There is an increasing interest in the production of monoglycerides (MAG) and diglycerides (DAG) rich in omega-3, such as DHA (C22:6n-3) and EPA (C20:5n-3). MAG offer several health benefits over the most commercialized ethyl esters. These partial acylglycerols can be produced by enzymatic glycerolysis reaction, often carried out in organic solvents. This work reports a lipase-catalyzed glycerolysis of sardine oil in supercritical carbon dioxide (SC-CO₂) as solvent medium. The use of SC-CO₂ offers some unique advantages. The lack of solvent residues and an atmosphere free of oxygen can help to keep the stability of the omega-3, which are very prone to oxidation. For this purpose both reactants, sardine oil and glycerol, were emulsified to improve their contact since they are completely immiscible. This microemulsion was placed in the reactor with a commercial immobilized lipase from Candida antarctica (Lipozyme 435) as biocatalyst. The experiments were conducted in a batch mode keeping constant the enzyme concentration at 5 wt% (by weight of substrates) and agitation of 800 rpm. The pressure and temperature were varied in the range 12.5 – 25.0 MPa and 40 – 80°C, respectively. The oxidative status of the final reaction products, as peroxide and anisidine values, was evaluated. MAG yields and the oxidation stability of the reaction products were compared with those obtained in solvent-free system and in tert-pentanol media. Results showed that lipase-catalyzed glycerolysis in SC-CO₂ might be a potential route to conventional methods, as high contents of MAG and DAG (65-70%), were achieved at mild temperature and pressure conditions (50°C and 150 bar) with glycerol to oil molar ratio of 3:1 and showing considerably less oxidation that without SC-CO₂.

INTRODUCTION

Fish oil is rich in omega-3 (n-3) polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid. The importance of omega-3 PUFAs in human nutrition and disease prevention was scientifically recognized some decades ago because they are involved in many important biological processes in the human body. Among the different types of lipid derivatives containing PUFAs concentrates, MAG and DAG have good bioavailability. In addition, MAG or its mixtures with DAG account for 75% of worldwide emulsifier production. The well-known drawbacks of the conventional chemical glycerolysis technique (energy intensive, low yields (30–40%), oxidized products) have prompted a growing interest in the development of alternative processes for the production of MAG and DAG rich in n-3 PUFAs. Enzyme-catalyzed reaction is an attractive alternative since the reaction can be carried out under mild conditions. Therefore, production of MAG rich in n-3 PUFAs from enzymatic glycerolysis has been shown to offer industrial potential as ingredients or compounds with improved functionality or a healthier nutritional profile. To overcome the problem of the immiscibility of glycerol and oil, different approaches have been used in the literature to improve the contact between the reactants and hence reduce mass
transfer limitation. Lipase-catalyzed glycerolysis has been carried out in different reaction media such as organic solvents, compressed fluids, and ionic liquids, in order to improve the mass transfer. Recently, the uses of different surfactants to increase the interfacial area, and ultrasound irradiation have been also proposed to reduce mass transfer limitation [1]. Among the latest, enzymatic concentration of n-3 PUFAs in supercritical fluids (SCFs) rises as an alternative for the prevention of oxidation during processing of fish oil. This technology is based on the use of lipases which are able to catalyze different reactions at supercritical conditions. Moreover, incorporating a SCF in the reaction media prevents oxidation due to displacement of oxygen and provides many other advantages over conventional organic solvents, such as higher diffusivity, lower viscosity and, thus, higher mass transfer rates. In addition, the solvating power of SCFs is strongly density-dependent and it can be easily controlled by variation of temperature and pressure. Supercritical carbon dioxide (SC-CO₂) is probably the most used SFC due to its additional benefits (non-toxic, non-flammable, readily available at high purities and low costs, and relatively mild critical conditions) that are appealing when choosing environmental replacement for organic solvents. Besides, SC-CO₂ can be easily separated from the reaction products by simple depressurization. Some previous studies of enzymatic reactions of different lipid sources in SC-CO₂ have been reported in the literature. However, in case of enzymatic glycerolysis, other compressed fluids as propane, n-butane, and acetone, have been used. Most of these studies employed the non-specific lipase Novozym 435 from Candida antarctica catalyst. Furthermore it can be found in literature some studies of glycerolysis of soybean oil in SC-CO₂ but without enzymatic catalyst using high temperatures [2]. 

In this work the kinetics of a lipase-catalyzed glycerolysis of sardine oil in SC-CO₂ have been studied. The formation of a microemulsion of the reactants as reverse micelles (glycerol-in-oil) helps to improve mass transfer rates. Microemulsions exhibit relatively ordered structure, are characterized by definite diameter, and provide an enormous interfacial area. It has been shown that lipases demonstrate high interfacial activity in micelle systems. Different operating pressures (12.5, 15, 20 and 25 MPa) and temperatures (40, 50, 65 and 80°C) have been studied to evaluate the effect on the kinetics. The experiments were conducted in a batch mode keeping constant the enzyme concentration at 5 wt% (by weight of substrates) and agitation of 800 rpm. Finally the oxidative status in the final reaction products was evaluated through the peroxide and anisidine values. MAG yields and the oxidation stability of the reaction products were compared with those obtained in solvent-free system and in tert-pentanol media.

**MATERIALS AND METHODS**

**Materials**

Refined sardine oil was kindly provided by Industrias Afines S.L. (Spain) with 18.3% of EPA and 7% of DHA and a water content of 0.2%. Glycerol was purchased from Sigma Aldrich with a purity of ≥ 99.5% and a water content of 0.18%. The food grade lipase Lipozyme® 435 from Candida antarctica (immobilized on a macroporous hydrophobic acrylic resin) was donated by Novozymes A/S (Bagsvaerd, Denmark). Carbon dioxide (99.9%) was supplied by Air Liquide S.A. (Spain). All other chemicals used in different analyses were of analytical or HPLC grade.

**Microemulsions preparation**
To prepare the microemulsion of the substrates as reverse micelles, the appropriate amount of glycerol was added drop by drop to the suitable amount of oil while are completely mixed at high speed. High speed blender (Micra D9 equipped with a DS-5/K-1 rotor–stator) at different speed from 16000 to 35000 rpm was used by pulses during 3 minutes. The characterization of the emulsions was performed 10 min after emulsification to avoid any creaming or coalescence effect. Droplet size distribution, mean droplet diameter and polydispersity index (PDI) of samples were measured by dynamic light scattering (DLS), using a Zetasizer Nano ZS apparatus (Malvern Instruments Ltd., UK) to evaluate the best conditions to produce a stable emulsion with smaller droplet size.

**Glycerolysis of sardine oil in SC-CO₂**

The glycerolysis reaction has been performed in a high pressure batch stirred tank reactor (HP-BSTR) made of stainless steel (SS-316) and having an internal volume of 100 mL. The reactor was then closed, connected to the pressure circuit and placed in a thermostatic water bath at the desired operating temperature. Subsequently, SC-CO₂ was fed into the reactor by means of a high pressure pump (ISCO 260 D) up to the desired pressure, which was maintained by a digital pressure controller. Once the established conditions have been reached, magnetic stirring (800 rpm) was connected and the reaction was initiated. Operating temperature and pressure has been varied in the range between 40-80ºC and 12.5-25 MPa. Samples were taken periodically during 8 h through a siphoned capillary equipped with a microfilter made of sintered steel, which prevented the withdrawal of the enzyme from the reaction mixture and, thus, stopped the reaction. Samples were collected in glass screw-top vials immersed in a cold trap and stored at -18ºC prior to analysis.

**Analysis of the reaction products**

The neutral lipid profile (TAG, DAG, MAG and FFA) was analysed and quantified by a normal phase high performance liquid chromatography (NP-HPLC). The method and calibration procedure were previously reported [3]. The regioisomers of DAG and MAG could not be distinguished by the applied analytical procedure. Therefore the total amount of MAG and DAG was shown in the study.

**Measurement of lipid oxidation**

The oxidation status can be estimated using two assays: the peroxide value (PV) and the anisidine value (AnV). Determinations of these values for the samples before and after the experiments have been performed in order to evaluate potential lipid oxidation processes during the glycerolysis reaction. Total oxidation of the oil can be estimated by the formula: 

\[ \text{TOTOX} = 2 \text{PV} + \text{AnV} \]

All determinations were performed according to standard methods [4-5].

**RESULTS**

**Emulsification process**

Some experiments to select the emulsification speed have been performed to define the rpm in which the microemulsion presents the best droplet size distribution. The time was kept
constant at 3 minutes. In a reverse micelle, the oil behaves as the continuous phase and glycerol represents the discontinuous one. In Figure 1, it can be seen that the smallest droplet diameter was obtained at 29000 rpm. Also, the polydispersity index was low at this speed, around 0.4. Although at 35000 rpm the polydispersity index is lower than at 29000, foaming was higher. Therefore a high speed blender of 29000 rpm was selected for further experiments.

Figure 1. Droplet diameter (○) and polydispersity index (△) obtained at different emulsification speeds for glycerol-in-oil micelle systems (molar ratio 3:1, glycerol:sardine oil).

Glycerolysis reaction in SC-CO₂ media with and without emulsification of the substrates is presented in Figure 2. As it can be observed, when the substrates were placed in the reactor without being emulsified mass transfer limitations lead to a poor initial contact of the substrates with the lipase and, hence low reaction rate. In case of the reverse micelle system, both hydrophilic and hydrophobic substances are available providing an enormous interfacial area, which favors lipase-catalyzed reactions. At longer reaction times, similar conversion can be reached, probably due to the MAG and DAG formation that can act as emulsifiers.

Figure 2. Lipase-catalyzed glycerolysis of sardine oil in SC-CO₂ media with (■) and without (△) substrates emulsification. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50°C, enzyme loading 5% wt. of substrates and 15.0 MPa.

Lipase-catalyzed glycerolysis of sardine oil in different systems

A comparative study of lipase-catalyzed glycerolysis reaction in three different reaction mediums: organic solvent (tert-pentanol) and emulsified substrates in a solvent free and in SC-CO₂ media is presented in Figure 3. It can be seen that highest MAG conversion was reached in TP media [3]. The homogenous system leads to higher mass transfer rates. Although very high yields of MAG have been obtained, the use of 56% of TP is an important disadvantage of this process from the environmental and economical point of view.
Figure 3. Effect of solvent media on the glycerolysis of sardine oil by Lipozyme 435. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

The oxidation status of the reaction products obtained in the experiments presented in Figure 3 has been also evaluated and compared with the initial oil. Lipid oxidation of fish oils which are rich in long chain n-3 PUFAs produce very complex mixtures of hydroperoxides, which are easy decomposed into a wide variety of secondary products and become very difficult to analyse quantitatively. Also high amount of volatile decomposition compounds are produced at extremely low levels of oxidation and these volatile compounds have not been analysed. In this work, two methods have been used, one to primary changes as the formation of hydroperoxides (peroxide value), and the second one evaluates the secondary changes that occur in oxidizing lipids, as the amount of aldehydes (anisidine value) [6]. The results are shown in Table 1. Although peroxide and anisidine values for the initial sardine oil do not exceed to the limit allowed (10 meq O₂/Kg oil by EPS) in case of PV is close to (8.8 meq O₂/Kg oil). On the other hand, AnV for the initial sardine oil (19.3) can be considered “acceptable”. Anyway the supplied refined oil was already partially oxidized (TOTOX = 37). From Table 1 it can be also observed that PV and AnV obtained in the sample from the reaction in SC-CO₂ are lower than those from the reactions performed at atmospheric pressure. The highest oxidation status was found when a conventional solvent ( tert-pentanol) was used as reaction medium.

Table 1. Oxidation status of the glycerolysis products obtained under the same conditions (3:1 as substrate molar ratio, 5% of Lipozyme 435, 50ºC) in different reaction media. In case of SC-CO₂, the reaction was carried out at 15.0 MPa. *According to EPS (European Pharmacopeia Standard).

<table>
<thead>
<tr>
<th>Sample</th>
<th>PV (meq O₂/Kg oil)</th>
<th>AnV</th>
<th>TOTOX (2PV + AV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial oil</td>
<td>8.80</td>
<td>19.30</td>
<td>36.9</td>
</tr>
<tr>
<td>Reaction Products in SC-CO₂</td>
<td>16.17</td>
<td>24.40</td>
<td>56.7</td>
</tr>
<tr>
<td>Reaction Products in Tert-pentanol</td>
<td>41.07</td>
<td>62.76</td>
<td>144.9</td>
</tr>
<tr>
<td>Reaction Products in Solvent free</td>
<td>20.24</td>
<td>55.91</td>
<td>96.4</td>
</tr>
<tr>
<td>Maximum in Legislation*</td>
<td>10</td>
<td>30</td>
<td>50</td>
</tr>
</tbody>
</table>

Lipase-catalyzed glycerolysis of sardine oil in SC-CO₂

The effect of various operating variables, such as pressure (p) and temperature (T) on the performance of the enzymatic glycerolysis of sardine oil in SC-CO₂ is presented. Besides, an evaluation of the oxidation status of the reaction products obtained at different temperatures (40-80 ºC) in SC-CO₂ media is performed. All the experiments were conducted in a batch
mode keeping constant the enzyme concentration at 5 wt% (by weight of substrates) and agitation of 800 rpm.

- **Pressure effect**

Loss *et al.* [2] have recently reviewed different applications of supercritical fluids as alternative solvent for biocatalysis processes, concluding that there seems to be no “rule of thumb” for predicting the effect of pressure on enzyme activity in SC-CO$_2$. Density-related changes in the physical parameters of SC-CO$_2$ may indirectly affect the enzyme catalytic activity, and thus the reaction performance. Figure 4 shows the pressure effect in the lipase-catalyzed glycerolysis of sardine oil in SC-CO$_2$. The results expose that pressure had no significant effect on conversion of TAG or yield of products in the pressure range from 12.5 to 25.0 MPa. In the substrates microemulsion, sardine oil behaves as the continuous phase. This means that the solubility of CO$_2$ in the oil at different pressures can modify the diffusivity in the system. According to literature, the solubility of the CO$_2$ in oil slightly increase with pressure at constant temperature, for instance at 40ºC solubility at 10 MPa is 25.8% mass and at 25 MPa is 33.7% mass [7]. Therefore an improvement in the diffusivity in the reaction system is not significant.

![Figure 4](image.png)

**Figure 4.** Effect of operating pressure (p) on the glycerolysis of sardine oil by Lipozyme 435. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

- **Temperature effect**

It has been reported that operating temperature significantly influences enzyme-catalysed reactions in SC-CO$_2$ media by affecting both the enzyme activity and stability and the physical properties of CO$_2$. Besides, the phase behaviour of the reaction system at different pressure and temperature conditions may also determine the efficiency of the reaction [8]. To assess the effect of temperature on the kinetics of the glycerolysis of sardine oil by Lipozyme 435 in SC-CO$_2$, operating temperature has been varied from 40 to 80ºC. Initial substrate molar ratio (3:1 glycerol:sardine oil), pressure (15 MPa) and enzyme loading (5% wt. of substrates) remained unchanged. Figure 5 shows the effect of the operating temperature on the TAG conversion rates and MAG production. The equilibrium conversion is essentially temperature independent. However, raising temperature from 40 to 80ºC resulted in an increase of the initial reaction rate, probably because of a higher kinetic energy of the molecules that leads to lower viscosity and higher diffusivity of the solvent and substrates. It can be also demonstrated that 8 hours was not sufficient time to achieve equilibrium
concentration at 40ºC, as well as the enzyme activity was not negatively affected by temperature even at 80ºC.

**Figure 5.** Effect of operating temperature (T) on the glycerolysis of sardine oil by Lipozyme 435 in SC-CO$_2$. Reactions were performed at MR = 3:1 (glycerol:oil), p = 15 MPa, enzyme loading 5 % wt. of substrates.

**Lipid oxidation.** In this work, peroxide and anisidine values have been determined for the supplied refined sardine oil and for the reaction mixtures obtained at the different temperatures assayed after 8 hours of reaction. Results obtained for PV and AnV are plotted in Figure 6. Temperature negatively affects the oxidation of the final mixture. Both, PV and AV increase with operating temperature [6]. The highest values were obtained at 80 ºC, the highest T assayed in this work. Although higher reaction rates were obtained with an increase in temperature from 40 to 80 ºC, lipid oxidation increases with temperature. Therefore according to the reaction rate and the oxidative status, it can be considered 50 ºC as the optimum operating temperature.

**Figure 6.** Effect of operating temperature on the oxidative status of the lipid mixtures obtained after 8 h of reaction at 15.0 MPa. Dashed line represents the recommended limit set by EPS (European Pharmacopeia Standard).

**CONCLUSION**

SC-CO$_2$ has been used as a green solvent in the lipase-catalyzed glycerolysis of sardine oil by Lipozyme 435, providing an environmentally benign reaction medium. Advantages of using SC-CO$_2$ include replacing organic solvents and preventing oxidation due to displacement of oxygen. The emulsification of glycerol and oil improves the initial contact of the substrates with the lipase providing higher reaction rates. It has been also demonstrated that oxidation level of the products obtained under SC-CO$_2$ is lower than in other systems, such as tert-
pentanol or solvent free. The results show that an increase in temperature from 40 to 80 °C produces higher reaction rates however lipid oxidation increases with temperature. Therefore, according to the reaction rate and the oxidative status, it can be considered 50 °C as the optimum operating temperature. Otherwise, pressure had no significant effect on conversion of TAG in the working pressure range. More research is required in emulsions phase behaviour to enhance our fundamental understanding into this complex system.

REFERENCES