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Production and Concentration of Monoacylglycerols Rich in Omega-3 Polyunsaturated Fatty Acids by Enzymatic Glycerolysis and Molecular Distillation

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Abstract

Production of monoacylglycerol (MAG) concentrate rich in omega 3 polyunsaturated fatty acids (n-3 PUFAs) was conducted through short path distillation (SPD) of an acylglyceride mixture produced by enzymatic glycerolysis. 67% yield of MAG was achieved in 2 h using sardine oil in a mole ratio of 1:3 with glycerol and 5% of immobilized *Candida antarctica* lipase B (Lipozyme 435) in *tert*-pentanol medium. For industrial interest and preservation of stability of the components, SPD under vacuum of 10^{-3} mbar was used as an appropriate approach for fractionation of MAG operated in a UIC KDL 5 system. A stepwise SPD process was designed: the first distillation at evaporator temperature (T_E) of 110ºC was performed to remove completely the glycerol and most of FFA; and the second distillation at optimized T_E 155ºC with feeding flow 1.0 mL/min; resulting in a stream distillate with MAG purity of 91% and 94% overall recovery of MAG. This work also demonstrated that SPD is able to concentrate n-3 PUFA in MAG form by distilling at proper T_E e.g. 125ºC, where n-3 PUFAs will be concentrated in the residues. Moreover, this work mapped out a complete processing diagram for scalable production of n-3 PUFAs enriched MAG as potential food emulsifier and ingredient.

Keywords: monoacylglycerol (MAG), glycerolysis, Lipozyme 435, omega-3 polyunsaturated fatty acids (n-3 PUFAs), short path distillation.

1. Introduction

Fish oil is rich in omega-3 (n-3) polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). n-3 PUFAs play important roles in human health, and epidemiological and clinical studies have shown that EPA and DHA reduce the risk of coronary heart disease and help in brain, neural, and retinal developments (De Deckere, Korver, Verschuren, & Katan, 1998; Kris-Etherton, Harris, & Appel, 2002; Nichols, McManus, Krail, Sinclair, & Miller, 2014). There are several reasons for producing concentrates of n-3 PUFAs, but their chemical form has to be taken into account, as n-3 PUFAs in free fatty acids (FFA), ethyl esters (EE), acylglycerides (AG), or phospholipids have different bioavailabilities. Studies have shown that human absorption of n-3 PUFAs in EE form is poor, primarily because EEs are poor substrates for pancreatic lipase. Although PUFAs in FFA form are absorbed more efficiently than PUFAs in EE or TAG form, they could present irritant effects and are very prone to auto-oxidation (Hernandez, 2014; Lawson & Hughes, 1988). Some studies have reported that the oxidative stability of PUFAs in TAG form is higher than PUFAs in EE form. Taking these considerations into account, MAG and DAG containing n-3 PUFAs are expected to have good application potentials, e.g. in functional foods, dietary supplements, or pharmaceuticals. Furthermore, these molecules, especially MAG, have excellent emulsifying properties, and MAG represent about 70% of the synthetic emulsifiers currently produced. For some applications, at least 90% pure MAG is required (L. V. Fregolente, Batistella, Maciel Filho, & Wolf Maciel, 2006). MAGs are usually produced by chemical glycerolysis employing inorganic alkaline catalysts at high temperatures. These processes are not suitable for heat-sensitive oils such as fish oils. Therefore, the enzymatic process has become an attractive alternative approach for polyunsaturated MAG production because the

reaction can be carried out under mild conditions (Bornscheuer, 1995; Feltes, de Oliveira, Block, & Ninow, 2013).

The most widely used immobilized lipases used in enzymatic glycerolysis reactions are from *Candida antarctica* (lipase B, Novozym® 435), *Rhizomucor miehei* (Lipozyme RM IM), *Pseudomonas fluorescens (*IM-AK), and *Thermomyces lanuginose* (Lipozyme TL IM) (Feltes, de Oliveira, Block, & Ninow, 2013; Kristensen, Xu, & Mu, 2005). Several researchers use Novozym® 435 as biocatalyst to mediate glycerolysis reactions due to its high MAG yield (Damstrup, Jensen, Sparsø, Kiil, Jensen, & Xu, 2006; Voll, Krüger, de Castilhos, Filho, Cabral, Ninow, et al., 2011; Yang, Rebsdorf, Engelrud, & Xu, 2005). This lipase is not water-dependent, and works successfully in glycerolysis systems, even in the presence of solvents (Feltes, de Oliveira, Block, & Ninow, 2013). This is important, as solvents are usually required in glycerolysis reactions due to the immiscibility of the reactants (glycerol and oil or free fatty acids). Damstrup et al. (Damstrup, Jensen, Sparsø, Kiil, Jensen, & Xu, 2005) evaluated various solvents and concluded that the highest MAG yield by glycerolysis were obtained with tertiary alcohols, *tert*-butanol (TB) and *tert*-pentanol (TP). The relatively low log P value of these tertiary alcohols (0.35 and 0.89, respectively) indicates both hydrophilic and hydrophobic characteristics, ensuring miscibility of the reactants. Moreover, *Candida antarctica* lipase B does not act on tertiary alcohols due to steric hindrance (Yang, Rebsdorf, Engelrud, & Xu, 2005). The liquid-liquid equilibria (LLE) of sardine oil + glycerol + tertalcohols have been determined previously (Solaesa, Bucio, Sanz, Beltrán, & Rebolleda, 2013). LLE data help to minimize the amount of solvent needed to create a homogeneous reaction system, which leads to an improvement in mass transfer, obtaining high reactions yields in short reaction time.

For commercial interests, several methods have been reported for concentrating n-3 PUFA derivatives. Generally, a combination of techniques is used, such as an enzymatic reaction (hydrolysis, ethanolysis or glycerolysis) followed by molecular distillation, also known as short path distillation (SPD), low-temperature crystallization, urea complexation or supercritical fluid fractionation (Gámez-Meza, Noriega-Rodríguez, Medina-Juárez, Ortega-García, Monroy-Rivera, Toro-Vázquez, et al., 2003; Lin, Chen, & Chang, 2006; Xuebing Xu, 2003). Crystallization at low temperature has some technical limitations, such as molecular association and mixed-crystal formation and use of large quantities of organic solvents (Brown & Kolb, 1955). On the other hand, urea complexation presents some technical advantages in fractionation of PUFAs such as high efficiency and better selectivity. However, for concentrating PUFAs for human consumption the use of urea should be avoided, where formation of ethyl carbamate, an animal carcinogen is reported (Canas & Yurawecz, 1999). In case of supercritical fluid fractionation, most studies are directed towards fractionating the FFAs or their esters, which are more soluble in supercritical fluids. To design an efficient process, more knowledge on the solubility and phase equilibria of other compounds in the supercritical fluid is still needed (Farvin & Surendraraj, 2012). Whereas, in SPD, compounds with different boiling points are separated under vacuum, which decreases the evaporation temperature and minimize the residence time, which enable heat-sensitive compounds to be separated with minimal thermal degradation. Therefore, this technology has been widely used in lipid areas. Most of the studies about the separation of MAG from DAG and TAG mixtures by SPD are focused on purification of saturated and monounsaturated compounds such as monoolein by glycerolysis from coconut oil (Zha, Chen, Wang, Wang, Chen, & Zheng, 2014), or high oleic sunflower oil (Zhu, Li, Wang, Yang, & Ma, 2011). Concentration of MAG and DAG by SPD from glycerolysis of

palm olein (Yeoh, Phuah, Tang, Siew, Abdullah, & Choong, 2014), soybean oil (P. B. L. Fregolente, Pinto, Wolf-Maciel, & Filho, 2010) or camellia oil (Zheng, Xu, Wang, Qin, Ning, Wang, et al., 2014) have been also reported. Processing of marine oils by SPD to remove pollutants and oxidation products has been reported (Oliveira & Miller, 2014; Oterhals & Berntssen, 2010), however, the studies focused on PUFA purification mainly obtained the PUFA concentrate in FFA or EE form (Breivik, Haraldsson, & Kristinsson, 1997; Kahveci & Xu, 2011; Rossi, Grosso, Pramparo, & Nepote, 2012; Zhang, Liu, & Liu, 2013). Other studies have used SPD to remove ethyl esters subsequently to ethanolysis in order to obtain an acylglycerol product (mainly DAG) rich in DHA and EPA (Lyberg & Adlercreutz, 2008; Martín Valverde, González Moreno, Rodríguez Quevedo, Hita Peña, Jiménez Callejón, Esteban Cerdán, et al., 2012; Valverde, Moreno, Callejón, Cerdán, & Medina, 2013). However, the preparation of a product with a high concentration of MAG rich in n-3 PUFA has not been well documented yet.

In this work, acylglycerols rich in n-3 PUFA were prepared by enzymatic glycerolysis of sardine oil using immobilized *Candida antarctica* Lipase B (Lipozyme 435) in TP. The MAG fraction was purified using SPD. Different evaporation temperatures were evaluated to maximize the MAG recovery at high purity in the distillate. The main purpose of this study was to develop an effective distillation process for concentration of n-3 PUFA in MAG form.

2. Materials and methods

2.1 Materials

Refined sardine oil was kindly provided by Industrias Afines S.L. (Pontevedra, Spain). The fatty acid (FA) composition of sardine oil and the mole percentage of FAs at the sn-

2 position of TAG is presented in Table 1 (Solaesa, Bucio, Sanz, Beltrán, & Rebolleda, 2014). More than 80% of DHA was found at sn-2 position.

Lipase B from *Candida antarctica* (Lipozyme 435, immobilized lipase) was generously supplied by Novozymes A/S (Bagsvaerd, Denmark). The water content of Lipozyme 435 was 3.5 ± 0.3% as determined by Karl-Fisher titration with a Mitsubishi CA-20 moisture meter in triplicate. Glycerol was purchased from Sigma Aldrich (St. Louis, MO) with a purity of ≥ 99.5% and a water content of 0.04 ± 0.01%. *tert*-Pentanol (TP) was purchased from Merck with a purity of ≥ 99% and a water content of 0.065 ± 0.023%.

The standards of MAG, DAG, FFA, and fatty acid methyl ester (FAME) for chromatography analysis were purchased from Sigma Aldrich Co. (St. Louis, MO). All other solvents and reagents were of analytical or chromatographic grades from VWR (Søborg, Denmark).

2.2 Enzymatic glycerolysis of sardine oil

Different vials containing one gram of mixture of sardine oil, glycerol and TP were incubated at 50 ºC in a water bath with orbital agitation. Different molar ratios of substrates and enzyme dosages were studied. The amount of TP added was calculated on the basis of previous studies on LLE (Solaesa, Bucio, Sanz, Beltrán, & Rebolleda, 2013). It was fixed at mass ratio of 1.5:1 (TP:substrates). At selected time intervals (from ten minutes up to eight hours), the reaction mixture was withdrawn and filtered through a microfilter (0.45 µm, Sartorius RC) to stop the reaction by removing the lipase. All samples were stored at −18 ºC prior to analysis. Samples were analyzed by HPLC to quantify the lipid compounds (TAG, DAG, MAG and FFA).

2.3 Fractionation of lipid classes by HPLC

The lipid compounds (MAG, DAG, TAG and FFA) were quantified by normal-phase high performance liquid chromatography (NP-HPLC). The chromatographic apparatus consisted of a HPLC system (Agilent Technologies 1200 Series Model, Santa Clara CA, United States) formed by a quaternary pump and an auto-injector. The chromatographic separation of the compounds was carried out at room temperature with a Lichrospher Diol column (5 mm, 4 mm×250 mm) and detection was performed in an evaporative light scattering detector (ELSD) at 35 ºC and 0.35 MPa. Gradient elution was achieved by mobile phases A (isooctane) and B (methyl *tert*-butyl ether:acetic acid = 99.9:0.1, v/v). The course of the gradient was as follows: first, solvent A was flowing for 1 min, after that, solvent B was added in three steps, up to 10% in 10 min, to 44% in 22 min and to 100% in 30 min. Subsequently, solvent B was decreasing down to 0% in 17 min to come back to the beginning conditions. Finally, the stationary phase was rinsed with solvent A for 2 min. Injection volumes of 10 µL and the elution flow-rate of 1 mL/min were used in all experiments. Individual compounds were identified and quantified using a calibration curve of the corresponding standard compound of DAG (mixture of dipalmitin and diolein), MAG (mixture of monopalmitin, 1-monoolein, 2-monoolein and monodocosahexaenoin) and FFA (oleic and palmitic acids) in *tert*-pentanol. In case of TAG, the calibration curve used was made with the refined sardine oil (TAG ≥99%) because the response factor of the oil as a complex mixture of TAG was very different than the pure standards of TAG. The fractions detected by NP-HPLC under optimal reaction conditions were collected according to their retention times. Separations were repeated at least six times in order to obtain enough sample to evaluate the FA profile of each compound (MAG, DAG and TAG).

2.4 Scaling up lipase-catalyzed glycerolysis

Eight hundred grams of reaction mixture with a reactant mole ratio 3:1 (glycerol:oil), with a TP:substrates weight ratio of 1.5:1 and 5 wt% of Lipozyme 435 were placed in a 1 L jacketed batch reactor and heated to 50 °C under 200 rpm stirring. The glycerolysis reaction took place for 2 h. After that, the enzyme particles were removed by double filtration. The first one was through the filter sited at the bottom of the reactor and the second filtration was under vacuum. The whole procedure was carried out four times to produce enough reaction mixture to perform the SPD experiments. Before SPD, the solvent was evaporated under vacuum using a rotary evaporator (Vacuubrand PC 3001 VARIOpro) at 55 °C. It could be reused by using molecular sieves to eliminate the water content.

2.5 Purification of MAG by SPD

The enzymatic glycerolysis reaction products were separated using SPD (KDL 5, UIC GmbH, Germany) at Aarhus University. To obtain a highly pure MAG fraction, two-step distillations were performed varying the evaporation temperature from 110 °C to 195 °C. The first step was optimized at 110 °C to remove glycerol and most of FFA. The residue obtained in the first distillation at 110 °C was the feeding material in the second distillation. The evaporator temperatures were varied in the second distillation from 135 to 195 °C to distillate the MAG fraction with the minimum of DAG possible. The other operation variables were fixed as follows: 375 rpm as rotation speed, 1 mL/min as feed rate, 10^{-3} mbar of vacuum, 45 °C as temperature of feed reservoir, and 50 °C as condenser temperature.

Moreover, once MAG was purified, a third distillation was carried out to distillate MC-SFA (medium chain-saturated fatty acid) MAG, leaving a concentrate fraction of LC-PUFA MAG in the residue.

The lipid profiles of the residue and distillate fractions were analyzed by thin layer chromatography coupled with a flame ionization detector (TLC-FID). The fractions from the third distillation and some fractions from the second one were also analyzed by GC-FID to determine their FA composition.

2.6 Acylglycerol composition analysis by TLC-FID

Samples from residue and distillate fractions obtained from SPD were analyzed by TLC-FID (Iatroscan MK-6 s, Bechenheim, Germany) at Aarhus University to quantify the reaction compounds (TAG, DAG, MAG, FFA and glycerol). The samples were diluted to 20 mg/mL in chloroform/methanol (3:1, v/v) and 1 µL was spotted onto silica-coated quartz rods. Samples were developed with the developing system of hexane/diethyl ether/acetic acid (40:30:1, v/v/v). The rods were dried for 5 min at 120 °C prior to analysis. The area percentages of lipid classes were used for the calculation of product yields.

2.7 Fatty acid composition by GC-FID

The samples were transferred to screw-capped tubes to carry out the conversion to methyl esters by the AOAC method ("Official Methods and Recommended Practices of the American Oil Chemists' Society, Determination of FAMES.," 1995) and subsequent analysis by gas chromatography (GC) with an Agilent gas chromatograph (6890N Network GC System, Santa Clara CA, United States) equipped with a flame ionization detector (FID). A fused silica capillary column (OmegawaxTM-320, 30 m×0.32 mm i.d.) was used. The separation was carried out with helium (1.8 mL/min) as carrier gas. The column temperature was programmed starting at a constant value of 180 ºC during 20 min, heated to 200 ºC at 1 ºC/min, held at 200 ºC during 1 min, heated again to 220 ºC at 5 ºC/min and finally held at 220 ºC for 20 min. A split injector (50:1) at 250 ºC was used. The FID was also heated at 250 ºC. The injection volume was 1 µL. Most

of the FAMEs were identified by comparison of their retention times with those of chromatographic standards (Sigma Chemical Co.).The method and the calibration procedure was developed previously and the compounds were quantified related to the area of an internal standard (methyl tricosanoate) as indicated by the AOAC method ("Official Methods and Recommended Practices of the American Oil Chemists' Society, Determination of FAMES.," 1995).

3. Results and discussion

3.1. Effect of substrate molar ratio and enzyme concentration in the production of MAG

To determine an optimum substrate mole ratio, the effect of glycerol:oil mole ratio on the reaction rate was studied at three levels (1:1, 3:1 and 5:1) at 50 ºC with 10 wt% of lipase. The results are shown in Fig. 1A. The substrate mole ratio can influence the reaction in different ways. An increase in glycerol amount will increase the theoretical equilibrium conversion, and shift the equilibrium towards MAG production. On the other hand, an excess of glycerol will affect the polarity as well as the stability of the system (Yang, Rebsdorf, Engelrud, & Xu, 2005). The MAG yield at a mole ratio of 1:1 was around 60%, whereas values up to 90% were reached at a mole ratio of 5:1. There was little difference in MAG yield between the 3:1 and 5:1 glycerol:oil mole ratios, reaching in both cases around 90 wt% of MAG yield. Based on these results, a glycerol:oil molar ratio of 3:1 was selected as optimum in the present study. This result agrees with previous studies on glycerolysis of different types of oils. Zhu et al. (Zhu, Li, Wang, Yang, & Ma, 2011) chose a substrate molar ratio of 3:1 using high oleic sunflower oil in a mixture of TP and TB medium. Zhong et al. (Zhong, Li, Xu, Cheong, Li, Hu, et al., 2009) selected 3.5:1 glycerol:soybean oil molar ratio in their study on glycerolysis reaction in a mixture of TB/isopropanol (80:20). Pawongrat et al.

(Pawongrat, Xu, & H-Kittikun, 2007) used methyl *tert*-butyl ether as solvent and showed that the optimum mole ratio of glycerol to tuna oil for MAG production was 3:1.

In the enzyme load study, glycerolysis of sardine oil was carried out using Lipozyme® 435 at a load of 5 and 10 wt% based on reactants weight, with a glycerol:oil mole ratio of 3:1. The results are shown in Fig. 1B. As expected, the MAG yield increased with increasing lipase concentration, which may be due to higher number of active sites. MAG production was around 90% and 80% (expressed free of glycerol and TP) at 10 and 5% of lipase loading, respectively. When using 10% lipase, around 80% of MAG was reached in less than 30 minutes. To achieve the same yield of MAG with 5% lipase, 4 hours was needed. The best enzyme load should be evaluated from an economical point of view, evaluating the effect of longer reaction time and higher enzyme loading. In this work, 5% Lipozyme® 435 and 2 hours of reaction was chosen to scale up the process with 67% of MAG.

3.2. Scale-up reaction under optimal conditions for MAG production

According to previous results (section 3.1), a high yield of MAG (67 wt%) can be reached in 2 hours of reaction time, with a reactants molar ratio 3:1 (glycerol:sardine oil) and a lipase load of 5% based on reactants weight. Large-scale reaction was performed at these conditions in a 1 L batch reactor at 50 °C under 200 rpm stirring. The final MAG content obtained by glycerolysis of 1 g and 800 g of reaction mixture were of the same order (67 and 70 wt%, respectively), hence, scaling up did not affect the yield of the enzymatic process. The slight increase in MAG yield at large scale could be due to the use of mechanic propeller agitation instead of orbital agitation in vials. The lipase and solvent were removed by filtration and evaporation, respectively, before SPD. The lipase and recovered TP could be reused for further reactions.

The FA composition of acylglycerols produced at optimal conditions can be seen in Table 1. These results revealed that the TAG fraction contained more palmitic, stearic and oleic acids, compared to the DAG and MAG fractions. On the other hand, EPA and DHA were higher in the DAG and MAG fractions. The produced MAG had a similar FA profile to the starting sardine oil, and the sum of EPA, DPA, and DHA was almost 27% as in the sardine oil.

3.3. Purification of MAG by SPD

The products from glycerolysis consist mainly of MAG and DAG, some glycerol, and a small amount of FFA and TAG. SPD was performed to obtain a highly pure MAG fraction after the glycerolysis reaction. The order of removal in the distillate stream would follow the same order as vapor pressure of different acylglycerols: Glycerol < FFA < MAG < DAG < TAG (Yeoh, Phuah, Tang, Siew, Abdullah, & Choong, 2014). However, the chain length and the number of double bonds in the fatty acid also influence the distillation order. The rate of fatty acids evaporation follows the order of 14:0 > 16:0 > 18:1 > EPA > DHA (Liang & Hwang, 2000; Rossi, Grosso, Pramparo, & Nepote, 2012). Due to the variability in the FA profile of fish oils, it is expected that different acylglycerols could be distilled at the same evaporator temperature; hence, MAG with the highest boiling points could be distillated together with DAG with the lowest boiling points.

Many parameters can influence the separation performance in SPD. Previous studies about SPD show that the operating parameters having greater effect on the separation efficiency are evaporator temperature, feed flow rate, and vacuum pressure (P. B. L. Fregolente, Pinto, Wolf-Maciel, & Filho, 2010; Yeoh, Phuah, Tang, Siew, Abdullah, & Choong, 2014). The feed temperature, the condenser temperature, and rotational speed have little influence on the separation (Zhang, Liu, & Liu, 2013; Zhu, Li, Wang, Yang,

& Ma, 2011), and in this work, these parameters were fixed at 45 °C, 50 °C and 375 rpm, respectively. For practical operation, the lowest vacuum should be used to maximize the separation efficiency (X. Xu, Jacobsen, Nielsen, Heinrich, & Zhou, 2002). In this case, the vacuum system included a diffusion pump and a rotary vane pump, achieving 10^{-3} mbar.

Feed flow rate is an important operating variable in SPD. High feed flow rate reduces the operating time but results in decreased separation and lower acylglycerol purity; while low flow rate fosters efficient separation, yet increases the processing time (Yeoh, Phuah, Tang, Siew, Abdullah, & Choong, 2014). In several studies, the feeding rate was around 1.5 mL/min for a UIC KDL1 system (Zha, Chen, Wang, Wang, Chen, & Zheng, 2014; Zhang, Liu, & Liu, 2013) and for MD-S80 distiller (Zhu, Li, Wang, Yang, & Ma, 2011). In this work, the feed flow rate for a UIC KD5 system was set to 1 mL/min.

Some studies showed that the evaporator temperature is the key factor in the separation of the compounds (L. V. Fregolente, Fregolente, Chicuta, Batistella, Maciel Filho, & Wolf-Maciel, 2007; Zha, Chen, Wang, Wang, Chen, & Zheng, 2014). In this work, a two-step distillation was performed to study the effect of T_E in the separation of acylglycerols. The first step was done at 110 °C to remove the glycerol and the FFA. In the second distillation, the temperatures were varied in the range from 135 °C to 195 °C.

When feeding the glycerolysis product mixture to the SPD, it was divided into two streams. Part of the feed (F) was collected in the distillate stream (D) while the rest remained in the residue stream (R). The yield and the recovery are important indexes to evaluate the performance of SPD process. The yield of R or D is calculated by the following equation (Eq. 1):

$$\text{Yield}_{\text{R/D}}(\%) = \frac{M_{R/D}}{M_{Feed}} \times 100 \quad [1]$$

where M is the amount in grams of R or D collected. The recovery of a certain component was determined by using Eq. (2):

$$\text{Recovery of a substance, i (\%)} = \frac{M_{R/D} \times C_{i,R/D}}{M_{Feed} \times C_{i,Feed}} \times 100 \quad [2]$$

where C is the concentration as area % measured by TLC-FID of each compound in the residue or distillate collected, and "i" represents TAG, DAG, MAG, FFA or glycerol. The acylglycerol compositions, yield, recovery and loss of the compounds in the D and R streams for different temperatures are shown in Table 2. The sum of R and D recoveries were sometimes above or below 100%, due to weight losses, which are expected in the process (Kahveci & Xu, 2011), and analytical errors.

As shown in Table 2, the sample initially fed to SPD at the beginning contains 10.4% TAG, 17.4% DAG, 64.6% MAG, 0.6% FFA, and 7.0% glycerol. As a general trend, higher yields of distillate were obtained at higher T_E. Increasing T_E leads to higher heat transfer rates, and therefore, more molecules could be evaporated (P. B. L. Fregolente, Pinto, Wolf-Maciel, & Filho, 2010). As the results shown in Table 2, the first step of SPD is a pre-distillation at 110 °C in order to remove the glycerol and part of FFA (D1 in Fig. 3). The glycerol was completely distillated at this temperature. However, the small vapor pressure difference between some FFA and some MAG make a complete separation of both components difficult. Therefore, a complete removal of FFAs was not possible if MAG would be not distillated at all. A T_E of 135 °C would be necessary to remove all FFAs, as it can be seen in Table 2, no FFA were detected in the residue stream under this temperature. This fact coincides with the study of Zhu et al (Zhu, Li, Wang, Yang, & Ma, 2011), where they concluded that most of the glycerol and FFA could be separated from their glycerolysis reaction mixture when the evaporation temperature was set over 140 °C, under vacuum of 1-5 Pa (operation pressure higher than in this work), a rotational speed of 200 rpm, and feed flow rate of 1.5 mL/min.

However, at this temperature the loss of MAG in the distillate is important. In this work, the recovery of FFA in the distillate stream at 110 °C reached values up to 63.5%. At this T_E, nearly all TAG and DAG were collected in the residue stream, while 8% of the MAG fraction was lost in the distillate stream. On the other hand, a good MAG recovery in the residue is obtained, almost 90%.

The variation in the lipid profile of D and R streams are represented as function of evaporation temperatures in Fig. 2A. It can be observed that TAG and DAG start to evaporate at 155 °C, so at this temperature, the purity of MAG in the distillate decreased. However, TAG in D fraction was lower than 5% for all temperatures; while DAG, being more volatile than TAG, steadily increases from 155 °C, and consequently a maximum in DAG profile with temperature in the R stream is observed. Fig. 2B shows the relation between MAG recovery in the distillate and MAG purity at different T_E. It can be seen that the MAG recovery increased until 155 °C, reaching more than 93%. Up from there, it kept constant because MAG distillation was complete. On the other hand, the MAG purity starts to decrease at T_E over 155 °C since mainly DAG starts to be distillated. At this temperature, the composition of the D stream was 91.0% MAG, 5.7% DAG, and 2.5% TAG, while just 5% MAG was lost in the R stream. At 165 °C, the MAG loss in the R stream was only 1.5%, but the recovery of DAG in the distillate was more than 20% decreasing MAG concentration in the D stream. With these results, it can be concluded that the optimal T_E to distillate MAG fraction is 155 °C. At this T_E, it was possible to concentrate MAG to 91% from a mixture that initially contained 63% of MAG.

Several authors demonstrated that SPD is useful to concentrate n-3 PUFA in EE form or even as FFA (Breivik, Haraldsson, & Kristinsson, 1997; Kahveci & Xu, 2011; Liang & Hwang, 2000; Zhang, Liu, & Liu, 2013). In this work, GC analyses were performed to

identify the FA profile of the residue and distillate fractions obtained in the second distillation at 135, 145 and 155 °C. No significant differences were observed in the FA profile of the residue fractions at these temperatures. The FA profile was found to be in the range of 28-30% myristic and palmitic acids as MC-SFA, and 24-28% of the sum of EPA, DPA and DHA as LC-PUFA. However, despite the similar purity of MAG found in the distillate fractions (around 90% of MAG in the three cases, as shows Fig.2B), there was a clear difference in their FA profiles, as can be seen in Fig. 4A. The distillate obtained at 135°C contained almost 40% of MC-SFA (C14:0 and C16:0), while the 155°C distillate contained only 23% of these fatty acids and more than 31% of LC-PUFA. It means that low T_E is more suitable to obtain a distillate rich in MC-SFA. On the other hand, the content of n-3 LC-PUFA (EPA, DPA and DHA) increased in the distillate at high $T_{E.}$ According to these results, a third distillation was carried out to remove MAG with MC-SFA in the D stream, while concentrating MAG with LC-PUFA in the R fraction, using fraction with more than 90% MAG as feeding material (D2 in Fig. 3).. The distillation was run at 125 °C as $T_{E.}$ As shown in Fig. 4a, the distillation step resulted in almost 50% of MC-SFA and only around 10% of n-3 LC-PUFA in D fraction. Accordingly, the most of the EPA, DPA and DHA were remained in the R fraction after distillation at 125 °C. A complete FA profile of R and D fractions collected in the third distillation is represented in Fig. 4b. The main differences between the FA compositions of the two streams were found in C14:0, EPA and DHA. In this way, almost all C14:0 was concentrated in the D fraction and on the contrary, EPA and DHA were found predominantly in the R fraction. In the case of the series of C18 FA with double bonds, as well as C18:0, no important differences between the streams were observed, because their concentration in the starting sardine oil was low. In summary, these results demonstrated that the third distillation process at 125 °C contributed to the

recovery of n-3 LC-PUFA in the residue. However, the level of concentration is highly affected by initial content of these FA (Kahveci & Xu, 2011). Further experiments with high initial content of n-3 LC-PUFA in the substrate should be done to achieve higher yield and purity of MAG with n-3 LC-PUFA.

4. Conclusion

This work reported the optimization of an SPD process for separating a mixture of acylglycerols, free fatty acids, and glycerol, obtained from an enzymatic glycerolysis of sardine oil, which produced high concentration of MAG (67%). The products (TAG, DAG, MAG, and FFA) contain n-3 PUFAs, and SPD is a suitable process to preserve their stability. A two-step distillation was applied to remove firstly at 110°C as T_E the glycerol and part of FFA (63.5%), and then a second distillation at 155°C produced a distillate with a MAG purity of 91% and 5.7% of DAG and with more than 31% of n-3 PUFA, which might be the emulsifier for some specific uses. This work further demonstrated that a third distillation process at 125 °C could contribute to the concentration of omega-3 LC-PUFA as MAG form in the residue. However, high initial content of n-3 LC-PUFA in the substrate is required to achieve higher yield and purity of MAG with n-3 LC-PUFA.

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Table 1. Fatty acid composition of sardine oil and at the sn-2[a] position of TAG (% mol) (Solaesa, Bucio, Sanz, Beltrán, & Rebolleda, 2014).

Fatty acid	Sardine oil		Reaction compounds[b]		
	TAG	*sn*-2[a]	TAG	DAG	MAG
14:0	12.4 ± 0.4	41.8 ± 0.1	12 ± 0.3	12.5 ± 0.4	12.4 ± 0.3
16:0	22.8 ± 0.2	41.9 ± 0.2	29.4 ± 0.7	23.2 ± 0.8	23.0 ± 0.6
16:1 n-7	12.5 ± 0.1	38.1 ± 0.1	11.9 ± 0.3	11.4 ± 0.4	13.1 ± 0.3
18:0	3.6 ± 0.1	6.7 ± 0.1	5.2 ± 0.4	3.2 ± 0.2	2.9 ± 0.3
18:1 n-9	9.8 ± 0.1	16.6 ± 0.1	12.3 ± 0.2	8.3 ± 0.5	10.7 ± 0.4
18:1 n-7	3.7 ± 0.1	10.1 ± 0.1	4.6 ± 0.2	2.8 ± 0.2	4.0 ± 0.2
18:2 n-6	2.5 ± 0.1	32.7 ± 0.1	2.1 ± 0.1	2.6 ± 0.1	2.1 ± 0.1
18:3 n-3	1.0 ± 0.1	29.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
18:4 n-3	3.3 ± 0.1	26.4 ± 0.1	2.0 ± 0.1	3.2 ± 0.2	2.9 ± 0.1
20:3 n-3	1.3 ± 0.3	19.6 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	1.2 ± 0.1
20:5 n-3	18.3 ± 0.3	12.1 ± 0.1	11.9 ± 0.6	19.4 ± 0.5	18.7 ± 0.6
22:5 n-3	1.8 ± 0.1	76.4 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	1.7 ± 0.1
22:6 n-3	7.0 ± 0.2	82.8 ± 0.1	5.5 ± 0.4	9.4 ± 0.6	6.4 ± 0.3

(a) % *sn*-2 = [mol % *sn*-2 fatty acid / (mol % fatty acid in TAG·3)] · 100
(b) Obtained at optimum conditions

Table 2. Acylglycerol compositions, yield, and recovery of all components obtained in two steps distillation at different evaporation temperatures.

Evaporator temperature (°C)	Streams	Yield (%)	TAG		DAG		MAG		FFA		Glycerol	
			Area %	Recovery %	Area %	Recovery %	Area %	Recovery %	Area %	Recovery %	Area %	Recovery %
1st distillation	Feed	-	10.4 ± 0.1		17.4 ± 0.3		64.6 ± 0.5		0.63 ± 0.08		7.0 ± 0.8	
110	Residue	85.4	11.9 ± 0.5	97 ± 5	20.0 ± 0.3	98.4 ± 3.2	67.8 ± 1.6	89.6 ± 2.8	0.33 ± 0.02	44.7 ± 8.4	-	-
	Distillate	14.6	4.5 ± 0.3	6.4 ± 0.5	7.0 ± 0.5	5.9 ± 0.5	36 ± 1	8.1 ± 0.3	2.74 ± 0.03	63.5 ± 8.8	49.8 ± 0.5	104 ± 13
2nd distillation	Feed	-	10.6 ± 0.5		25.6 ± 0.9		63.3 ± 0.7		0.52 ± 0.04			
135	Residue	76.5	16.3 ± 0.7	118 ± 11	32.9 ± 0.8	98.2 ± 5.8	50.9 ± 0.8	61.5 ± 1.5	-	-		
	Distillate	23.5	2.1 ± 0.2	4.6 ± 0.7	2.1 ± 0.3	1.9 ± 0.3	93.3 ± 0.7	34.6 ± 0.5	2.50 ± 0.04	114 ± 11		
140	Residue	65.0	18.8 ± 0.6	116 ± 9	45.6 ± 0.4	116 ± 5	35.6 ± 1.3	36.5 ± 1.6	-	-		
	Distillate	35.0	2.0 ± 0.2	7 ± 1	3.4 ± 0.2	4.6 ± 0.4	93.2 ± 0.9	51.5 ± 0.9	1.51 ± 0.02	102.6 ± 9.3		
145	Residue	51.2	23.5 ± 0.3	114 ± 7	56.1 ± 0.8	112 ± 6	20.4 ± 0.8	16.7 ± 0.8	-			
	Distillate	48.8	2.3 ± 0.1	10 ± 1	4.2 ± 0.4	8 ± 1	92.5 ± 0.4	71.3 ± 0.9	1.06 ± 0.05	100.4 ± 12.5		
155	Residue	34.9	30.1 ± 0.4	99 ± 6	60.8 ± 1.1	82.9 ± 4.4	9.1 ± 0.6	5 ± 0.4	-	-		
	Distillate	65.1	2.5 ± 0.2	15 ± 2	5.7 ± 0.4	14.5 ± 1.5	91 ± 1	93.6 ± 1.8	0.82 ± 0.04	106.8 ± 13.4		
165	Residue	29.6	34.3 ± 0.2	95.8 ± 5.1	62.5 ± 0.7	72.3 ± 3.4	3.2 ± 0.7	1.5 ± 0.3	-	-		
	Distillate	70.4	3.0 ± 0.6	19.9 ± 4.9	8.3 ± 0.7	22.8 ± 2.7	87.9 ± 0.6	97.7 ± 1.4	0.78 ± 0.04	109.8 ± 14.1		
175	Residue	22.2	38.3 ± 0.8	80.3 ± 5.5	60.4 ± 1.2	52.4 ± 2.9	1.3 ± 0.3	0.5 ± 0.1	-	-		
	Distillate	77.8	4.1 ± 0.5	30.1 ± 5.1	14.2 ± 0.6	43.1 ± 3.3	81 ± 1	99.5 ± 2.0	0.71 ± 0.02	110.5 ± 11.6		
185	Residue	18.3	44.3 ± 0.3	76.3 ± 4.1	55.3 ± 0.8	39 ± 2	0.4 ± 0.2	0.1 ± 0.1	-	-		
	Distillate	81.7	4.4± 0.5	33.9 ± 5.5	17.3 ± 0.3	55.2 ± 2.9	77.7 ± 1.4	100.3 ± 2.6	0.62 ± 0.03	101.4 ± 12.7		
195	Residue	14.9	50.4 ± 0.6	70.9 ± 4.2	49.2 ± 1.3	28.7 ± 1.8	0.4 ± 0.1	0.1 ± 0.1	-	-		
	Distillate	85.1	4.8 ± 0.4	39 ± 5	21.5 ± 0.6	71.5 ± 4.5	73.1 ± 0.2	98 ± 1	0.54 ± 0.04	91.9 ± 13.9		

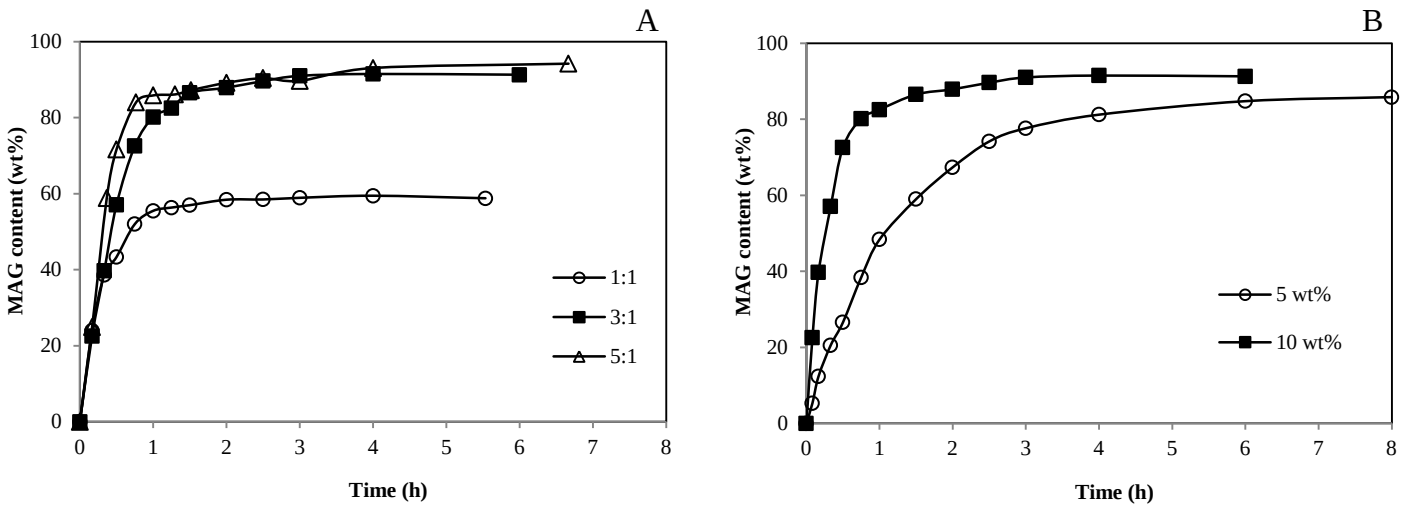

Fig. 1. Effect of kinetic parameters in MAG production by glycerolysis of sardine oil. (A) Effect of glycerol to oil molar ratio (1:1, 3:1, 5:1); reaction conditions: 10 wt% of Lipozyme 435, 50 °C and 1.5:1 (weight ratio. tert-pentanol:substrates). (B) Effect of lipase dosage (5 and 10 wt%, based on total substrates); reaction conditions: 3:1 glycerol to sardine oil molar ratio, 50 °C and 1.5:1 (weight ratio, tert-pentanol:substrates).

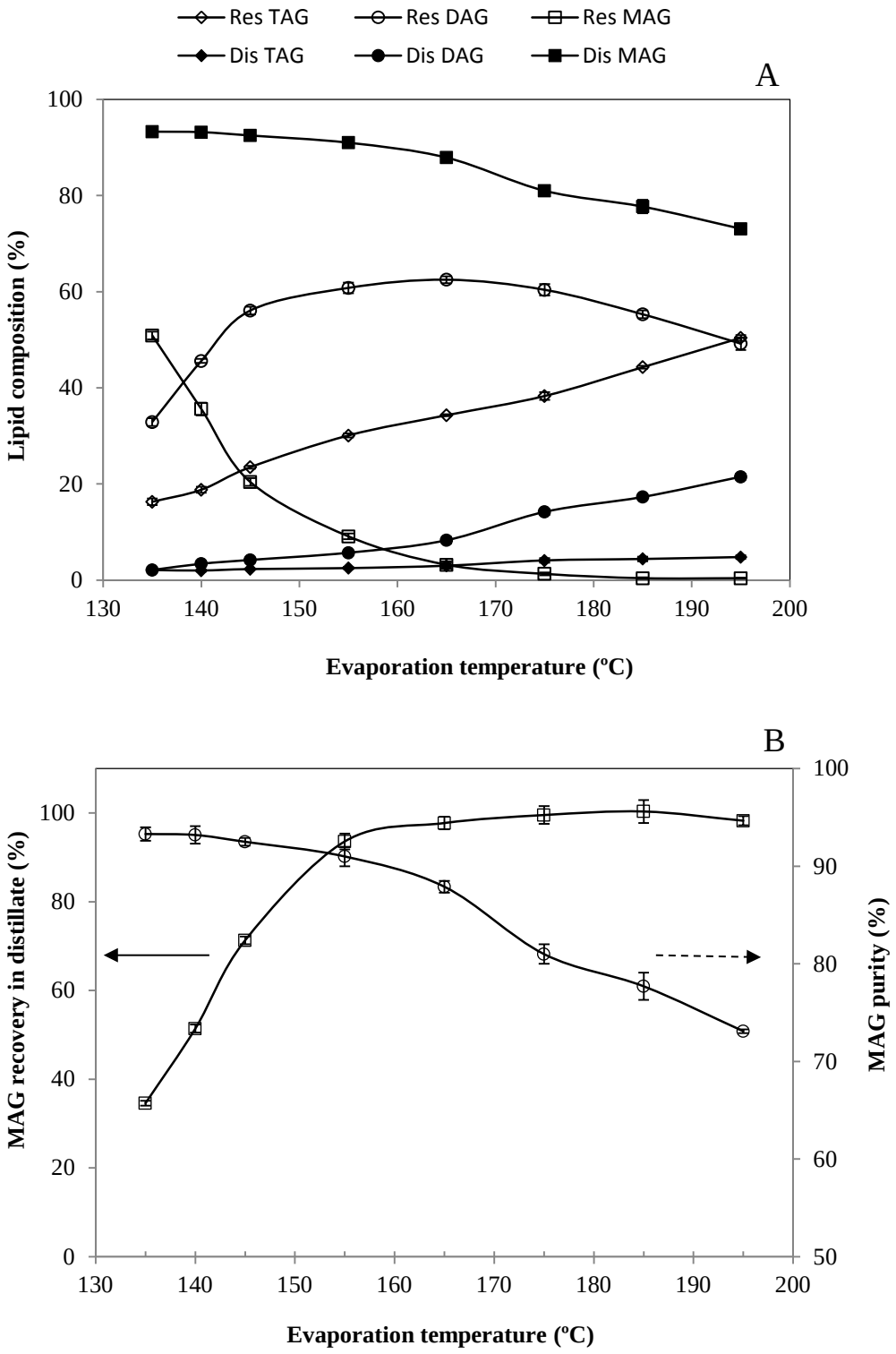

Fig. 2. (A) Acylglycerols composition in residue and distillate fractions at different evaporation temperatures. (B) MAG recovery and purity in distillate at different evaporation temperatures.

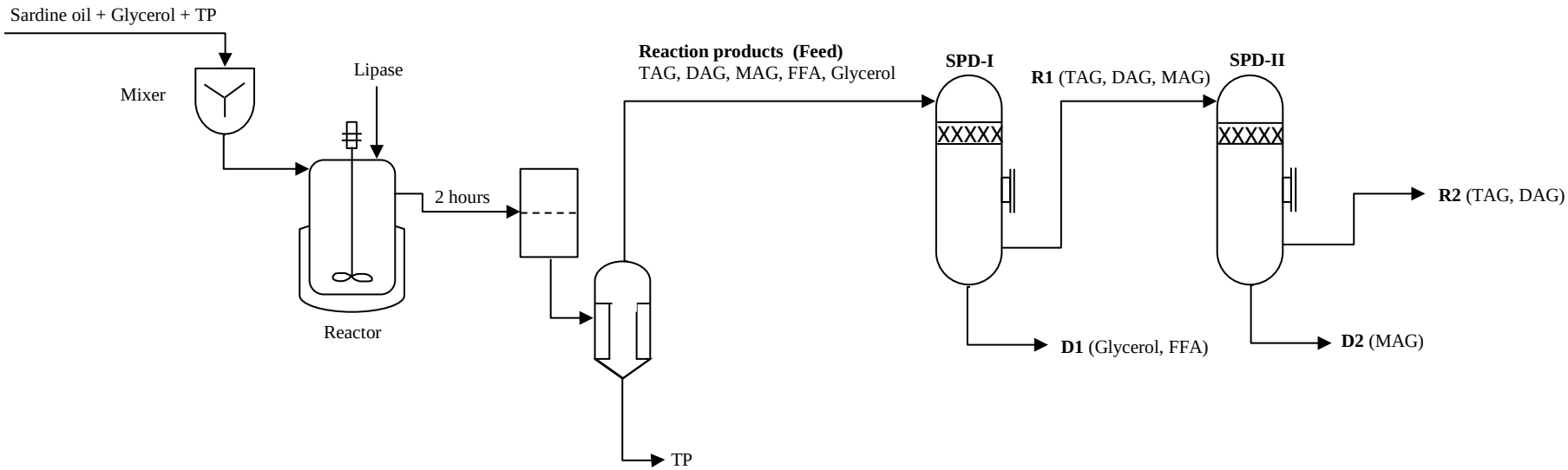

Fig. 3. Global scheme of the process to produce concentrated MAG fraction by glycerolysis reaction and double step distillation at selected evaporation temperatures with the main components in each stream.

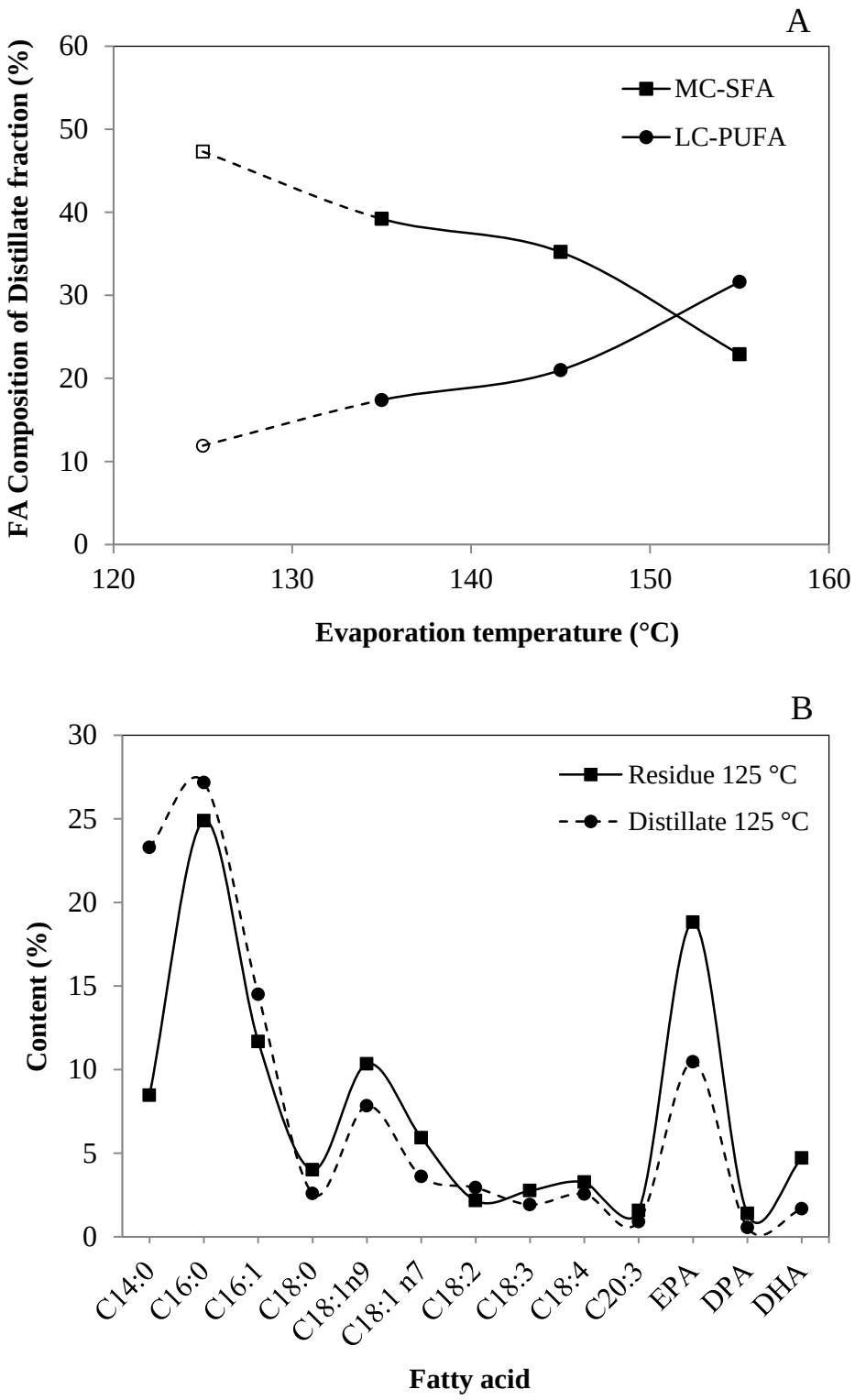

Fig. 4. (A) Fatty acid composition of distillate fractions from the second distillation at 135, 145 and 155 ºC (black symbols) and with MAG fraction as the feed material at 125 ºC (white symbols), MC-SFA included C14:0 and C16:0, while LC-PUFA included EPA, DPA and DHA. (B) Comparison of the fatty acid profiles of the residue and distillate fractions at 125 ºC.