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Substrates emulsification process to improve lipase-catalyzed sardine oil glycerolysis in different systems. Evaluation of lipid oxidation of the reaction products

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Abstract

Mono- and diacylglycerols rich in omega-3 have a great interest due to their good bioavailability and oxidation stability compared with other kind of omega-3 concentrates. The main drawback in mono- and diacylglycerols production by glycerolysis is the immiscibility of the substrates, oil and glycerol. To improve mass transfer rates, avoiding the use of organic solvents, emulsification of both reactants as reverse micelles (glycerol-in-oil) was carried out previous to lipase-catalyzed sardine oil glycerolysis. Substrate emulsification yielded higher reaction rates compared to kinetics with no previous emulsification, but still lower than in organic solvents. To avoid the use of organic solvent, SC-CO_2 was used as reaction medium but no kinetic advantages were demonstrated in the pressure range from 15 to 25 MPa. By increasing temperature, from 40 to 90 ºC, reaction rates increased both in a solvent-free system and in SC-CO_2 medium. It was also found that an increase in temperature does not lead to an increase in the final oxidation status of the reaction products. This behavior was due to the sorption capacity of the Lipozyme 435 support, giving lower oxidation status at the highest temperature, 80-90 ºC.

Keywords: fish oil, glycerolysis, microemulsion, SC-CO_2, peroxides adsorption.

Chemical compounds studied in this article: Glycerol (PubChem CID: 753); Eicosapentaenoic acid (PubChem CID: 446284); Docosahexaenoic acid (PubChem CID: 445580); Aerosol OT (PubChem CID: 23673837); Tween 80 (PubChem CID: 5281955).

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1. Introduction

The importance of omega-3 polyunsaturated fatty acids (n-3 PUFA), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in human nutrition and disease prevention is fully recognized scientifically (Kris-Etherton, Harris, & Appel, 2002; Riediger, Othman, Suh, & Moghadasian, 2009). n-3 PUFA supplements are available in different chemical forms. Among the different types of lipid derivatives containing n-3 PUFA concentrates, monoacylglycerols (MAG) and diacylglycerols (DAG) have good bioavailability and oxidation stability (Hernandez, 2014; Lawson & Hughes, 1988). Additionally, it must be also considered that dietary TAG are hydrolyzed in the small intestine to sn-2-MAG being the most favorable structure for n-3 PUFA to be adsorbed by intestinal mucosa (Bandarra et al. 2012). In addition, MAG or its mixtures with DAG account for 75% of the worldwide emulsifier production (Zhong et al., 2009). 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 PUFA. Enzyme-catalyzed reaction is an attractive alternative since the reaction can be carried out under mild conditions (Bornscheuer, 1995; Feltes, de Oliveira, Block, & Ninow, 2013).

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 (Damstrup et al., 2006), compressed fluids (Moquin, Temelli, King, & Palcic, 2005) and ionic liquids (Guo & Xu, 2006), in order to improve the mass transfer. The cost, toxicity and energy required for solvent removal from the product mixture, are important aspects to be considered when dealing with conventional solvent systems (Prat, Hayler, & Wells, 2014). Recently, the uses of different surfactants to increase the interfacial area, and ultrasound irradiation have been also proposed to reduce mass transfer limitation (Fiametti et al., 2012; Valério, Rovani, Treichel, De Oliveira, & Oliveira, 2010). Biocatalytic processing in microemulsion system has received attention in order to increase contact between substrates. The formation of a microemulsion of the reactants (glycerol-in-oil) as reverse micelles can help to improve mass transfer rates. Furthermore, lipases demonstrate high interfacial activity in micelle systems because the formation of the active site during the reaction

occurs at the interface between the substrates and the enzyme. Several food grade surfactants are able to stabilize the micellar system improving system homogeneity (Carvalho & Cabral, 2000; Stamatis, Xenakis, & Kolisis, 1999). Nevertheless, it must be taken into account that some food grade surfactants have chemical functions that could be modified by lipases. For instance, the lipase Novozym 435 presented activity at particular conditions towards some surfactants as soy lecithin and Tween in glycerolysis reactions (Camino Feltes, Villeneuve, Baréa, de Oliveira, & Ninow, 2012). To avoid this problem, other synthetic surfactants, such as sodium (bis-2-ethyl-hexyl) sulfosuccinate (aerosol-OT or AOT), have been used. AOT has been reported to form micelles in a great number of nonpolar substances and several other polar solvents such as glycerol (Fiametti et al., 2009). In this case, good results have been obtained in glycerolysis systems when adding more that 7.5% of AOT (Fiametti et al., 2009). However, the high amount of this surfactant may generate problems during removal processes (Stamatis, Xenakis, & Kolisis, 1994).

Another alternative to organic solvents is the use of the supercritical fluids (SCFs) as reaction medium. Supercritical carbon dioxide (SC-CO_2) is probably the most used SCF 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 (Matsuda, 2013; Rezaei, Temelli, & Jenab, 2007). SC-CO_2 has liquid-like density but gas-like viscosity resulting in high mass transfer being a clean alternative to replace organic solvents. Enzymatic concentration of n-3 PUFA in supercritical fluids (SCFs) is an interesting option for the prevention of oxidation during processing of fish oil (Lin, Chen, & Chang, 2006; Roh, Kim, & Choi, 2015). Besides, SC-CO_2 can be easily separated from the reaction products by simple depressurization and allows fractionation of the reaction products. Some previous studies of enzymatic reactions of different lipid sources in SC-CO_2 have been reported in the literature. However, in case of enzymatic glycerolysis, other compressed fluids such as propane, n-butane, and acetone, have been used (Esmelindro et al., 2008; Tai & Brunner, 2011; Valério et al., 2010). Some studies of glycerolysis of vegetable oils in SC-CO_2 at high temperatures can be found but with no enzymatic catalyst (Moquin et al., 2005; Temelli, King, & List, 1996).

In a previous work, a detail kinetic study of glycerolysis of sardine oil using Lipozyme® 435 form *Candida antarctica* B as biocatalyst in an optimized amount of tert-butanol was performed (Solaesa, Sanz, Beltrán, et al., 2016). Tert.butanol helped to create a

homogeneous phase and to reduce mass transfer limitations. However, organic solvents present different environmental concerns. In this work, to improve contact between substrates, avoiding the use of organic solvents, emulsification of glycerol and oil before glycerolysis reaction was considered. Glycerolysis reaction has been performed in a solvent free system at atmospheric pressure and in SC-CO_2 as reaction medium with previous susbstrates emulsification. The effect of adding a surfactant, AOT or Tween 80, to stabilize the emulsion, on glycerolysis performance has been also studied. Glycerolysis has been determined at different operating temperaturesat atmosphere pressure, 0.1 MPa, and in SC-CO_2 medium in the pressure range from 15 to 25 MPa. Since n-3 PUFA are highly susceptible to oxidation; the oxidative status of the final reaction products was evaluated through the peroxide and anisidine values. Reaction yields and the oxidation values of the reaction products were compared for both systems.

2. Materials and methods

2.1 Materials

Refined sardine oil was provided by Industrias Afines S.L. (Spain) with 18.3% of EPA and 7% of DHA and a water content of 0.2% (Solaesa, Bucio, Sanz, Beltrán, & Rebolleda, 2014). 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* B (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). Polyoxyethylene sorbitan monooleate (Tween 80) and sodium bis (2-ethylhexyl) sulfosuccinate (Aerosol AOT or AOT), used as food grade surfactants, were purchased by Sigma Aldrich. All other chemicals used in different analyses were of analytical or HPLC grade.

2.2 Emulsification process

Microemulsions of the glycerolysis system of sardine oil were prepared at a fixed mole ratio of 3:1 (glycerol:oil) since this mole ratio was found as the optimum in a previous kinetic study (Solaesa, Sanz, Beltrán, et al., 2016). A high-speed blender (Miccra D9 equipped with a DS-20/PF EMR rotor–stator) at different speeds, from 16000 to 35000 rpm, was used by pulses during 3 minutes. To prepare the surfactant-free emulsion as reverse micelles, the appropriate amount of glycerol (10 g) was added drop by drop to

the suitable amount of oil (30 g) while being completely mixed at high speed. Dispersed (glycerol) and continuous (sardine oil) phases were identified by the dilution test (Mize et al., 2013). Furthermore, different concentrations (0.5, 1 and 1.5% in glycerol or oil as indicated in Table 2) of two food grade surfactants, AOT and Tween 80, were tested in order to improve the stability of the emulsion. A defined quantity of each surfactant was dissolved in oil or in glycerol, depending on its solubility. The characterization of the emulsions was performed 10 min after emulsification to avoid any creaming or coalescence effect. Particle size distribution (PSD), 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 small (or the smallest) droplet size.

2.3 Lipase-catalyzed glycerolysis of sardine oil in different systems

A comparative study of lipase-catalyzed glycerolysis in different systems was carried out. All the experiments were conducted in a batch mode keeping constant the enzyme concentration at 5 wt% (by weight of substrates) and the substrate mole ratio (3:1, glycerol to oil) according to previous work (Solaesa, Sanz, Beltrán, et al., 2016). **Table 1** summarizes all glycerolysis reactions that have been done in this work. Experiments 1 - 6 have been carried out at atmospheric pressure in a solvent free system in a 100 mL jacketed batch reactor. First of all, experiments 1 and 2 were carried out to evaluate the effect of previous substrates emulsification on reaction rate. Experiments 3 and 4 were performed with emulsified substrates stabilized by adding a food grade surfactant, AOT and Tween 80 respectively, at the optimum concentration previously determine in section 2.2. Experiments 2, 5 and 6 were performed to evaluate the effect of reaction temperature, 50, 80 and 90ºC respectively. Glycerolysis reaction was carried out as follows. Once emulsion was prepared, it was charged into the reactor. Later, the lipase was added and a nitrogen stream was applied. The reactor was then closed and the stirring system by impellers was connected. A thermostatic water bath allows working at the desired temperature. The reactor was covered with foil paper to avoid the light exposure.

On the other hand, experiments 7-13 have been carried out in SC-CO_2 as reaction medium. They were performed in a high pressure batch stirred tank reactor made of stainless steel, having an internal volume of 100 mL (Melgosa et al., 2017). A freshly

prepared emulsion and the lipase were charged into the reactor provided with magnetic agitation and then it was closed, placed in a thermostatic water bath and connected to the pressure circuit. Subsequently, SC-CO_2 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. Operating pressure and temperature have been varied in the range between 15-25 MPa (Exp.7-9) and 40-90ºC (Exp. 7 and 10-13).

In both systems, samples were taken periodically during 8 h, filtered and stored at -18ºC up to analysis.

2.4 Analysis of the reaction products

The neutral lipid profile (TAG, DAG, MAG and FFA) was analyzed by a normal phase high performance liquid chromatography (NP-HPLC). The chromatographic apparatus consisted of a HPLC system (Agilent 1200) 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 µm, 4 mm × 250 mm) and detection was performed by an evaporative light scattering detector (Agilent 1200 series) at 35ºC and 0.35 MPa. Gradient elution was achieved by mobile phases A (isooctane) and B (methyltert-butyl ether:acetic acid = 99.9:0.1, v/v). The method and calibration procedure have been previously reported (Solaesa, Sanz, Falkeborg, et al., 2016). The regioisomers of DAG and MAG could not be distinguished by the applied analytical procedure, so the total amount of MAG and DAG was reported for the kinetic experiments. The lipid profile results were expressed in glycerol free basis.

2.5 Lipid oxidation analysis

The oxidation status has been determined using two assays: peroxide value (PV) and anisidine value (AV). The PV measures the concentration of hydroperoxides formed in the initial stages of lipid oxidation (primary oxidation). PV was determined following the AOAC Official Method 965.33 by an automatic titrator Methrom 905 Titrando (AOAC Official Method 965.33, 2000). The AV is an estimation of the concentration of non-volatile secondary oxidation products (mainly 2-alkenals and 2,4-dienals). The AV was measured according to AOCS official method (Cd 18–90), using a UV-Visible spectrophotometer (AOCS Official Method Cd 18-90, 2017). PV and AV allow calculating total oxidation (TOTOX) by the formula:

$$TOTOX = 2PV + AV \quad [1]$$

PV and AV have been determined for the supplied refined sardine oil and the final reaction mixtures obtained after 8 h at the different temperatures The lipid phase was separated for analysis from the lipase and the remained glycerol by centrifugation at 5000 rpm and 35ºC during 10 minutes. The upper phase, free of glycerol, formed by the lipid fraction (TAG, DAG, MAG and FFA) was collected under N_2 atmosphere and stored at -18ºC up to analysis.

2.6 Statistical analysis

All analyses were conducted using software Statgraphics X64. The results are presented as a mean ± standard deviation of at least three replicates. The significance of the differences was determined based on an analysis of the variance with the Tukey's honestly significant difference (HSF) method at p-value ≤ 0.05.

3. Results and discussion

3.1 Optimization of the emulsification process and characterization of the emulsion

3.1.1 Surfactant-free emulsions

The effect of emulsification speed on emulsion stability without the addition of surfactants has been evaluated by measuring the polydispersity index (PDI) and the droplet diameter of the emulsion obtained in the range from 16000 to 35000 rpm. At any of the emulsification speeds essayed, droplet diameter was lower than 2 µm but 29000 rpm were needed to obtain a PDI below 1. The lowest polydispersity index was obtained at the highest speed assayed in this work, 35000 rpm; however, foaming was observed. Therefore 29000 rpm was selected for further substrate emulsifications. At this speed the mean droplet diameter of the emulsion was 301 ± 34 nm and the PDI around 0.4. The surfactant-free emulsion presented a PDI lower than 1 only up to 20 minutes, although at longer times, still a translucent and homogeneous system was visually observed. In any case, the emulsion was prepared and immediately used as reaction media.

3.1.2 Surfactant stabilized emulsions

The use of a surfactant was also tested in this work to improve the emulsion stability and reaction rates. Two food grade surfactants, AOT and Tween 80, with hydrophilic

lipophilic balance (HLB) values of 10 and 15 respectively, were used at different amounts (0.5, 1 and 1.5%). PDI of the emulsions prepared adding these surfactants were measured at specific times to evaluate their stability **(Table 2).** Although a surfactant was added to stabilize the emulsions, PDI in the different emulsions increased with time in all cases (**Table 2**). The higher stability was observed when 0.5% of Tween 80 and 1.5% of AOT were previously dissolved in glycerol. In these cases the emulsion was found to be stable for at least 1 h. PSD was evaluated for emulsions with the highest stability formed by adding 0.5 % of Tween 80 and 1.5 % of AOT in glycerol and compared with those obtained in surfactant-freeemulsion. Smaller micelles were obtained when a surfactant was added to the system with medium particle sizes values of 67 ± 5 nm, 94 ± 4 nm and 301 ± 34 nm for AOT 1.5 % and 0.5 % of Tween 80 dissolved in glycerol and surfactant-free emulsion, respectively.

3.2 Glycerolysis reaction of sardine oil by Lipozyme 435

3.2.1 Effect of substrates emulsification on the reaction rate

Fig. 1 compares the kinetics of the glycerolysis reaction in a solvent free medium at atmospheric pressure with and without previous emulsification of the substrates (Exp 1 and 2 respectively). As it can be observed, when no previous emulsification of the reactants was carried out, mass transfer limitations lead to lower initial reaction rate. These limitations are reflected in the values of the initial slope of TAG composition as function of time being 0.15 ± 0.01 (mol TAG %·min^{-1}) without substrates emulsification and 0.279 ± 0.008 (mol TAG %·min^{-1}) for substrate emulsification. For a reverse micelle system, higher interfacial area is provided, which favors lipase-catalyzed reactions. At longer reaction times, reaction rates become similar due to the MAG and DAG formation as emulsifiers. The low HLB values of MAG and DAG mean that they tend to stabilize reverse micelles systems (O'Brien, 2004). Similar results were observed by Awadallak et al. (Awadallak, Voll, Ribas, Cardozo, & Edson, 2013) in the enzymatic palm oil hydrolysis under ultrasound irradiation to produce DAG. They also performed a control reaction (without ultrasound influence) to compare the degree of hydrolysis in both systems, being around 20% after 12 h in the control reaction and almost 40% when ultrasound was used before the reaction. But at longer reaction times (24 h) the degree of hydrolysis becomes similar. Therefore, they also

concluded that ultrasound used before the reaction to promote emulsification improved kinetics.

Fig. 2 represents the reaction time course of TAG when surfactants, AOT and Tween 80, were added to the system at the concentration reported in Table 2 for the most stable emulsions, 1.5% of AOT and 0.5% of Tween 80 in glycerol.Although , particle diameter was smaller when both surfactants, AOT or Tween 80, were added to the emulsions; this size reduction did not lead to significant improvement in the reaction rate or equilibrium conversion. Based on these results, further kinetics studies on glycerolysis were performed by previously emulsifying both reactants but with no addition of surfactant.

In the literature, surfactants have been also used to improve the contact between substrates, however much higher concentration was employed. Fiametti et al. (Fiametti et al., 2009) evaluated AOT concentrations from 5 to 20 % in the MAG production of olive oil, and obtained conversion values of around 60% for the AOT concentration of 20 wt%. On the other hand, Camino Feltes et al. (Camino Feltes et al., 2012) employed 10% of different surfactants (Tween 65, Tween 80, Tween 85 and soy lecithin) and they noticed that Tween as well as soy lecithin were partially modified by the lipase during the glycerolysis reaction. However, they acted as surfactants rather than as substrates at concentrations as low as 0.4–0.8%. Additionally, the use of high amounts of surfactants may imply additional steps for the desired products recovery.

In a previous work, lipase catalyzed glycerolysis have been performed in tert-butanol as organic reaction medium to avoid mass transfer limitations (Solaesa, Sanz, Beltrán, et al., 2016). **Fig. 3** compares the glycerolysis reaction time course in a lipid base for TAG, DAG, MAG and FFA when tert-butanol was used as reaction media (Solaesa, Sanz, Beltrán, et al., 2016) and in solvent free media with previous substrate emulsification (Exp. 2). It can be observed that reaction rates are much higher in tert-butanol than in a free solvent media. This proves that mass transfer controls the reaction process since tert-butanol helps to create an homogeneous system but also to reduce the viscosity of the medium (viscosity of glycerol, fish oil and tert-butanol at 50 ºC are 142, 20–30 and 1.421 mPa·s, respectively). Due to mass transfer limitations, reaction rates are slower and the intermediate, DAG, accumulates in a greater extent in a solvent free media. After 7 h DAG accounts for 17.2 %, compared to less than 5 % in tert-butanol. It seems that by controlling the viscosity of the medium selective formation of MAG and DAG can be obtained. In any case, although higher reaction rates and reaction yield

were obtained in tert-butanol, organic solvents present a number of environmental concerns and its use should be avoided.

3.2.2 Pressure effect

To avoid the use of organic solvents to improve the mass transfer and therefore increase the reaction rate and MAG conversion, the glycerolysis reaction has been carried out in SC-CO_2 medium with previous emulsified substrates. **Fig. 4** shows the effect of operating pressure (from 15 to 25 MPa) at 50ºC, on the reaction conversion and products yield after 7 h of reaction time. Based on these results it can be concluded that pressure has no significant effect on MAG and DAG yield or global TAG conversion in SC-CO_2 as reaction media in the pressure range studied in this work. TAG conversion ranged from 71 to 74% and MAG and DAG yield was 53 and 17 %, respectively. In this regard, the effect of pressure on enzyme catalysis in SC-CO_2 is difficult to predict since pressure affects the density and transport properties of SC-CO_2, but also has an effect on reaction rate since concentrations of reactants and products can be modified due to partitioning between the phases (Rezaei et al., 2007). As it has been previously explained, in a reverse micelle system, sardine oil behaves as the continuous phase. According to the literature, solubility of CO_2 in fish oil increases with pressure at constant temperature, for instance, at 40ºC, solubility at 15 MPa is 29.1% mass and at 25 MPa is 33.1% mass (Borch-Jensen & Mollerup, 1997). The increase in solubility with pressure could improve the diffusivity in the reaction medium. However, fish oil solubility also increase in SC-CO_2 by increasing pressure at constant temperature (Bucio et al., 2016). In the literature, it has been suggested (Temelli et al., 1996) that due to this solubility increment of TAG in the SC-CO_2, TAG could be in the supercritical phase, not being available in the liquid phase to react with glycerol that remains in the liquid phase due to its low solubility values in SC-CO_2 (Medina-Gonzalez, Tassaing, Camy, & Condoret, 2013; Nunes, Carrera, Najdanovic-Visak, & Nunes Da Ponte, 2013). These phenomenon's could cancel each other showing no effect of pressure on glycerolysis performance. Temelli et al. (Temelli et al., 1996) obtained similar results in the glycerolysis of soybean oil in SC-CO_2 at high temperatures (150-250ºC) in the pressure range from 20.7 to 62.1 MPa and Tao et al. in the enzymatic synthesis of dipalmitin from palmitic acid and glycerol at 65ºC in the pressure range from 8.5 to 18.5 MPa (Tao, Li, Qu, & Zhang, 2013).

Fig. 4 also presents the results obtained at atmospheric pressure at 50 ºC in a solvent-free system with previous substrate emulsification (section 3.2.1). Lipid products profile was similar in both systems, SC-CO_2 and in a solvent-free media. To compare reaction rates, **Table 3** summarizes the different initial reaction rates for TAG, DAG, MAG estimated by the initial slopes of the plot of the different lipid percentages profile as a function of time in both systems. Based on the properties of SC-CO_2, such as low viscosity and high diffusivity, kinetics are expected to be faster than in a solvent free system. Surprisingly, similar results were obtained at atmospheric pressure than when working in SC-CO_2. As previously explained, due to the increase of TAG solubility in SC-CO_2 with temperature, fish oil is less available in the liquid phase to react with glycerol. This could explain the similar results obtained at atmospheric pressure and in SC-CO_2. Therefore, from the reaction rate or product composition no advantages on the use of SC-CO_2 can be demonstrated. Although, it must be emphasized that the use of SC-CO_2 could be advantageous to the fractionation of the reaction products (Castillo, Marty, Combes, & Condoret, 1994).

3.2.3 Temperature effect

To assess the effect of temperature on the kinetics of the glycerolysis of sardine oil by Lipozyme 435 in SC-CO_2 media and in a solvent free system, operating temperature has been varied from 40 to 90ºC (experiments 8, 10-13) and 50 to 90 ºC (experiments 2, 5 and 6), respectively. Initial substrate molar ratio (3:1 glycerol:sardine oil) and enzyme loading (5% wt. of substrates) remained unchanged. **Fig. 5** shows the MAG + DAG production at different temperatures in SC-CO_2. For a solvent free system, as it has been described in section 3.2.2, the time reaction course overlap with those carried out in CO_2 medium and it has not been represented. Equilibrium conversion is essentially temperature independent, although, at 40ºC, 8 hours was not sufficient time to achieve equilibrium concentration. Rising temperature from 40 to 90ºC resulted in an increase of the initial reaction rate, due to the higher kinetic energy of the molecules that leads to lower viscosity and higher diffusivity of the solvent and substrates (Rezaei et al., 2007). It must be highlighted that enzyme activity was not negatively affected by temperature even at 90ºC.

In both systems, SC-CO_2 medium and solvent-free system, initial reaction rate followed and Arrhenius type dependence with temperature. From the slope of the

Arrhenius plot, an estimation of the activation energy can be evaluated as 52 ± 2 kJ/mol in SC-CO_2 medium and a similar value for the solvent free system, 49 ± 2 kJ/mol.

3.3 Comparison of lipid oxidation in both systems

In section 3.2.3 has been demonstrated that the enzyme Lipozyme 435 presents a high thermal stability since the highest reaction rate has been obtained at the highest reaction temperature assayed in this work, 90ºC. However, when working with easily oxidizable compounds, such as n-3 PUFA, the oxidation status of the final reaction products must be taken into account. In this work, PV and AV have been determined for the refined sardine oil and the reaction mixtures obtained at the different temperatures after 7 hours of reaction in solvent free and in SC-CO_2 media.

PV and AV for the supplied refined sardine oil were 4.8 ± 0.1 meq O_2/kg and 23.0 ± 0.1 respectively. These values can be considered "acceptable" because they do not exceed the limit allowed (10 meq O_2/Kg oil for PV and 30 for AV) according to European Pharmacopeia Standard (European Pharmacopoeia 5.0, 2005). **Fig. 6** shows the PV and the AV of the reaction products obtained from 40 to 90 ºC. This figure shows unexpected results since PV decreased as reaction temperature increased, obtaining values of around 3 meq O_2/kg at the highest temperatures assayed in this work (80-90ºC) which are even lower than that obtained for the supplied refined sardine oil. Similar results regarding oxidation status have been obtained in solvent free system in the temperature range from 50 to 90ºC. AV remained unchanged by increasing temperature (**Fig. 6**).

To determine the effect of temperature on autoxidation of sardine oil, oil samples were heated at 40, 65 and 90ºC for 7 hours and the oxidation status was analyzed. **Table 4** shows that, both PV and AV are strongly depend on temperature, increasing as temperatures increased; however, the opposite trend was found in the oxidation status of the reaction products at the same temperatures. The lipase used in this work, Lipozyme 435, is immobilized on a macroporous hydrophobic acrylic resin; therefore it was assumed that peroxides could be adsorbed on the resin. To verify this assumption, sardine oil was mixed with the immobilized lipase at the same ratio sardine oil:lipase loading as for the glycerolysis reaction (28:1 g sardine oil:g lipase) and heated at 40, 65 and 90ºC for 7 hours under low agitation. After that, PV and AV were determined. It can be clearly observed (see **Table 4**) that the amount of peroxides in the sardine oil decreased at each temperature when the immobilized lipase is present compared with

the control sardine oil. Furthermore, lower peroxide values were obtained by increasing temperature, proving that sorption process of lipid peroxide in the enzyme support is favored by increasing temperature due to improved diffusivity. Regarding the AV, lower values were also obtained in the sardine oil in contact with the immobilized lipase than in the sardine oil heated at the same temperature without lipase. Therefore, the support of the enzyme could also have capacity to adsorb secondary oxidation products. According to the PV and AV, TOTOX values also decreased in the sardine oil that has been in contact with Lipozyme 435 (**Table 4**). These results can explain the trend observed in the oxidation status of the reaction products with increasing temperature. In any case, it must be highlighted that during the glycerolysis reaction MAG and DAG are formed and sorption behavior could be modified.

4. Conclusions

In this work, it has been demostrated that the emulsification of substrates before enzymatic glycerolysis reaction improved process efficiency in solvent free and in SC-CO_2 media, reducing mass transfer limitations in the three-phase system glycerol/oil/lipase, giving around 75% of MAG and DAG in 4 hours. Furthermore, when food grade surfactants, AOT or Tween 80, were added to the emulsified system, no significant improvement was observed neither in the reaction rate nor in the equilibrium conversions. It has been also found that pressure has no significant effect on reaction conversion and reaction rate, showing no kinetic advantages on the use of SC-CO_2 in glycerolysis reaction, although SC-CO_2 could be used to fractionate the reaction products. An increase in temperature from 40 to 90 ºC produces higher reaction rates in both systems. Regarding the oxidation status of the reaction products, it has been concluded that higher reaction temperature results in a higher adsorption of the oxidation products on the support of the lipase, giving lower oxidation values.

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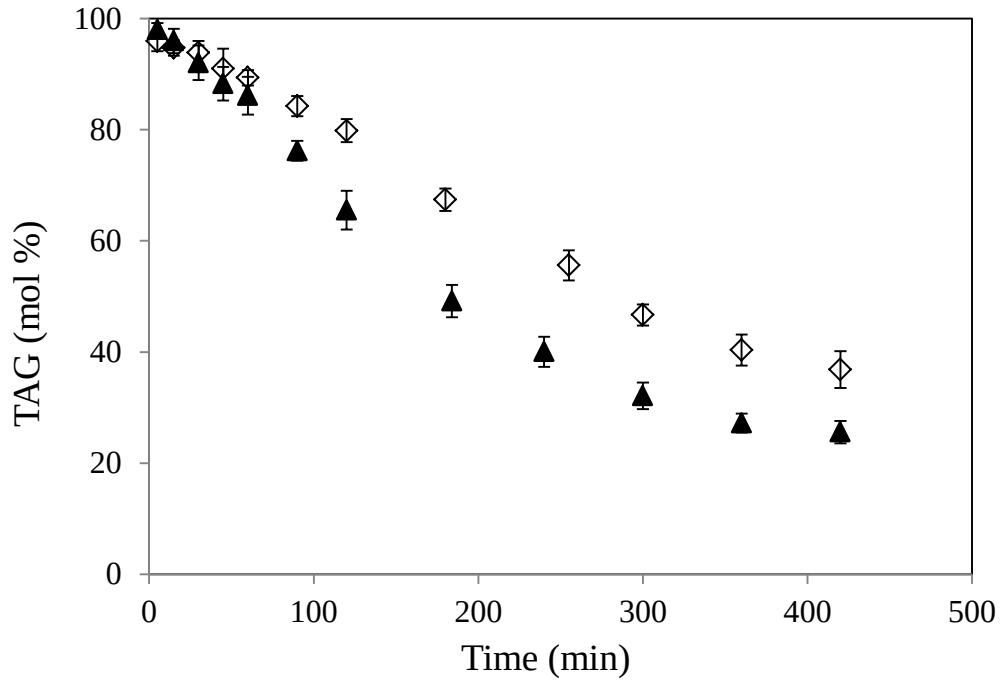


Fig. 1 .TAG composition as a function of time in the glycerolysis of sardine oil in solvent free system with (▲) and without (◊) substrates emulsification at atmospheric pressure (0.1 MPa). Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

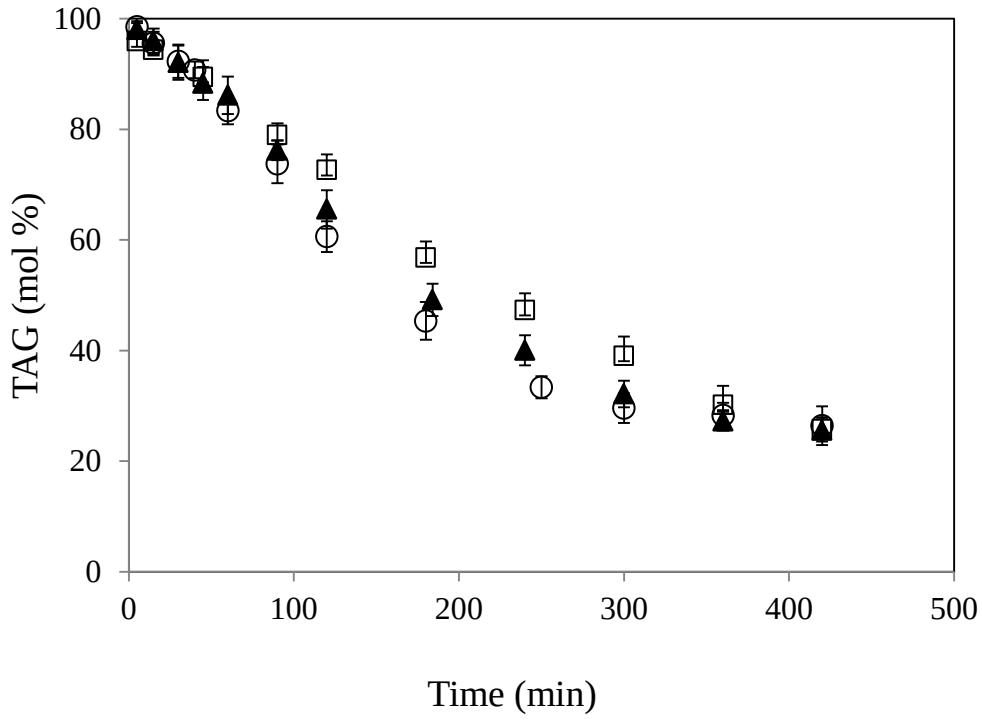


Fig. 2. TAG composition as a function of time in the glycerolysis of sardine oil previous substrates emulsification with 1.5% of AOT in glycerol (□), 0.5% of Tween 80 in glycerol (○) and surfactant free (▲) at atmospheric pressure (0.1 MPa).. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

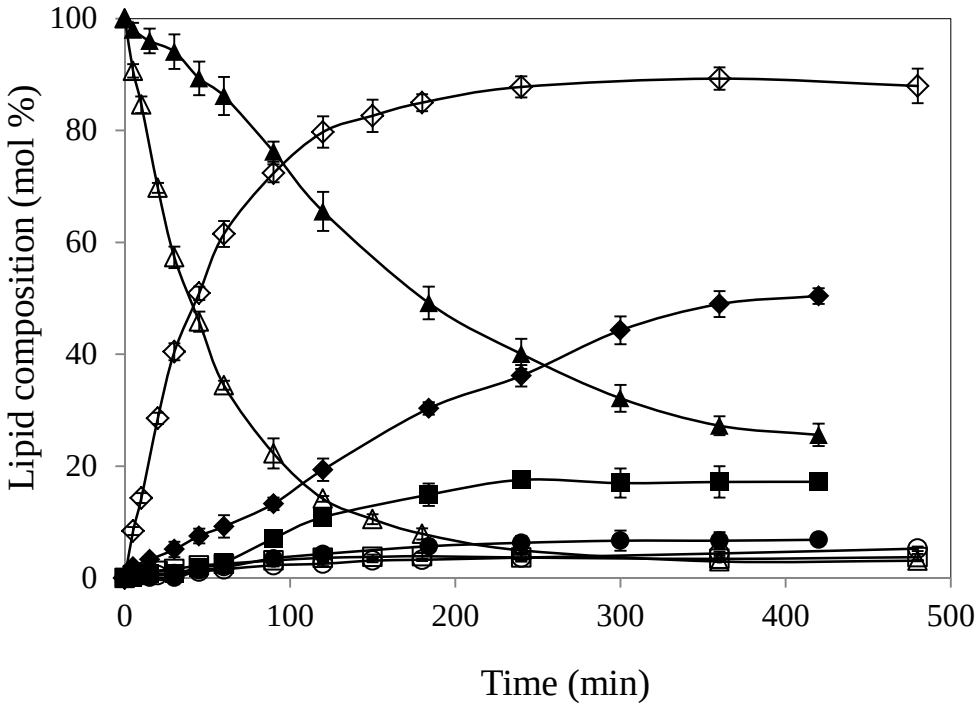


Fig. 3. Time course of lipase-catalyzed glycerolysis reaction of sardine oil in tert-butanol medium (hollow symbols) and in solvent free (solid symbols) with previous emulsification of the substrates at atmospheric pressure (0.1 MPa). Legend: TAG (Δ, ▲), DAG (□, ■), MAG (◇, ◆) and FFA (○, ●). Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

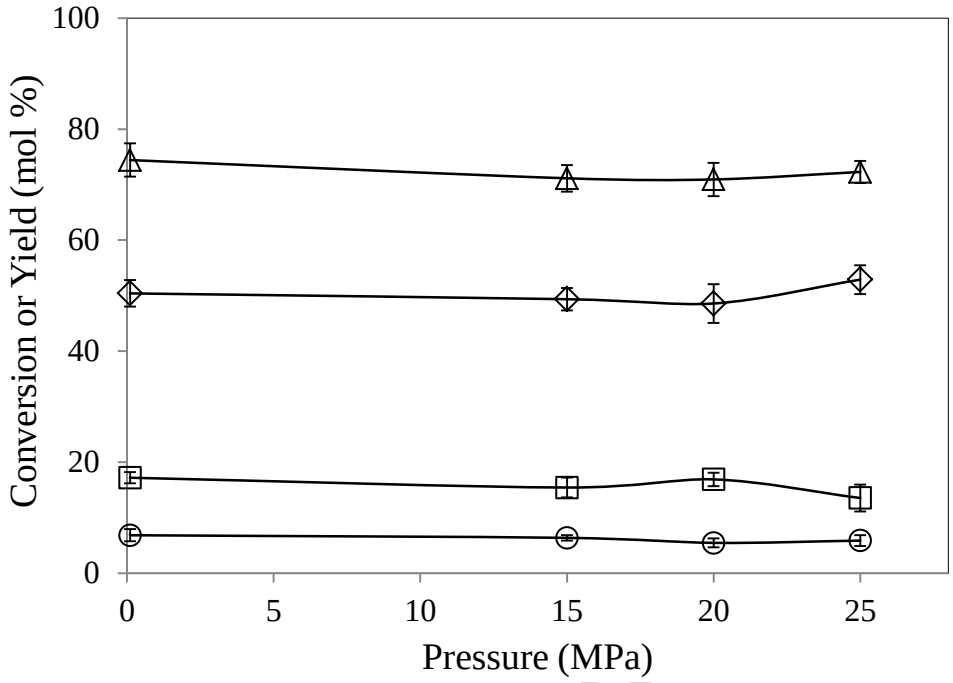


Fig. 4. Effect of pressure in SC-CO_2 as reaction medium on glycerolysis conversion of sardine oil and products yield with previous substrates emulsification . (Δ) conversion of TAG, (□, ◊, and ○) yields of DAG, MAG and FFA, respectively after 7 h of reaction time. Reactions were performed at MR = 3:1 (glycerol:oil), T = 50ºC, enzyme loading 5 % wt. of substrates.

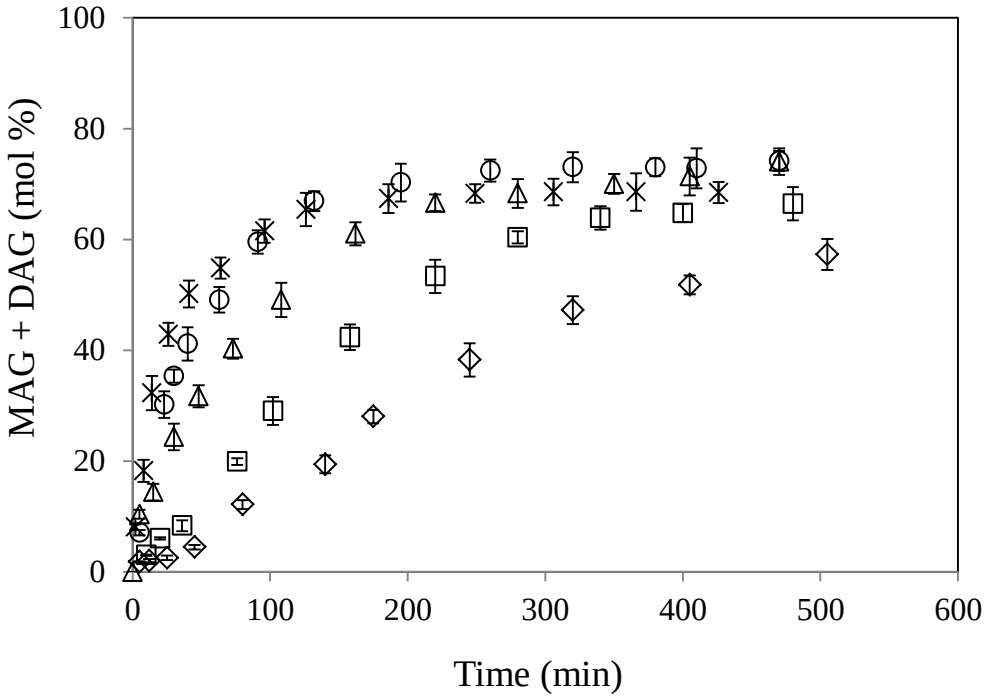


Fig. 5. Effect of temperature on MAG + DAG composition as function of time in the glycerolysis of sardine oil with previous substrates emulsification in SC-CO_2 at15 MPa: 40ºC (◊), 50ºC (□), 65ºC (Δ), 80ºC (○) and 90ºC (x). Reactions were performed at MR = 3:1 (glycerol:oil) and enzyme loading 5 % wt. of substrates.

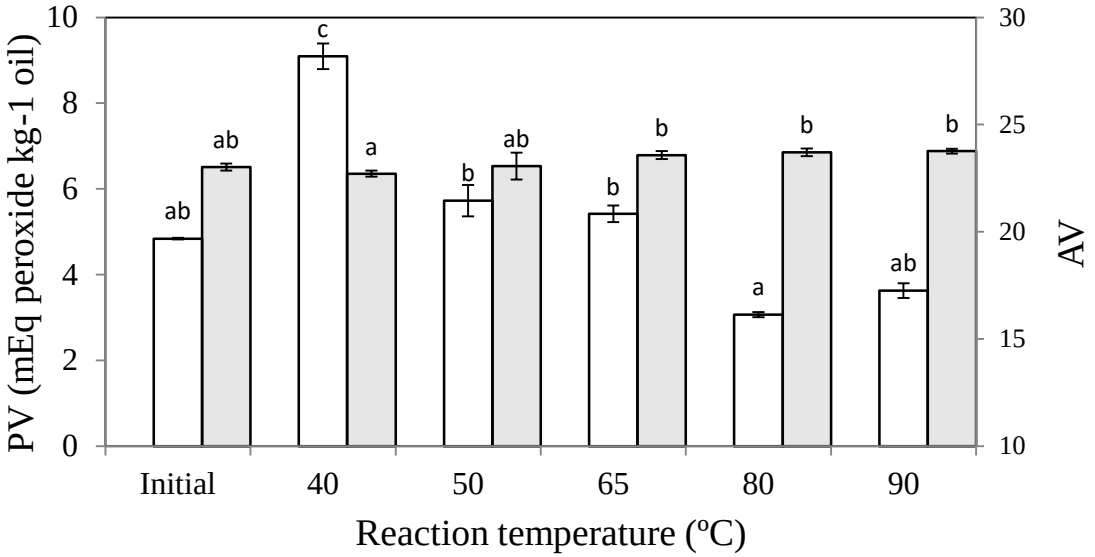


Fig. 6. Influence of reaction temperature on PV (white bars) and AV (grey bars) in the final reaction mixture after 7 h at 15 MPa in SC-CO_2 as reaction medium with previous substrates emulsficiation. Measurements given are mean values based on four determinations. Limit allowed is the maximum of each axis. Values with different letters in each type of analysis (PV or AV) are significantly different when applying the Tukey's honestly significant difference (HSD) method at p-value ≤ 0.05.

Table 1. Summary of the reaction conditions for lipase-catalyzed sardine oil glycerolysis reactions carried out in this work.

Exp.	Reaction medium	Pressure (MPa)	Temperature (ºC)	Emulsification	Surfactant
1	Solvent free	0.1	50	No	-
2				Yes	-
3				Yes	AOT
4				Yes	Tween 80
5			80	Yes	-
6			90	Yes	-
7	SC-CO_2 as solvent	15	50	Yes	-
8		20		Yes	-
9		25		Yes	-
10		15	40	Yes	-
11			65	Yes	-
12			80	Yes	-
13			90	Yes	-

Table 2. Polydispersity index (PDI) as measurement of reverse micelles stability when food grade surfactants are used at different concentrations at different times

Surfactant	Concentration of surfactant (wt%)[a]	PDI after different times		
		10 min	30 min	1 h
Tween 80 (HLB = 15)	0.5 in G	0.17 ± 0.04^{ab}	0.29 ± 0.01^{a}	0.78 ± 0.15^{b}
	1 in G	0.63 ± 0.09^{d}	0.83 ± 0.19^{b}	1
AOT (HLB = 10)	0.5 in G	0.51 ± 0.19^{cd}	0.79 ± 0.09^{b}	1
	1 in G	0.27 ± 0.06^{ab}	0.50 ± 0.01^{ab}	1
	1.5 in G	0.28 ± 0.04^{abc}	0.45 ± 0.13^{a}	0.43 ± 0.06^{a}
	0.5 in O	0.07 ± 0.02^{a}	0.64 ± 0.07^{ab}	1
	1 in O	0.34 ± 0.06^{bc}	0.43 ± 0.03^{a}	1
	1.5 in O	0.14 ± 0.05^{ab}	0.43 ± 0.09^{a}	1

[a]G = dissolved in glycerol; O = dissolved in oil

Values with different letters in each column are significantly different when applying the Tukey's honestly significant difference (HSD) method at p-value ≤ 0.05.

Table 3. Initial slopes of TAG, DAG and MAG composition at different operating pressures previous substrates emulsification in SC-CO_2 (pressure range: 15-25 MPa) and at atmospheric pressure in a solvent free system (0.1 MPa). Reaction conditions: 50ºC, 5 % enzyme loading based on substrate weight, MR = 3:1.

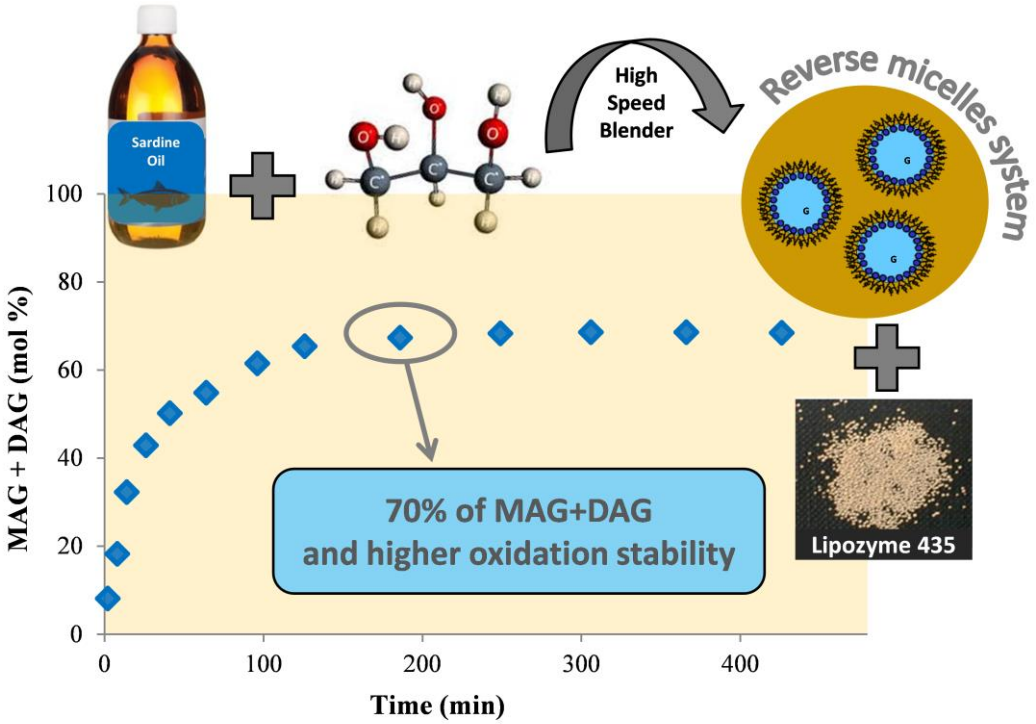
Pressure, MPa	Initial slopes (mol%·min^{-1})		
	TAG	DAG	MAG
0.1*	0.279 ± 0.008^{a}	0.045 ± 0.005^{a}	0.150 ± 0.006^{a}
15	0.296 ± 0.010^{a}	0.052 ± 0.002^{a}	0.202 ± 0.007^{b}
20	0.271 ± 0.008^{a}	0.062 ± 0.005^{a}	0.162 ± 0.009^{a}
25	0.284 ± 0.012^{a}	0.052 ± 0.008^{a}	0.204 ± 0.009^{b}

***** Solvent free. Values with different letters in each column are significantly different when applying the Tukey's honestly significant difference (HSD) method at p-value ≤ 0.05.

Table 4. PV, AV and TOTOX values obtained after 7 hours heating at different temperatures sardine oil and sardine oil in contact with Lipozyme 435.

T, ºC	PV, meqO_2/kg		AV		TOTOX	
	Sardine oil	Sardine oil + lipase	Sardine oil	Sardine oil + lipase	Sardine oil	Sardine oil + lipase
40	11.6 ± 0.1a	6.1 ± 0.1^{c}	24.4 ± 0.3^{a}	23.5 ± 0.7^{a}	47.6 ± 0.6^{a}	35.7 ± 0.9^{a}
65	22.3 ± 0.2^{b}	4.8 ± 0.1^{b}	30.2 ± 0.8^{b}	25.5 ± 0.6^{b}	75 ± 1^{b}	35.1 ± 0.8^{a}
90	47 ± 1^{c}	3.8 ± 0.1^{a}	54.5 ± 0.5^{c}	30.3 ± 0.2^{c}	148 ± 3^{c}	37.9 ± 0.4^{b}

Values with different letters in each column are significantly different when applying the Tukey's honestly significant difference (HSD) method at p-value ≤ 0.05.



Graphical abstract

Highlights

- Emulsified substrates improve the initial rate in solvent-free glycerolysis.
- The use of SC-CO_2 presents no kinetic advantages in glycerolysis reaction.
- Hydroperoxides are adsorbed on the lipase support.
- Sorption of hydroperoxide is favored at high reaction temperatures.