Masoodi, Kuda, Rossmeisl, Flachs, & Kopecky, 2015; Panikashvili et al., 2006). For these reasons, 2-MAGs rich in PUFA are expected to be applied in dietary supplements, ingredients, functional food or pharmaceuticals.

The preparation of MAGs can be conducted by esterification, alcoholysis or partial hydrolysis of TAGs (Feltes, Oliveira, Block, & Ninow, 2013). In industry, MAGs are primarily produced by the glycerolysis of oil with the catalysis of alkali. The advantages of chemical catalysis are its low cost and short reaction time. However, the chemical method also has many disadvantages, such as high reaction temperature, side reactions and toxic catalysts. These disadvantages limit its use in the synthesis of 2-PUFA-MAGs. Enzymatic catalysis has received

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https://doi.org/10.1016/j.foodchem.2018.01.027
Received 9 October 2017; Received in revised form 27 December 2017; Accepted 2 January 2018
Available online 03 January 2018

considerable attention due to the mild reaction condition, leading to decreased oxidation of heat-sensitive PUFA. In addition, the selectivity of most lipases enables the production of designer lipids. With the development of lipase, chemical and physical modification improves their catalytic properties and reuse, reducing process costs and developing a green pathway to produce structural lipids (Lee & Akoh, 1998).

Enzymatic ethanolysis of TAGs is an effective method for the synthesis of 2-MAGs. Wongsakul, Prasertsan, Bornscheuer, and H-Kittikun (2010) reported that the optimum conditions for the synthesis of 2-MAGs by alcoholysis of tuna oil were a water activity of 0.43, a temperature of 60 °C and catalysts of immobilized lipase preparations from *Pseudomonas* sp. and *Candida Antarctica* fraction B in methyl *tert*-butyl ether for approximately 12 h. After crystallization, up to 80% 2-MAGs containing 80% PUFA were achieved. Muñío et al. (2008) studied that 2-MAGs with 96% purity were obtained by ethanolysis of cod liver and tuna oils. The PUFA content of 2-MAGs was similar to the PUFA profile at the position 2 of the original TAGs (Esteban et al., 2009). For nutritional and pharmacological purposes, further purifications are required to concentrate 2-PUFA-MAGs.

This study aims to develop an effective method for synthesizing 2-MAGs and obtaining 2-MAGs rich in PUFA (EPA, DPA and DHA) by further purification. The flowchart of this study is shown in Fig. 1. For the synthesis of 2-MAGs, the effects of lipase type, reaction time, substrate weight ratio and lipase load on the content of 2-MAGs in the crude product were investigated. To obtain 2-MAGs rich in PUFA, two purification methods to increase PUFA content and avoid acyl migration were compared.

## 2. Materials and methods

### 2.1. Materials

Refined tuna oil was generously provided by NovoSana (Taicang) Co., Ltd. (Jiangsu, China). Novozym 435 (lipase B from *Candida Antarctica*, immobilized on macroporous polyacrylate resin beads), Lipozyme 435 (lipase B from *Candida Antarctica*, immobilized on acrylic resin), Lipozyme RM IM (a commercial immobilized 1,3-specific lipase from *Rhizomucor miehei*, immobilized on macroporous anion exchange resins) and Lipozyme TL IM (an immobilized 1,3-specific lipase from *Thermomyces lanuginosus*, immobilized on silica granulation) were generously donated by Novozymes (Beijing, China). 2-Oleoylglycerol (≥95%), 1-oleoylglycerol (≥99%), diolein (85% 1,3-diolein and 15% 1,2-diolein), standard mixtures of fatty acid methyl esters (FAME) (Supelco 37 component FAME mix) were purchased from Sigma–Aldrich Chemical Co., Ltd. (Shanghai, China). Hexane and isopropyl alcohol of HPLC-grade were purchased from Beijing J&K Scientific Co., Ltd. (Beijing, China). Other organic solvents were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

### 2.2. Optimization of the synthesis of 2-MAGs

Synthesis of 2-MAGs was conducted by enzymatic ethanolysis of tuna oil. The effects of lipase type, reaction time, reactant weight ratio and lipase load on the content of 2-MAGs in the ethanolysis were studied. The optimal conditions were obtained by maximizing the amount of 2-MAGs. In this reaction, the fatty acids located at the *sn*-1,3 positions of triacylglycerols (tuna oil) were removed by 1,3-specific lipase. Meanwhile, 1,2-diacylglycerols (1,2-DAGs), 2-MAGs and ethyl esters (EEs) were formed. The reaction was stated as the addition of lipase. At the end of reaction, the catalyst was removed by filtration, and the ethanol was removed by a vacuum evaporator. The crude reaction product was diluted with hexane and subsequently quantified by HPLC. When the reaction conditions were optimized, one factor was changed for each level, and the other factors were fixed. After one of the factors was optimized, the optimal value was used for the next factor optimization. All reactions were conducted in triplicate. The presented data are means ± standard deviations.

### 2.3. Purification of 2-MAGs in the crude product by liquid-liquid extraction

The 2-MAGs in crude product were purified though a liquid-liquid extraction method described by Wang, Liang et al. (2014) with minor modification. The crude ethanolysis product contained MAGs, DAGs, EEs and unreacted TAGs. After evaporating away the solvent, 1 g ethanolysis product was dissolved in 10 ml hexane and 10 ml 85% (v/v) ethanol aqueous solution. Subsequently, two layers were separated. The ethanol aqueous phase containing MAGs was collected and washed again with 10 ml hexane several times. Purer MAGs were obtained by removing ethanol and water under reduced pressure at 35 °C. The content of MAGs was analyzed by HPLC as described.

### 2.4. Concentration of 2-MAGs rich in PUFA

Two methods were employed to concentrate 2-MAGs rich in PUFA: low temperature solvent crystallization and molecular distillation. The methods were compared based on the content of 2-PUFA-MAGs.

#### 2.4.1. Low temperature solvent crystallization

First, after liquid-liquid extraction, the 2-MAGs were dissolved in a given solvent (1:10, w/v), including hexane, acetone, methanol and acetonitrile and then cooled at −40 °C for 12 h. After forming crystallization, the liquid phase containing most 2-PUFA-MAGs was separated from the solid phase by immediate vacuum filtration though a Buchner funnel, which was precooled to the same temperature applied to the mixture solution. The solid phase was discarded. The whole process was performed in triplicate for each solvent. The final content of PUFA in 2-MAGs form was confirmed by GC after methyl esterification.

#### 2.4.2. Molecular distillation

The MAGs derived from liquid-liquid extraction were separated using molecular distillation (KDL 1, UIC GmbH, Alzenau-Hoerstein, Germany). To obtain a high content of 2-PUFA-MAGs fraction, distillations were employed in different evaporation temperatures (125 °C, 135 °C and 150 °C). The other operation parameters were fixed as follows: 65 °C of feeding temperature, 1 ml/min of feeding rate, $10^{-3}$ mbar of vacuum, 55 °C of condenser temperature. The distillation at each temperature was conducted in triplicate. The 2-MAGs content and PUFA content of distillation product were identified by HPLC and GC, respectively.

### 2.5. HPLC analysis

Identifications of the reaction product (MAGs, DAGs, TAGs and EEs)

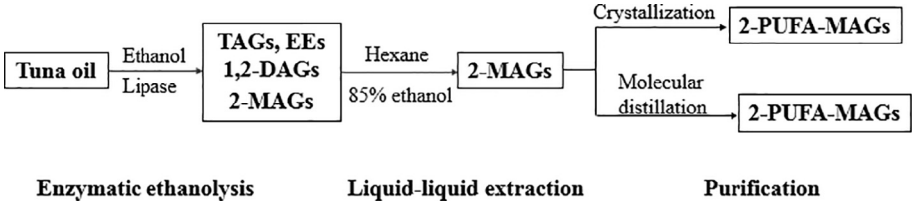


**Fig. 1.** Synthesis and purification of 2-MAGs. 2-MAGs, 2-monoacylglycerols; DAGs, diacylglycerols; TAGs, triacylglycerols; EEs, ethyl esters. PUFA, polyunsaturated fatty acids, including EPA, DPA and DHA.