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1 # Oxidation kinetics of sardine oil in the presence of
2 # commercial immobilized lipases commonly used as biocatalyst

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6 **Abstract**

7 Oxidation kinetics of sardine oil have been determined at 40, 65 and 90ºC by measuring
8 concentration of primary and secondary oxidation products in the presence of
9 commercial immobilized lipases (Lipozyme 435, Lipozyme RM and Lipozyme TL)
10 commonly used as biocatalyst in lipid modification reactions. Oxidation products
11 concentration was found to be lower when the immobilized lipases were added at the
12 highest temperatures studied. The lowest oxidation indices were observed in the
13 presence of Lipozyme RM.
14 Although the mechanism to explain this decrease in the oxidation products is not still
15 clear, these results might indicate that the use of these immobilized lipases in lipase-
16 catalyzed reactions of fish oils at high temperature (90ºC) will yield higher reaction
17 rates but also a reduction of the oxidation products formed due to oxidation of
18 polyunsatured fatty acids.

19 ***Keywords:*** *fish oil, omega-3, oxidation products, commercial immobilized lipases.*

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20 ## 1. Introduction

21 Fish oil has high amounts of omega-3 long-chain polyunsaturated fatty acids (n-3
22 PUFA), mainly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA,
23 22:6n-3) which have been reported to protect against the development of many diseases
24 (Solaesa, Sanz, Falkeborg, Beltrán, & Guo, 2016). However, despite the high nutritional
25 value of these products, the high degree of unsaturation makes fish oils very prone to
26 autoxidation. The resulting breakdown products cause off-flavors and rancidity, loss of
27 nutritional value and finally consumer rejection (Gómez-Alonso, Mancebo-Campos, &
28 Salvador, 2004). The degree and rate of lipid oxidation is influenced by the unsaturation
29 of fatty acids, oxygen concentration, temperature, surface area, water activity and
30 presence of anti– and prooxidants. Temperature has also an important impact on lipid
31 oxidation since an increase in temperature accelerates oxidation rates.

32 The process of lipid oxidation can be described in three general steps: initiation,
33 propagation and termination. The concentrations of primary and secondary oxidation
34 products can be measured quantitatively and therefore give an indication of the
35 oxidative status of the oil. The hydroperoxide content, as primary oxidation products, is
36 usually determined by the peroxide value (PV) assay. Secondary oxidation products can
37 be determined by anisidine value (AV) and thiobarbituric acid reactive substances
38 (TBARS) assays. AV measures mainly 2-alkenals and 2,4-dienals. On the other hand,
39 the cyclic peroxides formed by autoxidation from polyunsaturated fatty acids with three
40 or more double bonds are the most important precursors of malonaldehyde and hence
41 source of TBARS (Hoyland & Taylor, 1991). Polyene index (PI) is also used as a good
42 indicator of PUFA deterioration in fish oils, defined as (EPA + DHA): palmitic acid
43 ratio (Pazhouhanmehr, Farhoosh, Sharif, & Esmaeilzadeh, 2016).

44 Omega-3 concentrates can be produced by lipase-catalyzed reactions since they can be
45 carried out under mild conditions in short reaction times compared with chemical
46 processes. Some widely used commercial immobilized lipases used in enzymatic
47 reactions are from *Candida antarctica* (Novozym 435 and Lipozyme 435), *Rhizomucor*
48 *miehei* (Lipozyme RM IM) and *Thermomyces lanuginose* (Lipozyme TL IM). In a
49 previous work (Solaesa, Sanz, Melgosa, & Beltrán, 2017), it was found that the
50 oxidation status of the final reaction products of glycerolysis of sardine oil at 60 and
51 90ºC catalyzed by the commercial immobilized lipase, Lipozyme 435, was even lower
52 than the oxidation status of the initial sardine oil, determined as PV and AV. Especially,
53 hydroperoxide content decreased in a greater extend at the highest reaction temperature
54 assayed. Therefore, an increase in reaction temperature, not higher than the maximum
55 denaturation temperature for the lipase, was positive since reaction rate increased and
56 lower oxidation status of the final products was determined.

57 Based on those previous results, in this work, a systematic study of the oxidation
58 products formation of sardine oil in the presence of three commercial immobilized
59 lipases commonly used in lipid modification reactions has been carried out in the
60 temperature range where lipase catalyzed reactions usually take place. The commercial
61 lipases used, immobilized onto different supports, were Lipozyme 435, Lipozyme RM
62 and Lipozyme TL. Autoxidation kinetics of sardine oil, as a control, were first
63 determined at the temperatures selected in this work (40, 65 and 90ºC) to determine the
64 net formation rate of the primary and secondary oxidation products. Afterwards, sardine
65 oil was incubated at the same temperatures in contact with the different immobilized
66 lipases to evaluate the reduction of the oxidation products concentration in the presence
67 of these immobilized lipases.

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68 ## 2. Materials and methods

69 ### *2.1 Materials*

70 Refined sardine oil was provided by Industrias Afines S.L. (Spain). Three commercial
71 immobilized lipases were used in this work. Lipozyme 435 from *Candida antarctica* B
72 and Lipozyme TL IM from *Thermomyces lanuginosus* were kindly donated by
73 Novozymes A/S (Bagsvaerd, Denmark). Lipozyme RM IM, from *Rhizomucor miehei*,
74 was purchased from Sigma Aldrich. The support characteristics of these immobilized
75 lipases are summarized in **Table 1**. All other chemicals used in the different analyses
76 performed in this work, were of analytical or HPLC grade.

77 ### *2.2 Oxidation kinetics of sardine oil*

78 First, autooxidation kinetics of sardine oil were evaluated at three different temperatures
79 40, 65 and 90 ºC as control samples.
80 For each experiment, stoppered erlenmeyer glass flasks were filled with 70 g of refined
81 sardine oil and immediately applied a nitrogen stream in the flask. Afterwards they were
82 perfectly closed and covered with foil paper to avoid the light exposure. Sardine oil
83 samples were incubated in a water bath with orbital agitation at the corresponding
84 temperature. At different time intervals, during10 h, aliquots were withdrawn to follow
85 the oxidation kinetics.
86 When the oxidation kinetics were determined in the presence of the immobilized
87 lipases, the sardine oil was put in contact with the different immobilized commercial
88 lipases, Lipozyme 435, Lipozyme RM and Lipozyme TL, at 3.5% w/w concentration.
89 This concentration was found to be suitable for lipid modification reactions (Solaesa et
90 al., 2016). As for the autooxidation kinetics, samples were withdrawal at different time
91 intervals during 10 h.

92 All samples were stored at -18ºC up to oxidation products analysis. Duplicate
93 experiments were carried out at each temperature.

94 *2.3 Determination of primary oxidation products: peroxide value (PV)*

95 PV was determined by iodometric titration following the AOAC Official Method
96 (AOAC Official Method 965.33, 2000) by an automatic titrator Methrom 905 Titrando.
97 PV was expressed as milliequivalents of O_2 per kilogram of sample. All samples were
98 analyzed in triplicate.

99 *2.4 Determination of secondary oxidation products*

100 *2.4.1 Anisidine value (AV)*

101 The AV was measured according to AOCS Official Method (AOCS Official Method Cd
102 18-90, 2017), using a UV-Visible spectrophotometer at 350nm. All samples were
103 analyzed in triplicate.

104 *2.4.2 Thiobarbituric acid reactive substances (TBARS) assay*

105 TBARS were determined according to the spectrophotometric method described by
106 Norveel Semb in her Master's Thesis (Norveel Semb, 2012). The method is based on the
107 formation of a pink complex with strong absorbance at 532-535nm due to the presence
108 of thiobarbituric acid reactive substances (TBARS). TBARS is expressed as mg of
109 malondialdehyde (MDA) per kilogram of oil. All samples were analyzed in triplicate.

110 *2.5 Fatty acid analysis*

111 The initial sardine oil and the oil samples after 10 h of incubation at 40, 65 and 90ºC
112 were analyzed by the AOAC method (AOAC Official Method 991.39, 2000) to evaluate
113 the fatty acid profile and the polyene index (EPA+DHA/16:0). An Agilent gas
114 chromatograph (6890N Network GC System) equipped with a flame ionization detector

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115 (FID) and a fused silica capillary column (OmegawaxTM-320, 30 m x 0.32 mm i.d.)
116 was used. The method and the calibration procedure was developed previously (Solaesa
117 et al., 2016). The samples were analyzed in triplicate.

118 *2.6 Statistical analysis*

119 Statistical analyses were conducted using software Statgraphics X64. Control
120 experiments and oxidation status of sardine oil in contact with the immobilized lipases
121 were carried out in duplicate. For each oxidation product, analysis was performed in
122 triplicate. The results are presented as a mean ± standard deviation. The significance of
123 the differences was determined based on an analysis of the variance with the Tukey's
124 honestly significant difference (HSD) method at p-value ≤ 0.05. For each oxidation
125 product, significance difference has been determined among the three lipases at the
126 three operating temperatures at a specific contact time. ANOVA was also performed to
127 analyze the statistical significance of time by comparing oxidation product
128 concentration for each enzyme and temperature along time.
129 The estimation of the parameters for the models tested in this work was performed by
130 using the Marquardt algorithm (Statgraphics X64).

131 **3. Results and discussion**

132 *3.1 Chemical quality of initial sardine oil*

133 The fatty acid profile and some quality parameters of the supplied refined sardine oil
134 used in this work are presented in **Table 2**. The fatty acid profile was similar to those
135 previously reported for sardine oil (Homayooni, Sahari, & Barzegar, 2014; Noriega-
136 Rodríguez et al., 2009; Okada & Morrisset, 2007; Solaesa, Bucio, Sanz, Beltrán, &
137 Rebolleda, 2014). The polyene index (PI) of the supplied refined sardine oil used in this
138 work was 1.39 (**Table 2**) being similar to the PI value reported for sardine oil

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139 (Homayooni et al., 2014). Other important quality parameters such as acid value, PV
140 and AV for the supplied refined sardine oil were 0.45 ± 0.04 mg KOH/g, 4.8 ± 0.6 meq
141 O_2/kg and 19.7 ± 0.4, respectively. These values were very close to the limits allowed
142 by GOED Voluntary Monograph (GOED, 2015), which are 0.5 mg KOH/g for acid
143 value, 5 meq O_2/kg oil for PV and 20 for AV. Therefore, the supplied refined sardine oil
144 used in this work was partially oxidized and oxidation reaction rates could be faster due
145 to the presence in the medium of oxidation products. According to Toro-Vázquez et al.
146 (1993) the induction period was reduced as initial peroxide value increased, based on
147 their study of corn oil oxidation. The refined sardine oil used in this work presented a
148 value of 46 mg of MDA/kg. Regarding TBARS content, the maximum is not clearly
149 specified. A document by FAO establishes for fresh fish oil a TBARS value of 50 mg of
150 MDA/kg fish oil, although it is referred to fishes feed (Masson S, 1994). The induction
151 period (h) by Rancimat, determined at 70ºC and 20 L/h of air flow rate was 7.2 hours,
152 similar to that obtained by Noriega et al. (Noriega-Rodríguez et al., 2009) for crude and
153 deodorized sardine oil (10.4 and 17.7 h, respectively) using Rancimat at 60ºC and 7 L/h
154 of air flow rate.

155 *3.2 Oxidation kinetics of sardine oil at different temperatures*

156 *3.2.1 Kinetic aspects of oxidation products*

157 Peroxide values were determined over time during 10 h at 40, 65 and 90ºC (**Fig. 1a**). At
158 any incubation time, hydroperoxide content increased with incubation temperature. **Fig.**
159 **1a** also shows that peroxide value continuously increased with time during 10 h. This
160 means that hydroperoxide rate formation was still higher than hydroperoxide rate
161 decomposition in the period of time covered in this work for all the temperatures.

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162 The kinetics of lipid oxidation is not an easy task. Reactions rates are usually described
163 by a pseudo-zero, pseudo-first and pseudo second order reactions. In any case, the order
164 or the reaction rate does not comply with the stoichiometry of the reaction (Kamal-Eldin
165 & Yanishlieva, 2005). Labuza and Bergquist (Labuza & Bergquist, 1983) found that
166 lipid oxidation were half-order with respect to pure lipids in model systems. However,
167 in complex food systems the data sometimes fit zero order as well (Labuza & Bergquist,
168 1983). In this work, formation of primary oxidation products followed a pseudo-zero
169 order kinetic model:

170 $$PV = PV_o + k \cdot t \qquad [1]$$

171 where PV_o denotes the initial peroxide value and k is the reaction rate constant for a
172 zero order reaction (meq $O_2 \cdot kg\ oil^{-1} \cdot h^{-1}$). Reaction rate constants, k, were determined
173 from the slope of plotting the PV as a function of incubation time. **Table 3** lists the
174 reaction rate constant for the three temperatures assayed in this work, together with the
175 quality of the fitting. Pseudo-first and pseudo-second order kinetics were also tried, but
176 the fitting was worse than for pseudo-zero order reaction. Gomez-Alonso et al. (Gómez-
177 Alonso et al., 2004) also found that formation of primary oxidation products of olive oil
178 in the temperature range from 25 to 75ºC followed a pseudo-zero order kinetics.

179 **Fig. 1b** and **1c** show the variation of the AV and the TBARS content, respectively, over
180 time at the three temperatures studied in this work. It can be observed that, both AV and
181 TBARS content, continuously increased with time and temperature in the first 10 h of
182 incubation time, mainly at the highest temperatures, 90ºC and 65ºC. At 40ºC the
183 oxidation rates were slower and the secondary oxidation products concentration did not
184 increase in a great extent. Comparing **Fig. 1a, 1b** and **1c** it can be observed that
185 generation of secondary oxidation products was taking place simultaneously with

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186 hydroperoxides formation. In the temperature range covered in this work for 10 h of
187 incubation time, hydroperoxide formation was still higher than its decomposition into
188 secondary oxidation products. Similar behavior has been also observed for vegetables
189 oils, such as olive and rapeseed oil, in which it was observed that secondary oxidation
190 products begin to be formed nearly at the same time as hydroperoxides generation
191 (Guillen & Cabo, 2002). Gomez-Alonso et al. (Gómez-Alonso et al., 2004) proposed a
192 pseudo-first order reaction for the formation of secondary oxidation products,
193 determined as AV, in olive oil. However, similar to hydroperoxide formation, the best
194 fitting for AV and TBARS content was obtained for a pseudo-zero order reaction.
195 Reaction rate constants for this model are collected in **Table 3** together with the quality
196 of the fitting.

197 The kinetics of PUFA secondary oxidation products formation has not yet been well
198 investigated (Kamal-Eldin & Yanishlieva, 2005). From **Fig. 1b** and **1c** and the values of
199 the kinetic constant, it can be observed that TBARS content formation increased faster
200 than AV. Some authors support the theory that TBARS are formed in considerable
201 amounts mainly from PUFA's containing three or more double bonds (Hoyland &
202 Taylor, 1991). Therefore, the faster increase of TBARS content in comparison with AV
203 could be probably due to the high PUFA content of the refined sardine oil.

204 The polyene index was determined at the end of the kinetics at the three temperatures. It
205 did not change significantly compared to the initial composition of sardine oil. Small
206 variations were observed but those were within the variability associated to the
207 chromatographic method. This result agrees with the work of Sullivan et al. (Sullivan,
208 Suzanne, Jovica, & Jean, 2015) in the study of oxidation rates of triacylglycerols and
209 ethyl esters of fish oil. These authors suggested that GC-FID might not be sensitive

210 enough to detect changes in EPA and DHA concentration that could take place as a
211 consequence of oxidation.

212 *3.2.2 Thermodynamic aspects of sardine oil oxidation*

213 The dependence of the kinetic rate constant on temperature is usually represented by an
214 Arrhenius type relationship:

215 $$k = k_o \cdot \exp\left(-\frac{E_a}{RT}\right) \qquad [2]$$

216 where k is the reaction rate constant, k_o is the pre-exponential factor, E_a the activation
217 energy and R the universal gas constant. **Fig. 2a** shows the Arrhenius plot for the
218 primary and secondary oxidation products of sardine oil. The activation energy for each
219 oxidation product formation is reported in **Table 3**. The errors associated to the
220 activation energy values for each oxidation product have been evaluated through the
221 slope-error of a least square line-fitting algorithm.
222 For the hydroperoxide formation, an activation energy of 37.1 kJ/mol was obtained.
223 This value was compared with other studies found in the literature, which are
224 summarized in **Table 4**. Yin et al. (Yin & Sathivel, 2010) obtained a similar value for
225 the E_a (30.9 kJ/mol) in their study for hydroperoxides formation in the unrefined
226 menhaden oil in the temperature range from 45 to 85 ºC and Sathivel et al. (Sathivel,
227 Huang, & Prinyawiwatkul, 2008) also reported similar E_a (33.2 kJ/mol) for the
228 hydroperoxides formation in the unrefined pollock oil. **Table 4** also reports E_a for some
229 vegetable oils being more temperature-dependent than fish oils autooxidation. The
230 lower E_a for fish oils is expected because less energy should be required to initiate
231 oxidation due to the higher n-3 PUFA content compared to vegetable oils. Furthermore,
232 vegetable oils have high content of natural antioxidants, such as tocopherols (Lee, Lee,
233 & Choe, 2007).

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The dependence on temperature of the reaction rate constants of the secondary oxidation products formation followed also an Arrhenius type relationship with activation energy of 55.9 kJ/mol when they were determined as AV and 35 kJ/mol when they were determined as TBARS. The higher activation energy for the formation of non-volatile secondary oxidation products determined as AV demonstrates that is more temperature sensitive than hydroperoxides and TBARS formation. For formation of both, hydroperoxides and TBARS, similar values of E_a were obtained, according to the mechanism proposed by some authors for malonaldehyde formation from peroxides which are produced from fatty acids containing three or more double bonds (Hoyland & Taylor, 1991).

Enthalpy ($\Delta H^{\neq}$) and entropy ($\Delta S^{\neq}$) of activation were also estimated by applying the equation of Eyring (Eq. 3), by plotting ln (k/T) vs 1/T:

$$ln\frac{k}{T} = ln\frac{k_B}{h} + \frac{\Delta S^{\neq}}{R} - \frac{\Delta H^{\neq}}{RT} \quad [3]$$

where k_B is the Boltzmann constant ($1.38065 \cdot 10^{23}$ J/K) and h is the Planck's constant ($6.62608 \cdot 10^{-34}$ J·s). **Fig. 2b** shows the Eyring plot for kinetics of the primary and secondary oxidation products of sardine oil. According to units of constants in Eyring equation, units of k should be s^{-1}. However, primary and secondary oxidation products formation was good described by zero order reaction rate. Although units of Erying equation won't cancel each other, in this work, it was assumed that comparison of temperature dependence among the different oxidation products could be established since all the oxidation products determined in this work were fitted to zero order reaction rate. In any case, for the Eyring plot, time units in the reaction rate constants were expressed as s. A straight line was obtained with negative slope ($-\Delta H/R$) and a y-intercept ($\Delta S/R + \ln(k_B/h)$). Therefore, from the slope and the intercept, the values of $\Delta H^{\neq}$

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258 and $\Delta S^{\neq}$ have been calculated, and presented in **Table 3**. $\Delta H^{\neq}$ is the energy involved in
259 the process during oxidation, the positive value of $\Delta H^{\neq}$ for the primary and secondary
260 oxidation products indicated that the oxidation process is endothermic. The higher value
261 of $\Delta H^{\neq}$ for AV, compared to PV and TBARS indicated lower reactivity.

262 $\Delta S^{\neq}$ is related to the degree of disorder of a system, in a spontaneous process the energy
263 of the system tends to decrease, while the total entropy tends to increase (Silva
264 Rodrigues et al., 2017). The negative value for $\Delta S^{\neq}$, in all the oxidation reactions, was a
265 result of association mechanism between reacting species to form the transition state
266 during the reaction. The Gibb's free energy of activation ($\Delta G^{\neq}$) was calculated for each
267 temperature by the thermodynamics fundamental equation:

268 $$\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq} \quad [4]$$

269 Negative values of $\Delta G^{\neq}$ indicate a spontaneous reaction, while positive values indicate
270 nonspontaneous reaction. **Table 3** also reported the $\Delta G^{\neq}$ at the three temperatures for all
271 the oxidation kinetics. The positive values of the Gibbs's free energy of activation also
272 showed the non-spontaneity of this process. The increase of $\Delta G^{\neq}$ with temperature
273 observed was related to the endothermic oxidation mechanism (Pazhouhanmehr et al.,
274 2016).

275 The errors associated to the values of the different thermodynamic parameters presented
276 in **Table 3** have been evaluated through the slope or intercept error of a least square
277 line-fitting algorithm and in the case of $\Delta G^{\neq}$ combining the uncertainties from $\Delta H^{\neq}$ and
278 $\Delta S^{\neq}$ according to the error propagation theory.

279 *3.3 Oxidation status of sardine oil in contact with different immobilized lipases*

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280 **Table 5** presents the different PV, AV and TBARS over time for sardine oil in contact
281 with the three immobilized lipases selected in this work, at different incubation
282 temperatures, 40, 65 and 90ºC.

283 When sardine oil was in contact with the immobilized lipases, PV were much lower
284 than the values corresponding to the control samples (**Fig. 1a**) at the highest
285 temperatures studied in this work (65 and 90 ºC), being even lower than the initial PV
286 for sardine oil. This decrease in primary oxidation products can be the result of a
287 complex balance between formation, decomposition, adsorption capacity of the
288 immobilized lipase support and also reaction of primary oxidation products with the
289 own lipase. This complex balance seems to be favored by an increase in temperature
290 (**Table 5**). At 10 h of contact time, at the highest temperature, 90 ºC, PV of sardine oil
291 in contact with Lipozyme RM, immobilized on a weak base anion exchange resin
292 (Rodrigues & Fernandez-Lafuente, 2010), reached the lowest value, followed by
293 Lipozyme 435, immobilized on a macroporous resin of poly-methylmethacrylate. The
294 same trend was observed at 65 and 40ºC. However, at 40ºC the PV of sardine oil in
295 contact with the immobilized lipases was no significantly different than those reported
296 previously for oxidation of sardine oil at 40ºC in the control samples for Lipozyme RM
297 and Lipozyme 435, while for Lipozyme TL, even higher values were obtained.
298 Therefore, at 40ºC hydroperoxides formation was still probably promoted.

299 Regarding secondary oxidation products content, determined as AV and TBARS,
300 sardine oil incubated with the immobilized lipases presented also lower levels than the
301 corresponding to the control samples at the highest temperatures, 90 and 65ºC (see **Fig.**
302 **1b** and **1c**). AV was slightly lower than the initial value of the sardine oil, 19.7, for
303 samples in contact with Lipozyme RM after 10 h, 17.4 at 90ºC. For the other two

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304 immobilized lipases, AV continuously increased, although at slower rate than in the
305 control samples in the absence of immobilized lipases.

306 In the case of TBARS content, at the highest temperatures, 90 and 65ºC, much lower
307 values were obtained compared to the values reported for the control oil in the absence
308 of immobilized lipases (see **Fig 1c**). Similar to PV, oil samples in contact with
309 Lipozyme RM at 90 ºC and 65 ºC, reduced their TBARS content below the initial value
310 for sardine oil, from 46 mg MDA/kg of oil to 15 mg MDA/kg of oil, almost a 70% less.
311 On the contrary, for Lipozyme TL, at 40 ºC, values of the same order or even higher
312 were obtained than for the control sardine oil in the absence of immobilized lipases.

313 In general, the oxidation products concentration of sardine oil bulk in contact with the
314 commercial immobilized lipases assayed in this work was lower than the values
315 obtained in the autooxidation kinetics following the order Lipozyme TL > Lipozyme
316 435 > Lipozyme RM. This finding is important in the design of biocatalysis by these
317 immobilized lipase of oils rich in PUFA since high temperatures can be selected as
318 optimum due to higher reaction rates and also lower oxidation products concentration in
319 the reaction bulk. The decrease of oxidation status was also observed by Solaesa et al.
320 (2017) in a previous work on glycerolysis reaction of sardine oil catalyzed by Lipozyme
321 435 after 7 hours of reaction time. Xu et al. (2005) also observed a decrease in the
322 primary oxidation products, measured as PV, after acidolysis of fish oil with caprylic
323 acid catalyzed by Lipozyme RM IM in a packed reactor at 60ºC. These authors
324 proposed that peroxide compounds could be retained in the enzyme bed.

325 In any case, when considering high operation temperatures in biocatalysis, although it
326 could be positive from the quality product point of view, denaturation temperature of
327 the lipase must be also taking into account as well as reusability of the lipase. This

328 regard, Wang et al. (1991) in the study of transesterification reaction of triolein with
329 lauric acid catalyzed by Lipozyme IM 20 observed that, when testing the operation
330 stability of the immobilized lipase at 65 ºC over 10 number of reaction batches, 10 h
331 each batch, hydroperoxide content decreased through the repeated batches, but so did
332 the lipase activity. These authors proposed that hydroperoxide decomposition products
333 could cause inactivation of the enzyme.

334 Although, mechanism is not still clear, among the different phenomenon that could be
335 taking place, adsorption of the oxidation products on the lipase support could play an
336 important role. Lipozyme RM is immobilized onto a weak-base anion exchange resin,
337 having good capability for adsorption process. Its hydrophilic nature could result in a
338 higher affinity for hydrophilic oxidation products. On the contrary, Lipozyme 435 was
339 immobilized onto a hydrophobic support (Chen et al., 2008). The secondary oxidation
340 products, as well as hydroperoxides, are considered polar molecules and, it is well
341 known that the higher the degree of oxidation of an oil, the more polar the oxidation
342 products usually are (Kamal-Eldin & Yanishlieva, 2005). This could partially explain
343 the difference observed among both immobilized lipases. In case of Lipozyme TL,
344 although it was immobilized on silica gel, a hydrophilic material, its particle size range
345 was bigger (250-1000 µm) as well as the true density of the support (1830 kg/m^3,
346 Zhang, 2007). Therefore, its surface area was considerably lower. In **Table 1** it can be
347 observed that the average value of Lipozyme RM particle size was smaller (200~600
348 µm) than the particle size of the others two immobilized lipases (300-1000 µm).
349 Therefore, the adsorption could be increase due to a higher frequency of collisions
350 between adsorbate and adsorbent. In any case, it must be highlighted that the average
351 particle of these immobilized lipases was much higher than the particle size for

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352 commercial absorbents such as Tonsil® bleaching earths which range from 25 to 150
353 µm (Strieder et al., 2017).

354 The oxidation status for sardine oil in contact with the immobilized lipases ($C_{immob\text{-}lipase}$)
355 has been related to the corresponding data for the control sardine oil previously
356 determined in section 3.2 at the same temperature and incubation times ($C_{control}$), $C_{immob\text{-}lipase}/C_{control}$,
357 for each type of oxidation product. **Figures 3a, 3b, 4a, 4b, 5a** and **5b** show
358 the ratio $C_{immob\text{-}lipase}/C_{control}$ for the PV, AV and TBARS level change at 90 and 65ºC,
359 respectively for the three immobilized lipases. The oxidation status of sardine oil in
360 contact with the immobilized lipases at 40ºC showed no difference with that of the
361 sardine oil in the absence of immobilized lipases, or even slightly higher oxidation
362 status. Therefore, the results have not been plotted. For the three immobilized lipases
363 tested in this work at 65 and 90ºC, the ratio $C_{immob\text{-}lipase}/C_{control}$ decreased over time.
364 Based on the shape of the **Fig. 3-5** the following equation has been used to correlate the
365 data over time:

366 $$C_{immob-lipase,t}/C_{control,t} = \exp(-kt^{n}) \quad [5]$$

367 where $C_{immob\text{-}lipase,t}$ is the concentration of oxidation compounds in sardine oil in contact
368 with immobilized lipases at a certain contact time, t, $C_{control,t}$ is the concentration of
369 oxidation compounds in control sardine oil (in the absence of immobilized lipases) at
370 the same incubation time, t, n is an adjustment parameter and k is an empirical constant.
371 This equation was based on the modified model of Brimberg proposed by Monte et al.
372 (Monte et al., 2015) in the study of reduction of color and oxidation products of carp oil
373 with blends of bleaching earth and activated carbon. The dependence of k parameter on
374 temperature was assumed to follow an Arrhenius type relationship (Eq. 2), although

375 only the highest temperatures, 90 and 65º C were considered in the fitting procedure.
376 **Table 6** lists the parameters obtained for equation 5 to relate the oxidation products
377 concentration in contact with the three immobilized lipases and the control oil for the
378 three measured parameters, PV, AV and TBARS content. For all the oxidation products,
379 the highest E_a was found for Lipozyme TL proving that this immobilized lipase is more
380 temperature-dependent on the reduction of oxidation products in the bulk oil. The
381 continuous lines in **Fig. 3-5** represent the equation 5, showing good fitting.

382 ## 4. Conclusions

383 The oxidation kinetics of sardine oil revealed that hydroperoxide and TBARS formation
384 was higher than that of secondary oxidation products determined as AV, due to the high
385 PUFA content. Oxidation status was lower in the presence of three commercial
386 immobilized lipases, in the same temperature range, especially at high temperatures, 65
387 and 90ºC. However, at 40ºC, oxidation products formation seemed to be still promoted.
388 Lipozyme RM yielded the lower oxidation indices, quite below to those of the initial
389 fish oil.

390 Although exact mechanism of reduction of oxidation products in the presence of
391 commercial immobilized lipases is not yet clear. It is an important finding since high
392 temperatures are usually avoided when dealing with lipid modification of fish oil due to
393 its high content of PUFA, very prone to oxidation. By using these type of lipases,
394 temperatures, at least, up to 90ºC can be used having a double benefit of temperature;
395 on one hand higher reaction rates and, on the other hand lower oxidation status.
396 However, reusability of the enzyme should be further considered.

397 **Acknowledgements**

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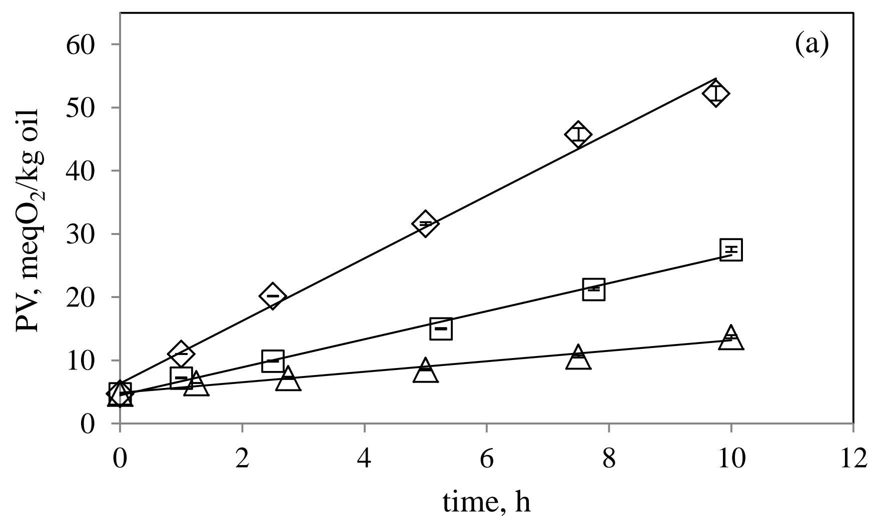

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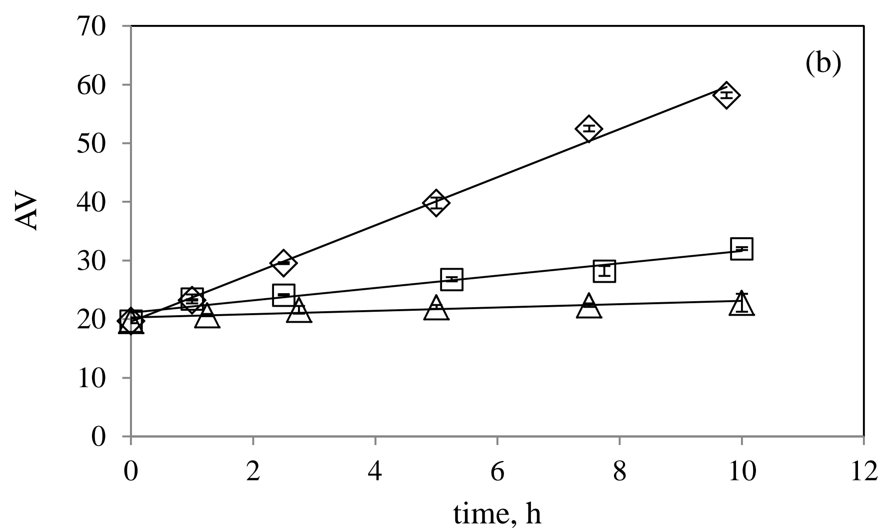

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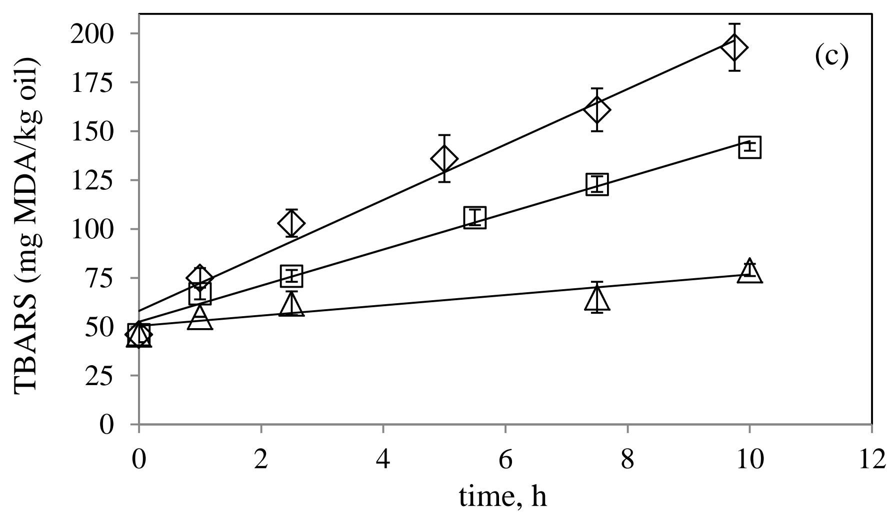

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498 **Fig. 1**. (a) PV, (b) AV and (c) TBARS of sardine oil during incubation time at 40ºC (Δ),
499 65ºC (□) and 90ºC (◊)**.** Continuous lines represent the pseudo-zero order kinetic model (Table
500 3).

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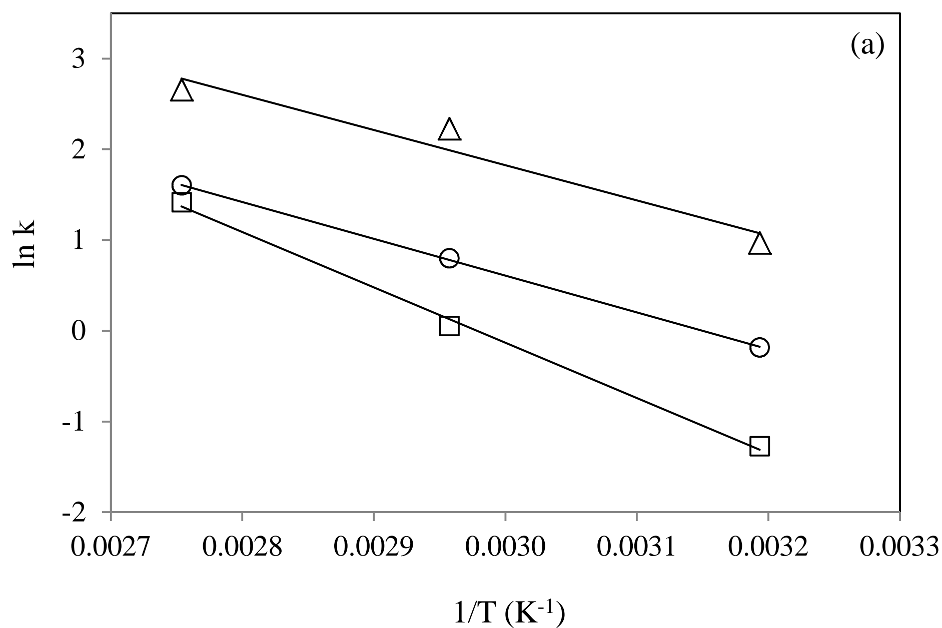

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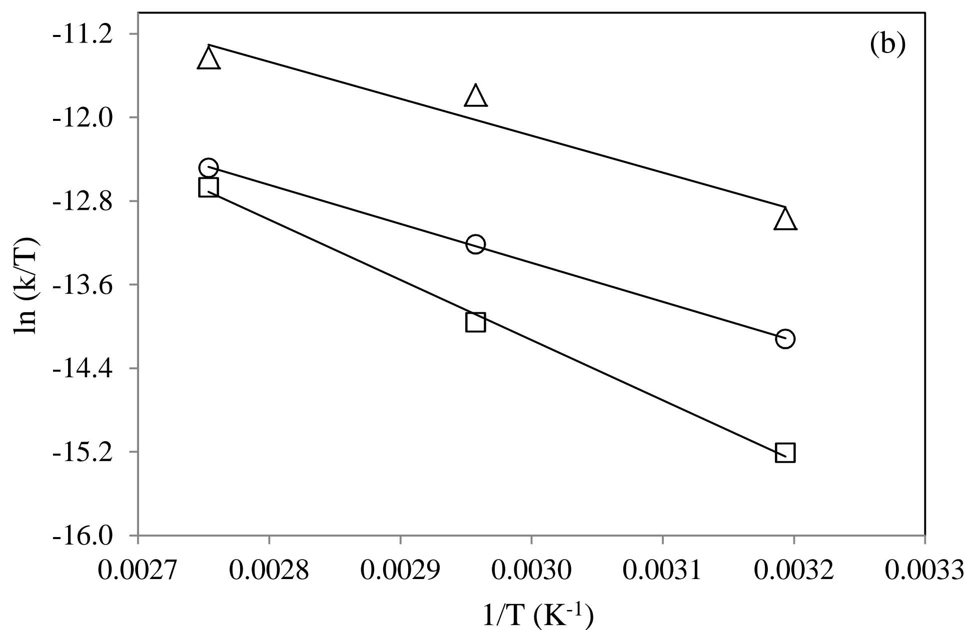

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505 **Fig. 2.** (a) Arrhenius plot and (b) Eyring plot of the oxidation of sardine oil, PV (○), AV (□),
506 TBARS (Δ). Units of k are listed in Table 3. Time units in Eyring plot for k values have been
507 expressed as s.

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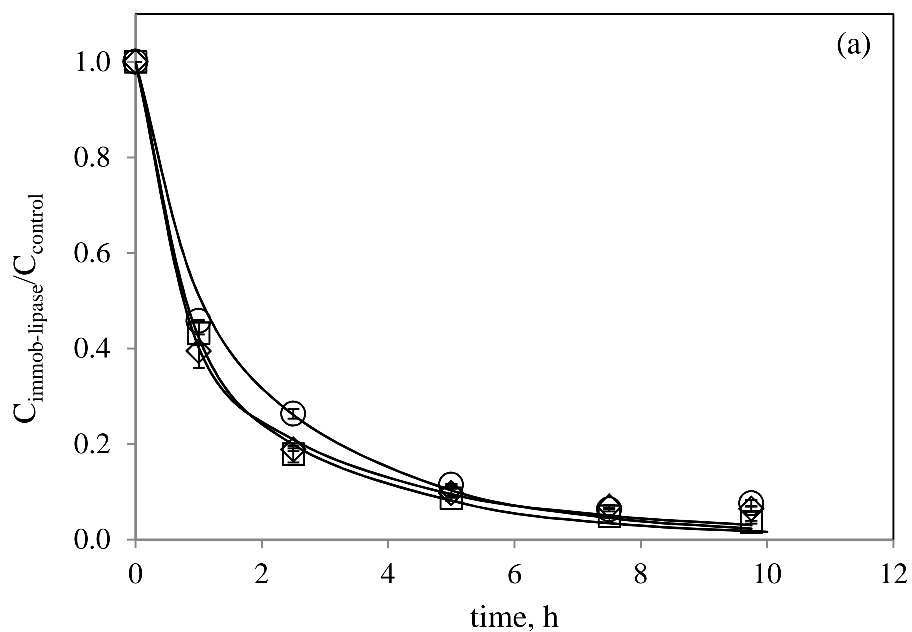

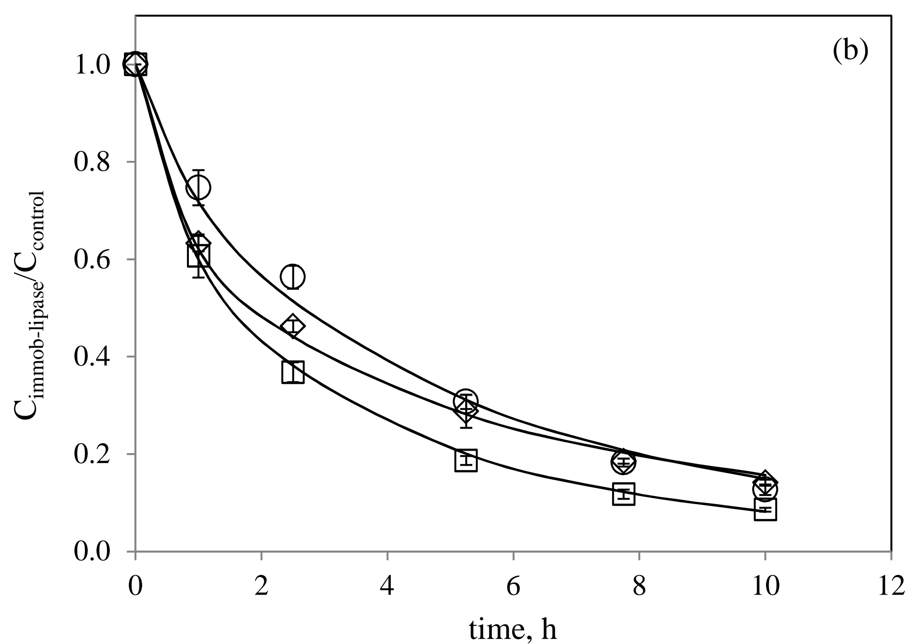

514 – 533

534 **Fig. 3.** PV ratio between samples in contact with the immobilized lipases and control, (◊)
535 Lipozyme 435, (□) Lipozyme RM, (○) Lipozyme TL, (a) at 90ºC and (b) 65ºC. Continuous
536 lines represent the values obtained by the equation 5.

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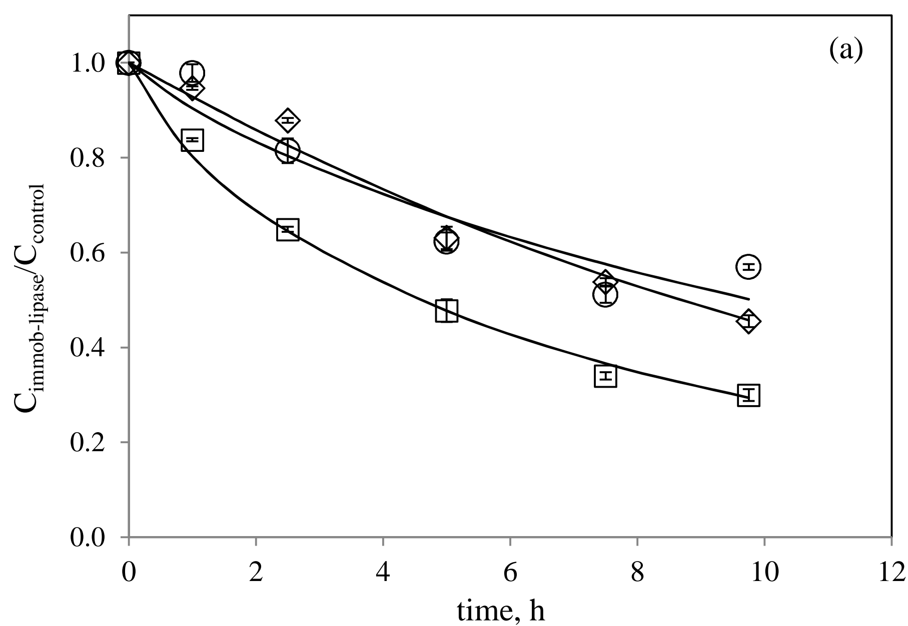
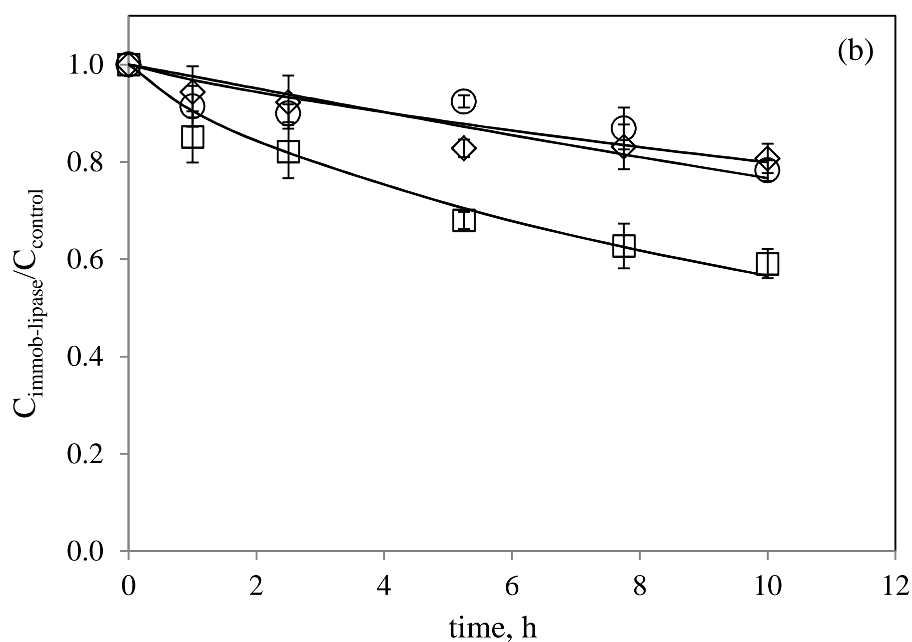
539 

549 

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560 **Fig. 4.** AV ratio between samples in contact with the immobilized lipases and control, (◊)
561 Lipozyme 435, (□) Lipozyme RM, (○) Lipozyme TL, (a) at 90ºC and (b) 65ºC. Continuous
562 lines represent the values obtained by the equation 5.

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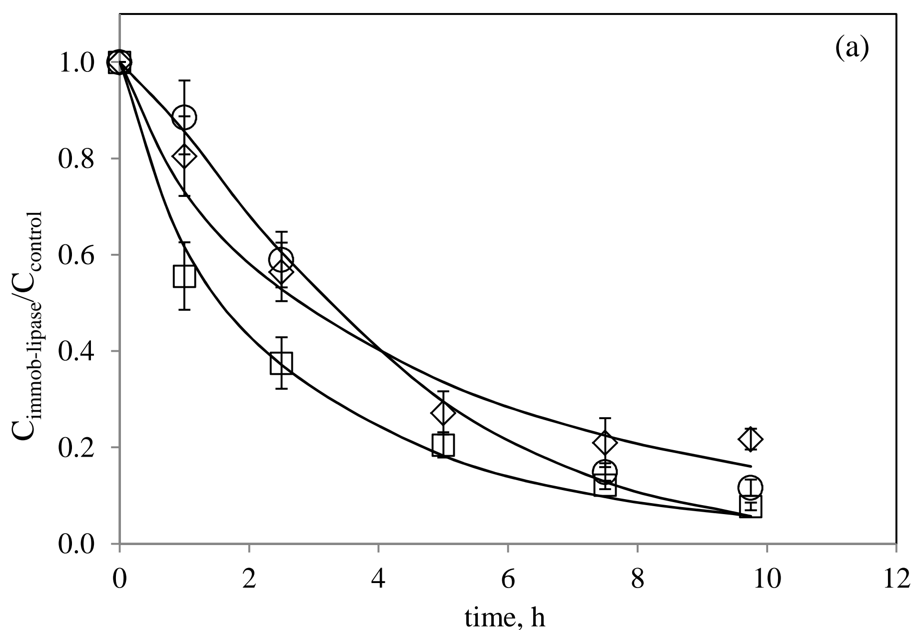

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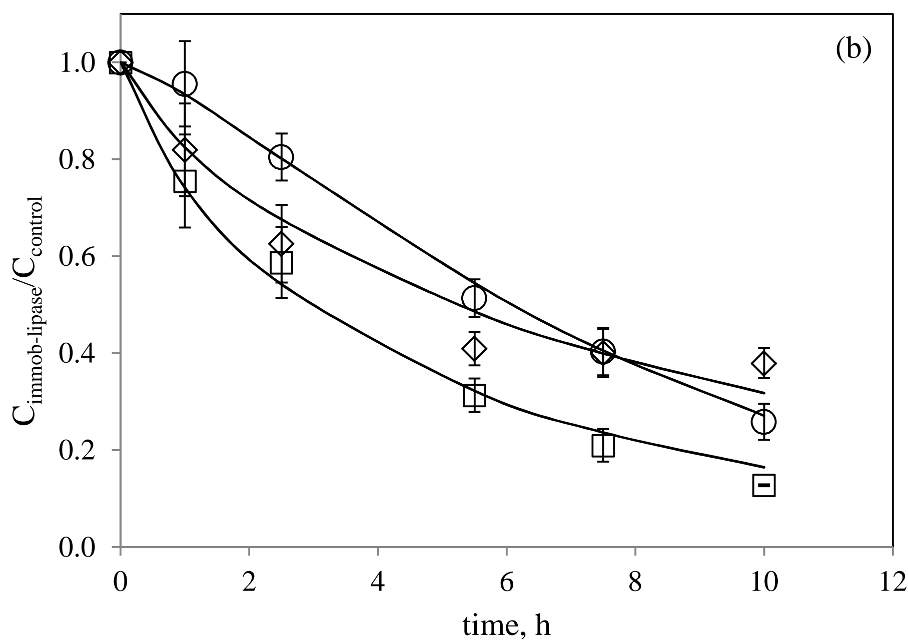

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589 **Fig. 5.** TBARS ratio between samples in contact with the immobilized lipases and control, (◊)
590 Lipozyme 435, (□) Lipozyme RM, (○) Lipozyme TL, (a) at 90ºC and (b) 65ºC. Continuous
591 lines represent the values obtained by the equation 5.

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597 **Table 1.** Characteristics of the immobilized commercial lipases used in this work.

Immobilized lipase	Support	Particle size	Reference
Lipozyme 435 *(Candida antarctica* B)	Macroporous resin of poly-methyl methacrylate (Lewatit VP OC 1600)	315~1000 µm	(Chen et al., 2008)
Lipozyme RM (*Rhizomucor miehei)*	Weak base anion-exchange resin based on phenol–formaldehyde copolymers (Duolite ES 562)	200~600 µm	(Rodrigues & Fernandez-Lafuente, 2010)
Lipozyme TL (*Thermomyces lanuginosus)*	Granulated silica	250~1000 µm	(Zhang, 2007)

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618 **Table 2.** Fatty acid composition and chemical characteristics of the
619 refined sardine oil used in this work.

Fatty acid profile (wt%)	C14:0	6.3 ± 0.2
	C16:0	17.3 ± 0.3
	C16:1n-7	8.4 ± 0.1
	C16:2n-4	1.1 ± 0.0
	C16:3n-4	1.2 ± 0.0
	C16:4n-1	2.1 ± 0.0
	C18:0	3.7 ± 0.0
	C18:1n-9	13.9 ± 0.1
	C18:1n-7	3.7 ± 0.0
	C18:2n-6	2.9 ± 0.0
	C18:4n-3	2.1 ± 0.0
	C20:1n-9	2.6 ± 0.0
	C20:3n-3	1.1 ± 0.0
	C20:5n-3	15.7 ± 0.3
	C22:1n-11	1.9 ± 0.0
	C22:5n-3	1.9 ± 0.1
	C22:6n-3	8.2 ± 0.3
	Others <1%	6.0 ± 0.1
PUFA %		39.6 ± 0.8
n3 %		31.2 ± 0.7
EPA+DHA		23.9 ± 0.6
Polyene index		1.39 ± 0.01
Acid value (mg KOH/kg)*		0.45 ± 0.04
Peroxide value (meqO_2/kg)		4.8 ± 0.6
Anisidine value		19.7 ± 0.4
TBARS (mg MAD/kg oil)		46 ± 4
Induction period (h) by Rancimat (70 ◦C)		7.2 ± 0.7

620 *According to AOCS, Ca 5a-40.

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Table 3. Kinetic rate constant (k), activation energy (E_a), enthalpy ($\Delta H^{\neq}$), entropy ($\Delta S^{\neq}$) and Gibb's free energy ($\Delta G^{\neq}$) of activation for primary and secondary oxidation of the sardine oil at different temperatures

	PV			AV			TBARS		
T, ºC	k (meqO_2/kg oil·h)	R^2	$\Delta G^{\neq}$ (kJ·mol^{-1})	k (h^{-1})	R^2	$\Delta G^{\neq}$ (kJ·mol^{-1})	k (mg MAD/kg oil·h)	R^2	$\Delta G^{\neq}$ (kJ·mol^{-1})
40	0.831 ± 0.06	0.9797	98.5 ± 4	0.280 ± 0.05	0.8690	101.4 ± 1	2.631 ± 0.60	0.8669	95.2 ± 19
65	2.217 ± 0.09	0.9933	103.6 ± 4	1.053 ± 0.13	0.9444	105.2 ± 1	9.245 ± 0.53	0.9869	100.2 ± 20
90	4.951 ± 0.23	0.9912	108.8 ± 4	4.109 ± 0.15	0.9944	109.1 ± 1	14.202 ± 1.04	0.9791	105.3 ± 21
E_a (kJ·mol^{-1})	37.1 ± 2			55.9 ± 0.5			35± 10		
$\Delta H^{\neq}$ (kJ·mol^{-1})	34 ± 2			53 ± 1			32 ± 10		
$\Delta S^{\neq}$ (J·mol^{-1}·K^{-1})	-206 ± 6			-155 ± 2			-202 ± 30		

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Table 4. Activation energy from the Arrhenius equation for the formation of hydroperoxides in different kind of oils found in literature.

Type of oil	T range (ºC)	E_a, kJ/mol	Reference
Refined sardine oil	40 - 90	37.1	This work
Unrefined menhaden oil	45 - 85	30.9	(Yin & Sathivel, 2010)
Unrefined pollock oil	24 - 90	33.2	(Sathivel et al., 2008)
Refined soybean oil		73.6	
Refined sunflower oil	25 - 80	79.5	(Lee et al., 2007)
Virgin olive oil		52.3	

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Table 5. Oxidation products concentration for sardine oil samples in contact with Lipozyme 435, Lipozyme RM and Lipozyme TL at 40, 65 and 90ºC at different incubation times.

Oxidation Index	Immobilized lipase	T (ºC)	Contact time (h)				
			1	2.5	5	7.5	10
PV	Lipozyme 435	90	$^{B}4.3 \pm 0.4_{a}$	$^{AB}3.8 \pm 0.1_{ab}$	$^{A}3.1 \pm 0.1_{ab}$	$^{A}3.1 \pm 0.1_{ab}$	$^{A}3.3 \pm 0.3_{b}$
		65	$^{A}4.6 \pm 0.1_{a}$	$^{A}4.6 \pm 0.1_{bc}$	$^{A}4.3 \pm 0.5_{cd}$	$^{A}3.9 \pm 0.1_{b}$	$^{A}3.9 \pm 0.1_{c}$
		40	$^{A}4.6 \pm 0.2^{a}$	$^{B}5.2 \pm 0.0^{cd}$	$^{C}6.5 \pm 0.0^{e}$	$^{B}5.3 \pm 0.5^{c}$	$^{AB}5.0 \pm 0.1^{d}$
	Lipozyme RM	90	$^{B}4.8 \pm 0.3_{a}$	$^{AB}3.6 \pm 0.3_{a}$	$^{A}2.7 \pm 0.2_{a}$	$^{A}2.2 \pm 0.1_{a}$	$^{A}1.9 \pm 0.1_{a}$
		65	$^{C}4.4 \pm 0.3_{a}$	$^{B}3.6 \pm 0.2_{a}$	$^{A}2.9 \pm 0.1_{ab}$	$^{A}2.5 \pm 0.2_{a}$	$^{A}2.4 \pm 0.1_{a}$
		40	$^{A}4.6 \pm 0.1_{a}$	$^{A}5.4 \pm 0.2_{cd}$	$^{B}8.3 \pm 0.3_{f}$	$^{B}7.4 \pm 1.0_{d}$	$^{A}5.1 \pm 0.4_{d}$
	Lipozyme TL	90	$^{C}5.0 \pm 0.0_{a}$	$^{C}5.3 \pm 0.2_{cd}$	$^{B}3.6 \pm 0.0_{bc}$	$^{A}2.9 \pm 0.1_{ab}$	$^{B}4.0 \pm 0.3_{c}$
		65	$^{C}5.4 \pm 0.2_{a}$	$^{C}5.6 \pm 0.2_{d}$	$^{B}4.6 \pm 0.2_{d}$	$^{A}3.9 \pm 0.1_{bc}$	$^{A}3.5 \pm 0.2^{bc}$
		40	$^{A}5.2 \pm 0.0_{a}$	$^{C}11.9 \pm 0.7_{e}$	$^{D}15.0 \pm 0.1_{g}$	$^{C}11.9 \pm 0.5^{e}$	$^{B}9.1 \pm 0.0_{e}$
AV	Lipozyme 435	90	$^{A}22.0 \pm 0.0_{de}$	$^{BC}26.0 \pm 0.0_{e}$	$^{B}25.1 \pm 0.4_{d}$	$^{D}28.2 \pm 0.2_{e}$	$^{C}26.5 \pm 0.5_{c}$
		65	$^{A}22.1 \pm 0.5_{de}$	$^{A}22.3 \pm 1.2_{cd}$	$^{A}22.2 \pm 0.2_{c}$	$^{AB}23.4 \pm 0.6_{bc}$	$^{B}25.9 \pm 0.8_{c}$
		40	$^{A}21.0 \pm 0.4_{cd}$	$^{AB}21.5 \pm 0.3_{bc}$	$^{AB}21.6 \pm 0.2_{c}$	$^{AB}21.5 \pm 0.7_{b}$	$^{B}22.1 \pm 0.9_{b}$
	Lipozyme RM	90	$^{B}19.5 \pm 0.4_{ab}$	$^{AB}19.2 \pm 0.7_{a}$	$^{AB}19.0 \pm 0.3_{ab}$	$^{AB}17.9 \pm 0.7_{a}$	$^{A}17.4 \pm 0.2_{a}$
		65	$^{B}20.0 \pm 0.3_{bc}$	$^{B}19.8 \pm 0.3_{ab}$	$^{A}18.2 \pm 0.1_{a}$	$^{A}17.7 \pm 0.7_{a}$	$^{AB}18.9 \pm 0.5_{a}$
		40	$^{A}18.8 \pm 0.1_{a}$	$^{A}19.4 \pm 0.2_{a}$	$^{A}19.7 \pm 0.1_{b}$	$^{A}18.2 \pm 1.5_{a}$	$^{A}19.2 \pm 0.0_{a}$
	Lipozyme TL	90	$^{A}22.8 \pm 0.1_{e}$	$^{A}24.1 \pm 0.1_{de}$	$^{AB}24.8 \pm 0.1_{d}$	$^{B}26.8 \pm 0.7_{de}$	$^{C}33.1 \pm 1.2_{d}$
		65	$^{A}21.4 \pm 0.3_{d}$	$^{A}21.7 \pm 0.2_{bc}$	$^{B}24.8 \pm 0.5_{d}$	$^{B}24.5 \pm 0.6_{cd}$	$^{B}25.1 \pm 0.5_{c}$
		40	$^{A}21.4 \pm 0.5_{d}$	$^{A}22.0 \pm 0.3_{c}$	$^{A}23.1 \pm 0.8_{c}$	$^{A}22.3 \pm 0.5_{bc}$	$^{A}22.4 \pm 0.2_{b}$
TBARS	Lipozyme 435	90	$^{B}60 \pm 4_{cd}$	$^{B}58 \pm 4_{cd}$	$^{A}37 \pm 3_{ab}$	$^{A}34 \pm 6_{b}$	$^{A}42 \pm 2_{b}$
		65	$^{A}55 \pm 4_{abcd}$	$^{A}48 \pm 4_{abc}$	$^{A}43 \pm 2_{bc}$	$^{A}49 \pm 5_{c}$	$^{A}54 \pm 4_{c}$
		40	$^{AB}57 \pm 3_{bcd}$	$^{AB}56 \pm 3_{bcd}$	$^{A}54 \pm 0_{c}$	$^{AB}64 \pm 0_{d}$	$^{B}70 \pm 2_{d}$
	Lipozyme RM	90	$^{D}42 \pm 4_{a}$	$^{CD}39 \pm 4_{a}$	$^{BC}28 \pm 1_{a}$	$^{AB}20 \pm 0_{a}$	$^{A}15 \pm 1_{a}$
		65	$^{C}51 \pm 4_{abc}$	$^{C}45 \pm 4_{ab}$	$^{B}33 \pm 2_{ab}$	$^{A}22 \pm 3_{ab}$	$^{A}14 \pm 0_{a}$
		40	$^{A}43 \pm 3_{ab}$	$^{B}59 \pm 3_{cd}$	$^{CD}73 \pm 4_{d}$	$^{D}85 \pm 2^{e}$	$^{BC}69 \pm 3_{d}$
	Lipozyme TL	90	$^{B}66 \pm 3_{d}$	$^{B}61 \pm 4_{d}$	$^{A}30 \pm 1_{a}$	$^{A}24 \pm 1_{ab}$	$^{A}22 \pm 2_{a}$
		65	$^{C}64 \pm 3_{cd}$	$^{BC}61 \pm 1_{d}$	$^{BC}54 \pm 2_{c}$	$^{B}50 \pm 4_{cd}$	$^{A}37 \pm 5_{b}$
		40	$^{A}52 \pm 9_{abcd}$	$^{BC}110 \pm 11_{e}$	$^{D}143 \pm 7_{e}$	$^{C}120 \pm 5_{f}$	$^{B}105 \pm 2_{e}$

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

Values with different capital letters in the same row for each lipase at a certain incubation temperature are significantly different when applying the Tukey's honestly significant difference (HSD) method at p-value ≤ 0.05.

Table 6. Parameters of equation 5 with an Arrhenius type dependence on temperature for k parameter for oxidation products quantified by PV, AV and TBARS for the samples in contact with Lipozyme 435, Lipozyme RM and Lipozyme TL.

Immobilized lipase	PV				AV				TBARS			
	k_o	E_a, kJ/mol	n	R^2	k_o	E_a, kJ/mol	n	R^2	k_o	E_a, kJ/mol	n	R^2
Lipozyme 435	$5.9 \cdot 10^3$	26.5	0.59	0.998	$2.3 \cdot 10^5$	45.2	1.03	0.968	$2.2 \cdot 10^2$	19.9	0.78	0.973
Lipozyme RM	$9.6 \cdot 10^2$	21.2	0.68	0.999	$8.9 \cdot 10^4$	32.03	0.76	0.989	$3.2 \cdot 10^2$	19.6	0.78	0.993
Lipozyme TL	$9.0 \cdot 10^3$	28.7	0.76	0.992	$5.3 \cdot 10^5$	46.7	0.84	0.919	$1.0 \cdot 10^4$	33.5	1.28	0.995

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Highlights

- PV and TBARS increase faster than AV in the autoxidation of sardine oil.
- Oxidation products concentration decreased in the presence of immobilized lipases.
- The decrease was higher for PV and TBARS than anisidine reacted compounds.
- Lipozyme RM presented the best results to obtain lower oxidation indices.

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