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Optimization of pure linoleic acid 13-HPX production by enzymatic reaction pathway: unravelling oxygen transfer role

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Highlights

- _ Relevant determination of SBLOX1 kinetic parameters taking into account k_{La} .
- _ Sufficient oxygen supply leads to pure LA 13-HPX production.
- _ The use of k_{La} and Damköhler number is essential to optimize SBLOX-1 reactor.
- _ The enzymatic reactor allows a green production of 13-HPX with high added-value.

Abstract

This work is, to our knowledge, the first to have investigated the role of gas-liquid oxygen transfer on the determination of the kinetic parameters of an oxidase, and on oxidase reactor sizing for pure hydroperoxides production using soybean lipoxygenase-1. The addition of a small amount of ethanol (2% v/v) was found suitable for simple linoleic acid handling and probably a better dispersion of the latter in the reaction medium. Then, the kinetic parameters of the enzyme in a pH 9 buffered medium were determined and compared to the literature data. Following a simple methodology which accounts for gas-liquid oxygen transfer, the dissolved oxygen kinetics were reprocessed to correct the initial reaction rates. k_{La} was thus found essential to determine accurate reaction rates. The latter were found consistent with the hydroperoxide production and linoleic acid consumption rates, confirming that the data reprocessing method was necessary. This sheds new light on several literature results on lipoxygenase activity characterizations, where a discrepancy is often observed between the apparent oxygen kinetics and the hydroperoxide production rates. Based on these results and the Damköhler number (Da) in relation to oxygen uptake and transfer, a reactor sizing procedure was proposed and led to the almost full conversion of linoleic acid into pure 13-hydroperoxide.

Keywords

Oxygen transfer; Lipoxygenase; Linoleic acid; Oxidation; 13-Hydroperoxide; Reactor sizing.

Graphical abstract

1. Introduction

Oxidation of poly-unsaturated fatty acids (PUFAs) has been widely studied for decades [1], particularly the first aerobic step of the oxidation reaction cascade, leading to the production of fatty acid hydroperoxides (HPX). These molecules have an important contribution in flavor formation and taste modification of food [2]. They are commonly used as analytical standards for lipid characterization and as reaction precursors for the lipid oxidation investigations, or for controlled flavor synthesis [3-5]. Fatty acid hydroperoxides are also used to investigate the cyto-toxicity and pathogenicity of lipid oxidation in mammals [6, 7].

Currently, commercial PUFA hydroperoxides are produced by chemical synthesis and are moderately pure. Linoleic acid (LA – *cis,cis*-9,12-octadecadienoic acid) is the most commonly used PUFA for lipid oxidation investigations in the literature [4, 8]. Its chemical oxidation in the presence of oxygen and metal ions (such as Fe^{2+}) leads to a mixture of 13- and 9-linoleic acid hydroperoxides, with *cis-trans* and *trans-trans* conjugated dienes for the first one and *trans-cis* and *trans-trans* conjugated dienes for the second one [9, 10]. Consequently, most of the commercialized fatty acid hydroperoxide standards are composed of a mixture of these isomers. In order to obtain a high purity isomer, complementary purification steps are required to separate the isomers and the resulting product is thus extremely expensive.

To simplify the production of a pure hydroperoxide isomer and reduce its cost, an enzymatic production by the widespread enzymes lipoxygenases (LOX - EC 1.13.11.12; iron as co-factor) can be considered, given the high region-selectivity and enantio-selectivity of the lipoxygenase. After enzymatic oxidation of linoleic acid, a mixture of 9 and 13 – hydroperoxides is obtained. However, according to the isoform activity and the ionization of the considered PUFA, some of these enzymes can produce a specific isomer[11].

In the case of linoleic acid oxidation by soybean lipoxygenase-1 (SBLOX-1, the most used LOX for HPX formation investigation), it is well established that pH has an important effect on the isomeric

composition of the hydroperoxides produced. As a matter of fact, the pKa for linoleic acid was experimentally determined at 7.9 by Bild *et al.* [12]. At pH higher than this pKa value, most of the linoleic acid is in its linoleate form and SBLOX-1 is known to favor the formation of the 13-hydroperoxy-*cis*-9-*trans*-11 form [11, 13, 14] (Figure 1). At pH 9, the most commonly used pH in SBLOX-1 reaction investigations, this leads to 92.6% of the linoleate form. Moreover, most of literature data show that the 13-*cis*-*trans*-hydroperoxide is formed at pH 9 with SBLOX-1 at a purity higher than 90% [13, 15, 16].

Additionally, this reaction also requires non-limiting amounts of dissolved oxygen in the reaction medium to occur. The lack of dissolved oxygen switches the reaction pathway to an anaerobic (non-enzymatic) pathway, generating dimers, oxodienes, hydroxyacids and *n*-pentane (see Figure S.1 in the Supplementary Information for reaction pathways). The hydroperoxides present in the medium are then used as substrates for anaerobic reactions [1]. Therefore, if an enzymatic oxidation of linoleic acid to produce high purity hydroperoxides is considered, a sufficient supply of oxygen must be provided. However, several published studies show enzymatic oxidation kinetics with different lipoxygenases for which the reactions are only monitored through (i) either the consumption of PUFA (by HPLC analyses) [17]; (ii) or hydroperoxides synthesis (by UV absorbance measurements) [16, 18-20], regardless oxygen availability.

Oxidation kinetics are seldom monitored through the dissolved oxygen (DO) evolution [6]. However, the change in DO is a balance between enzymatic oxidation (Oxygen Uptake Rate, OUR) and dissolution of oxygen from the atmosphere into the liquid medium (Oxygen Transfer Rate, OTR). In the above-mentioned investigations, the oxygen transfer from atmosphere into liquid phase was not taken into account during the oxidation reaction. Consequently, the OUR may have been underestimated. The consideration of this phenomenon is particularly important when initial LA amount and/or the studied enzymatic activity are high. Likewise, Aziz *et al.* [5] demonstrated that increasing the stirring speed leads to an improvement of the reaction velocity. The authors attributed this result to a better homogenization of LA substrate. It would be interesting to check if this is not rather the result of a better oxygen transfer with the increase of stirring speed.

Consequently, most of the available investigations lead to an apparent affinity constant value of the enzyme for PUFAs and rarely investigate its affinity for oxygen: only Glikmann and Klinman [6] and Knapp [11] experimentally determined the affinity constant of SBLOX-1 towards oxygen. Under the same experimental conditions (i.e. in 0.1 M borate buffer pH 9), they found a value between 33.3 μ M and 40.5 μ M and a value of 11 μ M respectively (values recalculated from the data in the article). Both studies performed the dissolved oxygen monitoring with a Clark electrode, but neither considered the impact of oxygen transfer from ambient air during stirring on their recorded curves. The obtained oxygen uptake rates are then underestimated in both cases. This not only makes it difficult to extract the real kinetic parameters of the used enzymes but also complicates the comparison between the different results available in the literature, as the oxygen transfer conditions always differ from one experimental device to another.

Generally, this leads to an unwise sizing of the enzymatic production of the fatty acid hydroperoxides. Gardner *et al.* [21] reported this issue from the work of Glikman and Klinman [6] without explanation. Indeed, Glikman and Klinman [6] compared the reaction constants of linoleic acid oxidation by SBLOX-1, measured spectrophotometrically (HPX production) and with a Clark electrode (oxygen consumption). They noticed that both values did not match (they differed by an order of magnitude of 3). They attributed this difference to the stirring, which leads to an underestimation of the reaction kinetics measured by the Clark electrode compared to those measured by UV-spectra. Authors did not give a robust explanation. Anyhow, they did not attribute it to oxygen transfer at the gas/liquid interface, but suggested that premicellar fatty acid aggregates are probably the preferred substrate for LOX and are disrupted by the force of stirring. A similar observation was reported by Pourplanche *et al.* [15] without suitable explanation. Although this issue is valid for all oxidases, only Van Hecke *et al.* [22] have appropriately considered oxygen transfer kinetics to study enzymatic oxidation of saccharides.

In the present work, SBLOX-1 is used to oxidize linoleic acid to the 13-hydroperoxide isomer in sodium tetra borate buffer (pH 9) at 5 °C. The choice of low temperature is justified by a better solubility of

oxygen, especially since Elshof *et al.* [16] have demonstrated that the oxidation of LA by LOX is not negatively affected by the decrease of temperature. They even demonstrated that, over a temperature range of 5 °C to 40 °C, the highest oxidation rate is obtained at 5 °C. Additionally, several investigations have shown that SBLOX-1 remains active in certain organic solvent media[23], of which ethanol is the least toxic. It is well known that the presence of ethanol in an aqueous medium significantly reduces the surface tension [24] and thus improves the transfer of oxygen from the gas to the aqueous media[25]. Moreover, LA is easier to handle when dissolved in ethanol. Therefore, in the present work, the effect of ethanol on the SBLOX-1 activity is also studied and a suitable amount is used in the reaction medium. After the characterization of SBLOX-1 kinetics towards LA, the main aim of this work is to propose a simple and economical methodology to monitor the enzymatic production reaction of pure LA 13-hydroperoxide based on the systematic characterization of both oxygen transfer and uptake kinetics.

2. Material and Methods

2.1. Chemicals

All chemicals used were listed in Table 1. All solutions were prepared in ultrapure water. The soybean lipoyxygenase-1 solution was prepared in sodium tetraborate buffer 0.1 M pH 9 at 4 mg.mL⁻¹ and stored at -20 °C as 1 mL aliquots until use. The enzyme used contains mainly SBLOX isoform 1 (SBLOX-1) representing 75% of the total activity, and isoform 2 (SBLOX-2) representing about 25% of the total activity. At pH 9, SBLOX-1 expresses 100% of its activity while SBLOX-2 expresses only around 1% [26]. Moreover, according to the supplier, the enzyme preparation is free of other enzyme activities such as lipase and peroxidase. Consequently, all reactions presented in the present work and occurring at pH 9 can be attributed to SBLOX-1 isoform.

To facilitate the accurate addition of the substrate to the reaction medium, a concentrated ethanoic solution of LA was prepared at 500 mM and stored at -20 °C. This high concentration was useful to minimize the change in the amount of ethanol in the reaction medium upon addition of the substrate.

Indeed, for LA concentrations from 20 μ M to 100 μ M in the reaction medium, the volumes of ethanol added were of 10 μ L to 100 μ L, in a total reaction volume of 500 mL. This leads to a percentage of ethanol of 0.002% to 0.02% (v/v) in the reaction medium, which can be neglected for its impact on the volumetric transfer coefficient of oxygen at the gas-liquid interface (k_{La}).

2.2. Enzymatic oxidation set up and monitoring

Enzymatic reactions were performed in a 500 mL jacketed reactor with magnetic stirring, equipped with a Clark electrode (Heito BOT2, adapted for temperature effect correction) connected to an OSD23 oximeter for measuring dissolved oxygen (DO). DO data were recorded with the Data Studio 1.9.8r6 software.

The reaction medium was a 0.1 M sodium tetraborate pH 9 buffer containing different amounts of ethanol to improve substrate solubility and facilitate LA addition. All enzymatic reactions were monitored at 5 °C. Soybean lipoxygenase-1 solution was used at 0.1% v/v in the reaction medium, unless otherwise stated.

Based on the pKa value of LA of 7.9 determined experimentally by Bild *et al.* [12], 92.5% of linoleic acid is in its linoleate form (sodium linoleate) in sodium tetraborate buffer pH 9. The use of air bubbling to deliver oxygen to the reaction medium is therefore risky because of foam formation leading to a decrease in the substrate availability. To avoid this, a special and simple procedure based on exclusive stirring without bubbling (oxygen transfer through the upper surface of the liquid medium) was applied and presented in section 2.5.

2.3. Quantification of oxidation products and verification of the purity

The kinetics of hydroperoxide formation were monitored by UV absorbance at 234 nm (UVIKON xs Secoman, software UVxLite). HPX concentrations were calculated after calibration of the UV absorbance with pure LA hydroperoxides. The extinction coefficient of HPX was determined to be 24.000 $M^{-1}.cm^{-1}$.

For verification of product purity, HPX and LA were analyzed with RP-HPLC. The HPLC analysis were performed on a Dionex Ultimate-3000 HPLC system equipped with a DAD detector and a ThermoFisher C30 Accucore column (2.6 μm , 150 mm x 2.1 mm) was used. The mobile phase was water (A)-acetonitrile (B), with 0.1% formic acid and 5% THF added to each solvent. Gradient elution was adopted and the detailed program was as follows: initial 5% B linearly increased to 63% B in 3.0 min, kept at 63% B from 3.0 to 10.0 min, then linearly increased to 85% from 10.0 to 11.0 min and maintained at 85% B from 11.0 to 15.0 min, and returned to 5% in 1.0 min and maintained 5.0 min. The flow rate used was 0.25 mL.min⁻¹. All the separations were performed at room temperature. LA was identified from its retention time (17.5 min) with UV absorbance at 205 nm. 13-HPX showed a retention time of 12.5 min and was detected at 234 nm. 9-HPX eluted slightly later than 13-HPX, with a retention time of 13 min and was detected at the same wavelength of 234 nm. The main oxodiene peak co-eluted with 13-HPX but showed no significant absorbance at 234 nm and was detected at 278 nm.

2.4. Effect of ethanol content on enzymatic activity

The enzymatic reaction procedure described above was tested with a fixed initial LA concentration of 50 μM and a varying ethanol concentration between 0% (v/v) and 10% (v/v) to study the effect of ethanol on the enzymatic activity. The buffer medium with the enzyme at each ethanol content was incubated for different durations (0, 24 and 72 h) at 5 °C before addition of LA and monitoring the reaction kinetics through HPX formation.

2.5. Determination of gas-liquid oxygen transfer coefficients

To take into account the oxygen transfer, the oxygen mass balance during the LOX reaction is written according to equation (1):

$$\frac{d[O_2]}{dt} = OTR - OUR \quad (1)$$

where *OTR* is commonly expressed as equation (2):

$$OTR = k_L a \times ([O_2]^* - [O_2]) \quad (2)$$

with k_{La} the volumetric transfer coefficient of oxygen at the gas-liquid interface and $[O_2]^*$ the solubility of oxygen in the reaction medium at the fixed temperature.

The volumetric oxygen transfer coefficient between gas and liquid phases (k_{La}) was determined for each oxidation reaction experiment in this way:

Before the additions of enzyme and LA, the reaction medium was deaerated with nitrogen. LA was then added as a concentrated ethanoic solution and the reaction medium was let under controlled stirring to reaerate without air bubbling. The k_{La} was determined from the slope of the curve $\ln([O_2]^* - [O_2]) = f(t)$.

Once SBLOX-1 has been added to the reaction medium, the decrease of dissolved oxygen concentration corresponds to the balance between oxygen uptake by lipoxygenase and oxygen transfer from atmosphere, as described in the equation (1).

2.6. Determination of the kinetic parameters of the enzyme

In a first set of experiments, initial dissolved oxygen was fixed at saturation (theoretically 407 μM at 5 °C in pure water) to ensure its supply in large excess. The initial concentration of LA was varied between 10 and 100 μM , considering a Michaelian model (equation (3)) to determine the kinetic constant of SBLOX-1 towards LA:

$$v_i = \frac{V_{max} \times [LA]}{K_{M(LA)} + [LA]} \quad (3)$$

where $K_M(LA)$ is the affinity constant and V_{max} the maximum reaction rate.

In a second set of experiments, the initial concentration of LA was set to 20 μM or 90 μM and the initial dissolved oxygen was varied between 24 μM and 407 μM to check its possible effect on the initial reaction rate. A small reaeration was technically unavoidable before the start of the reaction due to the incompressible experimental delay between the end of deaeration step and the addition of the enzyme.

The concentrations of HPX and DO were monitored to evaluate the kinetics of the reaction. The recorded DO values were analyzed as mentioned in 2.5 to take into account the oxygen transfer kinetics (*OTR*) and determine the oxygen uptake values (*OUR*).

3. Results and discussions

3.1. Effect of ethanol content on enzymatic activity

The effect of ethanol on SBLOX-1 activity was investigated. Enzyme was incubated (0 to 72 h) with different ethanol contents ranging from 0% (v/v) to 10% (v/v), then 50 μ M LA were added to measure SBLOX-1 activity. Figure 2 shows the evolution of the initial reaction rates of LOX towards LA as a function of the ethanol concentration.

The initial reaction rates are calculated from the HPX production rates, which correspond to the increase in absorbance at 234 nm. Given the low initial concentration of LA, dissolved oxygen is in large excess in all the experiments shown in Figure 2. This figure shows that between 0% and 3% ethanol, there is no negative effect on the initial reaction rates with a plateau reached between 2% and 3%. There is even a positive effect, which could be due to an improvement of dispersibility or solubility of LA due to the presence of ethanol, leading to a better availability of substrate for LOX. It also could be due to a better dispersion of the enzyme in the aqueous medium with ethanol. Figure 2 also shows that the inactivation effect is enhanced for longer contact times with ethanol. Above an ethanol content of 3%, a decrease in the initial reaction rate is observed and may be due to a partial inactivation of the enzyme.

Therefore, an ethanol content of 2% seems to be a very good compromise between a high dispersion of LA in the reaction medium, the amount of organic solvent added, and the inactivation of SBLOX-1, especially if the reaction time is limited to a few hours. This percentage of ethanol was chosen for the following experiments.

3.2. SBLOX-1 kinetic parameters towards LA

For the determination of enzyme kinetic parameters towards LA, experiments were performed with 2% (v/v) ethanol, at 5 °C by fixing initial dissolved oxygen at saturation (407 μ M) and by varying initial

201 LA concentration between 10 μM and 2500 μM . The kinetics of the reaction can then be modeled as a
202 one-substrate (LA) reaction kinetics because the initial DO concentration is in large excess compared
203 to $K_M(\text{O}_2)$ reported in literature[6, 11]. The results are given in Figure 3 as Michaelis-Menten (a) and
204 Lineweaver-Burk representations (b).

205 It appears from Figure 3b that the Lineweaver-Burk curve is linear only for low LA concentrations
206 ($[\text{LA}] \leq 100 \mu\text{M}$) and that increasing LA initial concentration above this order of magnitude (red
207 symbols) did not lead to an improvement of the initial reaction rates. No results in literature
208 demonstrate an inhibition by excess substrate (LA). As an example, Berry *et al.* hypothesized such
209 inhibition [27] but we emphasize that this is a lack of oxygen or a poor substrate accessibility (micellar
210 form). The evidence of our statement is that a simple addition of oxygen or an increase of its solubility
211 (by adding pure oxygen instead of compressed air) is sufficient to overcome this limitation and an
212 improvement of the enzymatic activity is noticed, revealing that there was no inhibition but a simple
213 lack of oxygen as substrate. This is in full agreement with the results of Berry *et al.* [27]. Consequently,
214 it can be considered that in the present case, amounts of substrate above the observed limit of
215 100 μM range are not accessible to the enzyme and the most likely explanation is the micellar form of
216 the LA substrate at these high concentrations. Thus, from data at low LA concentrations (blue symbols,
217 $[\text{LA}] \leq 100 \mu\text{M}$), V_{max} and $K_M(\text{LA})$ values are calculated with the Michaelis-Menten model (dotted line
218 in Figure 3a) and the corresponding values were obtained: $V_{\text{max}} = 11.4 \mu\text{M}.\text{min}^{-1}$ and $K_M(\text{LA}) = 45 \mu\text{M}$.
219 This $K_M(\text{LA})$ value is of the same order of magnitude as the values reported in literature (Table 2).
220 However, the operating conditions differ between the literature and the present work, with respect to
221 two parameters: a lower temperature and the presence of ethanol. If only data from experiments with
222 borate buffer are considered for comparison, the $K_M(\text{LA})$ value obtained in the present work is higher.
223 This may be explained by (i) the negative effect of a lower temperature on the access of LA to the
224 internal active site of the enzyme due to lower mobility and (ii) the likely negative effect of ethanol on
225 the conformation of the active site.

Above $[LA] = 100 \mu M$, initial reaction rates stop fitting the Michaelis-Menten model. It is well known that the solubility of LA in aqueous media is very low. Micellar structures are often present even at low concentrations between $20 \mu M$ and $200 \mu M$ [28-32], depending on the ionic composition and pH of the medium, but also on the analytical method used to determine the solubility or the critical micelle concentration (CMC). In the case of the presence of micelles, special kinetic models are used, to describe enzymatic reactions towards micellar PUFA, but these models assume that the CMC values are known. Firstly, the literature does not provide consistent data on CMC values of LA in aqueous media [30-32] and secondly, data on media containing ethanol are really scarce [32].

Therefore, for the following sections, the experiments are performed with initial LA concentrations within the range fitting with Michaelis-Menten model ($< 100 \mu M$), in order to guarantee the LA solubility condition and to simplify the present kinetic investigation, with the aim of studying the oxygen transfer.

3.3. Effect of dissolved oxygen concentration on enzyme kinetics

To investigate the effect of dissolved oxygen on SBLOX-1 activity, experiments were performed at two LA concentrations lower than $100 \mu M$, with 2% ethanol and varying the initial dissolved oxygen concentration.

Mass balances must be written on dissolved oxygen by taking into account transfer and uptake rates, according to the equation system (4):

$$\begin{cases} \frac{d[O_2]}{dt} = OTR - OUR \\ \frac{d[LA]}{dt} = -\frac{V_{max}[LA]}{K_{M(LA)} + [LA]} = -OUR \\ \frac{d[HPX]}{dt} = OUR \\ OTR = k_L a \times ([O_2]^* - [O_2]) \end{cases} \quad (4)$$

The assumption made to facilitate the interpretation of oxygen kinetics is that the $k_L a$ value remains constant during conversion of LA to HPX. The applied experimental methodology is presented in Figure 4.

The buffer medium containing 2% ethanol was poured in the jacketed reactor and its temperature was regulated at 5 °C. It was partially deaerated by nitrogen bubbling before the addition of LA (step not shown in Figure 4). After the addition of LA at two concentrations lower than 100 µM, the reaction medium was let under controlled stirring to reaerate. This step is mentioned as “zone 1” in Figure 4 and is used to calculate k_{La} in the initial reaction medium. Under these conditions, the maximum k_{La} value is determined to be 10 h⁻¹.

When the desired DO concentration was reached, LOX was added and the resulting decrease in dissolved oxygen is used to calculate the enzymatic reaction rate, taking into account the previously calculated transfer rate (OTR). This is referred to as “zone 2” in Figure 4 where the oxygen mass balance is the result of both oxygen uptake rate (OUR) and oxygen transfer rate (OTR).

At the end of the conversion step in the mentioned “zone 3” of Figure 4, it can be noticed that the amount of dissolved oxygen increased again. During this new reaeration phase, k_{La} can be evaluated and compared to the value obtained in “zone 1”. The former (zone 1) corresponds to a medium loaded with LA while the latter (zone 3) corresponds to a medium loaded with HPX. This comparison validates the modeling parameters used for “zone 2” and the above-mentioned assumption of a constant k_{La} value.

A series of experiments was performed with an initial LA concentration of 20 µM and the initial dissolved oxygen concentration was adjusted from 10% to 80% of saturation (i.e. 40 µM to 325 µM) to check if it influences the reaction kinetics and/or the product purity. k_{La} measurements in “zone 1” range between 0,99 and 1,5 h⁻¹ for the different experiments and show a variation of less than 10% between “zone 1” and “zone 3” for each experiment, which confirms the hypothesis of a rather constant value along each experiment.

Figure 5 shows the variation of dissolved oxygen from the initial aeration step to the end of the enzymatic reaction, after enzyme addition (zones 1 to 3). Based on the k_{La} values in “zone 1” and the DO change in “zone 2”, the initial oxygen consumption rates are calculated and lead to a common

mean value of $3.9 \pm 0.2 \mu\text{M} \cdot \text{min}^{-1}$. Even for the lowest initial dissolved oxygen concentration, no significant decrease in the initial reaction rate is found, indicating that dissolved oxygen is in large excess. The v_i value found is consistent with that presented in section 3.2, Figure 3, for $20 \mu\text{M}$ LA ($3.5 \mu\text{M} \cdot \text{min}^{-1}$).

Furthermore, since the stoichiometry of enzymatic oxidation is one mole of O_2 for one mole of LA, the initial rate of HPX production should equal the initial oxygen uptake rate. Indeed, Figure 6 shows that the initial HPX production rate is the same regardless of the initial dissolved oxygen concentration and its value is $3.92 \pm 0.04 \mu\text{M} \cdot \text{min}^{-1}$. Likewise, Figure 7 shows that for an initial LA concentration of $90 \mu\text{M}$, regardless of the initial DO amount (25% to 95% of the saturation, i.e. $101 \mu\text{M}$ to $387 \mu\text{M}$), the initial HPX production rate is constant and equal to $8.6 \pm 0.5 \mu\text{M} \cdot \text{min}^{-1}$, which corresponds to the value obtained at oxygen saturation, presented in section 3.2. This excellent correspondence between oxygen consumption rates and HPX production rates validates the k_{La} estimates and confirms that an accurate measurement of oxygen transfer leads to a valid determination of the enzyme kinetic parameters.

The relatively constant oxygen uptake rate for different initial concentrations results from a very low affinity constant $K_{\text{M}}(\text{O}_2)$ of the enzyme for oxygen, which would be lower than the minimum amount used ($24 \mu\text{M}$). The few available literature data on $K_{\text{M}}(\text{O}_2)$ ($33\text{--}45 \mu\text{M}$ and $11 \mu\text{M}$) [6, 11] are consistent with this observation although they may be overestimated because of the oxygen transfer not taken into account (the authors only followed the change in dissolved oxygen concentration and assumed it to be equal to the reaction rate). Also, due to the accuracy limitations of Clark electrode, it is not possible to determine a precise $K_{\text{M}}(\text{O}_2)$ value if it is very low. In any case, this observation confirms that for Figure 3, it was relevant to model the kinetics by considering LOX as a single-substrate enzyme, since the concentration of dissolved oxygen was significantly above the $K_{\text{M}}(\text{O}_2)$. Therefore, the values of V_{max} and $K_{\text{M}}(\text{LA})$ estimated in section 3.2 are also valid and represent the true kinetic constants and not just the apparent ones.

Thus, when the SBLOX-1 kinetic parameters and the oxygen transfer constant are taken into account, the zones 1, 2 and 3 previously defined in Figure 4 can be modeled into curves fitting the experimental data very well, as shown in the example in Figure 8. Similarly, the evolution of DO, LA and HPX concentrations is also well modeled, as shown in Figure 9.

However, it is obvious that the phenomena described above and the corresponding kinetic model are only relevant when dissolved oxygen is available. Figure 10 shows a typical case where dissolved oxygen was rapidly consumed and its renewal slower than consumption. This experiment was performed in a 1 L jacketed reactor, with a k_La value of 1.1 h^{-1} , an initial LA concentration of 2 mM and 3 mL of the SBLOX-1 solution added after k_La measurement.

For this experiment, Figure 10 shows that the concentration of DO decreased in the first 15 minutes and remained null from this point until the end of recording (100 min). Also at the 15th minute, the rate of HPX accumulation decreased dramatically, confirming that the absence of dissolved oxygen had a direct effect on the enzymatic reaction. Given the amount of enzyme used (3 mL.L^{-1}), the initial reaction rate is expected to be about $34 \text{ }\mu\text{M.min}^{-1}$. The results for HPX production and LA decrease shown in Figure 10 give initial rates of $32.8 \text{ }\mu\text{M.min}^{-1}$ and $32 \text{ }\mu\text{M.min}^{-1}$ respectively, which is consistent with this expected value. After the 15th minute, the HPX accumulation rate is equal to $2.7 \text{ }\mu\text{M.min}^{-1}$ while the OTR is constant and equal to $7.3 \text{ }\mu\text{M.min}^{-1}$. Since there was no oxygen accumulation, this means that all oxygen transferred to the liquid medium was directly consumed by the oxidation reactions ($\text{OTR}=\text{OUR}$). Therefore, the difference between these two calculated rates may be due to the conversion of a significant part of the HPX produced into degradation products, with a net rate of $4.6 \text{ }\mu\text{M.min}^{-1}$. Simultaneously, the LA concentration decreased linearly in this second period. The calculated rate between 15 and 50 minutes is about $15 \text{ }\mu\text{M.min}^{-1}$, which is twice the OTR value, confirming thus the presence of secondary anaerobic reactions.

The modeled curves presented in Figure 10 show that the modeling equations (4) cease to fit the experimental data from 15 min. This is due to the secondary reactions mentioned above, that are not taken into account in the modeling procedure and require specific investigations to be characterized.

To conclude, this study reveals the importance of the integration of gas-liquid oxygen transfer in SBLOX-1 kinetics studies, if a correct evaluation of oxygen consumption kinetics is aimed. It also reveals that even very low amounts of residual dissolved oxygen (a few percentages of saturation) are not limiting for the SBLOX-1 activity, which confirms that $K_M(O_2)$ is very low. However, anaerobic conditions should be avoided to preserve the medium from secondary and uncontrolled reactions.

From the expression of oxygen transfer rate, it can be noticed that the maximum transfer rate is obtained with the lowest amount of residual DO in the reaction medium, according to equation (5):

$$OTR_{max} = k_L a \times ([O_2]^* - 0) \quad (5)$$

The previous results show that this case is however not recommended. In fact, it is well known that in the absence of dissolved oxygen, SBLOX-1 goes through the anaerobic pathway. The anaerobic reaction starts, like the aerobic reaction, with the formation of radicals due to hydrogen abstraction from the LA. Without sufficient oxygen for the oxygenation step, this results in the formation of various carbonyl compounds: LA dimers, pentane, 13-oxotridecadienoic acid, AL epoxides, AL-epoxide dimers [33]. Some of these compounds are not detectable in UV and mass balances are therefore impossible to establish on the sole basis of the present analyses.

Therefore, it is necessary to identify or at least encompass the lowest permitted (or critical) DO amount, especially if significantly higher LA concentrations are used for large-scale HPX production.

3.4. Enzymatic reactor set up for large scale HPX production and product purity

For a proper scaling of an enzymatic HPX production process, it is obvious from the previous section that gas-liquid oxygen transfer must be controlled. One relevant criterion for scaling up is the

346 Damköhler number[34], $Da = \frac{\text{maximum reaction rate}}{\text{maximum transfer rate}}$. Ideally, Da value should be less than 1 to
347 ensure sufficient oxygen transfer, especially for long duration reactions. The maximum transfer rate
348 can be calculated using a low residual oxygen concentration.

349 Therefore, two production experiments were performed in a 1 L jacketed reactor. Together with the
350 previous experiment shown in Figure 10, this led to a set of three production experiments with a k_{La}
351 between 1.1 h^{-1} and 5.3 h^{-1} , and an initial LA concentration of 2 mM. 3 mL of the SBLOX-1 solution were
352 added after k_{La} determination. The evolution of the DO concentration over time for three different k_{La}
353 values is shown in Figure 11. If the lowest DO reached concentration during reaction, used in section
354 3.3, is considered, the Da numbers obtained are 4.5, 1.3 and 0.9 for k_{La} values of 1.1, 3.7 and 5.3 h^{-1}
355 respectively. For k_{La} values of 1.1 and 5.3 h^{-1} , the Da values confirm the obtained results in terms of
356 lack and availability of oxygen, respectively. For the intermediate k_{La} value of 3.7 h^{-1} , there was no
357 observed lack of oxygen for the only reason that the duration of the reaction was short enough. For a
358 higher initial LA concentration, thus for a longer duration reaction, a lack of oxygen would have been
359 noticed around the 20th minute as predicted by the Da number value.

360 For $k_{La} = 3.7 \text{ h}^{-1}$ and 5.3 h^{-1} , the DO amounts remain above $20 \text{ }\mu\text{M}$ and $170 \text{ }\mu\text{M}$ respectively. Even for
361 that very low residual DO concentration, pure HPX was obtained (Figure 11). The purity of HPX (234
362 nm and retention time 12.5 min in the chromatograms) and mainly the absence of oxodienes, is
363 verified by the absence of peaks on the chromatograms recorded at 278 nm. Additionally, in the
364 chromatograms at 234 nm, no peaks are identified at 13 min attesting to the absence of 9-
365 hydroperoxide. Even with a very low residual DO ($20 \text{ }\mu\text{M}$), almost 100 % LA is converted ($>99.9 \%$ after
366 6h for k_{La} 3.7 and 5.3 h^{-1}). The level of HPX produced ($\frac{\text{total HPX}}{\text{Initial LA}}$) reaches 95 %. Complementary results
367 on the purity of the obtained product are given in [35].

368 We show that even extremely low DO concentrations ($[\text{O}_2]_{\text{lim}} \sim 20 \text{ }\mu\text{M}$) allow almost total conversion
369 of LA ($>95\%$) and pure 13-HPX production. However, it is difficult to determine an accurate $[\text{O}_2]_{\text{lim}}$ value
370 due to Clark electrode sensitivity.

In comparison, commercial hydroperoxides used as standards contain a significant amount of oxodienes (additional peaks in the chromatograms in Figure 12). This may be due to a lack of oxygen during the oxidation reactions (usually chemical oxidations) or to the presence of reactive pollutants interacting with the hydroperoxides produced.

In conclusion, it is possible to produce pure 13-HPX with very low residual amounts of DO. However, once the latter is null, the anaerobic SBLOX-1 pathway is activated, and both LA and HPX are used as SBLOX-1 substrates to produce secondary degradation products. To predict this effect, several numerical simulations are performed with different experimental conditions, based on the equation system (4). Some examples are provided in the Supplementary Material.

In summary, to practically control the process by Damköhler number, the following procedure is necessary:

- a. The value of $k_L a$ can be precisely predicted by dimensional analysis, knowing the specification of the reactor and stirrers, as well as the physicochemical properties of the solution (viscosity and oxygen solubility $[O_2]^*$); it can also be simply measured experimentally as described in the present work;
- b. Once the value of $k_L a$ is known, the amount of enzyme to be added can (and should) be adjusted so that $V_{max} < k_L a [O_2]^*$ is obtained (one should have $Da < 1$ so $\frac{\text{maximum reaction rate}}{\text{maximum transfer rate}} < 1$).

4. Conclusions

Currently, PUFA hydroperoxides are chemically synthesized and the commercial products available are of moderate purity, often containing significant amounts of oxodienes and other secondary reaction products. Moreover, they are very expensive, which limits their use as reaction substrates in lipid oxidation research. Soybean lipoxygenases are known to be highly regio-selective and are therefore

very attractive for the production of pure fatty acid hydroperoxides, especially pure isomer by controlling pH and dissolved oxygen concentration.

The present work has demonstrated the need to consider oxygen transfer for satisfactory reactor sizing, as lack of oxygen induces the production of secondary oxidation and scission products. Once the oxygen uptake kinetics are corrected with the transfer rates, simple, economical and accurate inline characterization of the kinetics is possible, with oxygen uptake rates that were consistent with the LA and HPX kinetics.

The use of an optimal amount of ethanol allows an easier handling of the substrate and improves the dispersion into the reaction medium. A precise study on solubility of LA, its CMC and micelle sizes in such a reaction medium is in progress and will be published later in order to consolidate and clarify the data available in the literature. The use of low temperature improves the oxygen solubility and stability of the enzyme in a hydro alcoholic medium.

The proposed strategy results in almost full conversion of LA and very high purity of 13-HPX, which is currently used as a standard in our laboratory, and as a pure precursor for the study of lipid oxidation in food matrices. When high purity 13-HPX is produced, without any degradation products in the reaction medium, no free radical reactions are possible during storage, and the resulting product can be thus stored longer.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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418 **Figure captions**

419 *Figure 1* : Linoleic acid and its two main hydroperoxide products obtained by SBLOX-1 oxygenation
420 under aerobic conditions

421 *Table 1* : List of chemicals

422 *Figure 2* : Effect of ethanol content and incubation time on initial reaction rates of LOX towards LA
423 (initial concentration 50 μM) calculated as HPX production rates

424 *Figure 3* : Michaelis-Menten (a) and Lineweaver-Burk (b) curves for SBLOX-1 conversion of LA into HPX
425 in borate buffer pH 9 (2% (v/v) ethanol)

426 *Table 2* : SBLOX-1 $K_M(\text{LA})$ values reported in literature

427 *Figure 4* : Illustration of the different steps (« zones ») followed to determine k_{La} and calculate initial
428 enzymatic reaction rate.

429 *Figure 5* : Modification of dissolved oxygen concentration during enzymatic reaction with 20 μM of
430 initial LA concentration and various initial DO amounts. Zones 1 to 3 are represented. The sudden slope
431 change corresponds to the enzyme addition for each curve.

432 *Figure 6* : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm)
433 in reaction media containing 20 μM of initial LA and different initial DO amounts.

434 *Figure 7* : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm)
435 in reaction media containing 90 μM of initial LA and different initial DO amounts.

436 *Figure 8*: Example of experimental and modeled DO curves for the successive steps of the proposed
437 methodology (zones 1, 2 and 3) taking into account all transfer and reaction constants

438 *Figure 9* : Example of experimental and modeled data (equations 4). DO are monitored with Clark
439 electrode, HPX and LA are quantified by HPLC-DAD.

440 *Figure 10* : DO, LA and HPX kinetics for $k_{La}=1.1 \text{ h}^{-1}$

441 *Figure 11* : a) DO concentration curves for the production tests at three different k_{La} values. Only zones
442 2 and 3 are present b) Chromatogram at 234 nm of the final product obtained with production test at
443 $k_{La} 3.7 \text{ h}^{-1}$. Included curve represents the UV spectrum of the HPX peak.

444 *Figure 12* : Chromatograms of fresh commercial linoleic acid 13-hydroperoxide at 234 nm and 278 nm.
445 Included curve represents the UV spectrum of the main peak. 234 nm is the maximum absorbance
446 wavelength for HPX and 278 nm the maximum absorbance wavelength for oxodienes.

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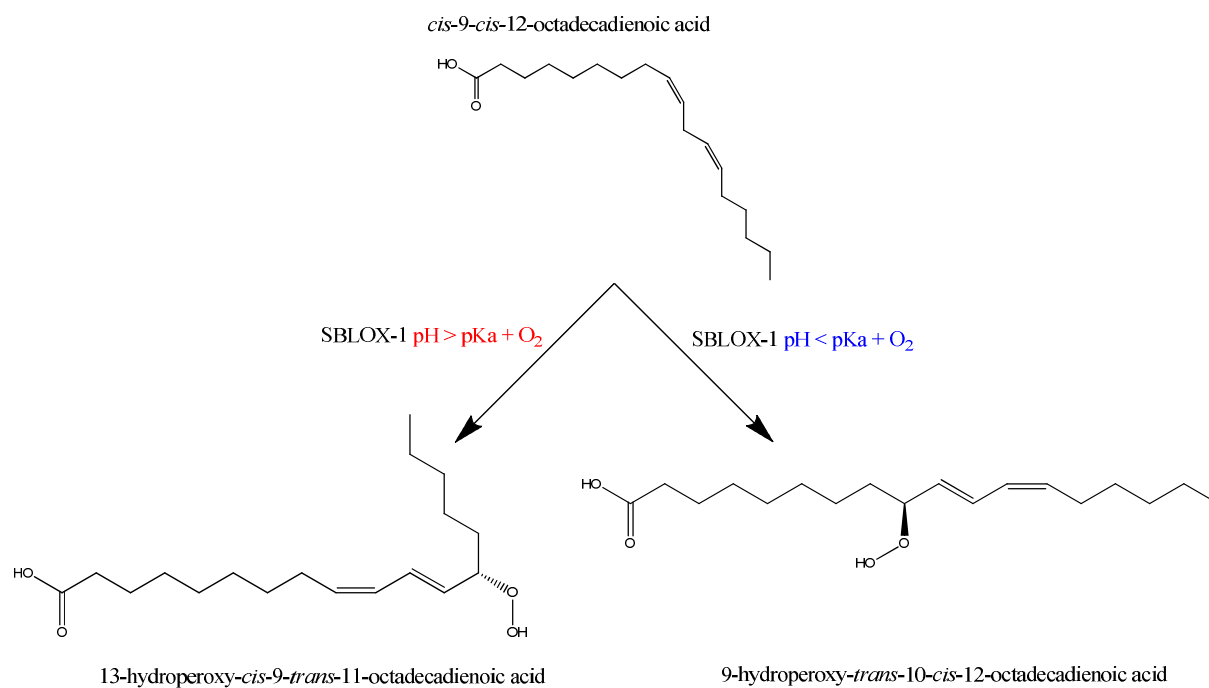


Figure 1 : Linoleic acid and its two main hydroperoxide products obtained by SBLOX-1 oxygenation under aerobic conditions

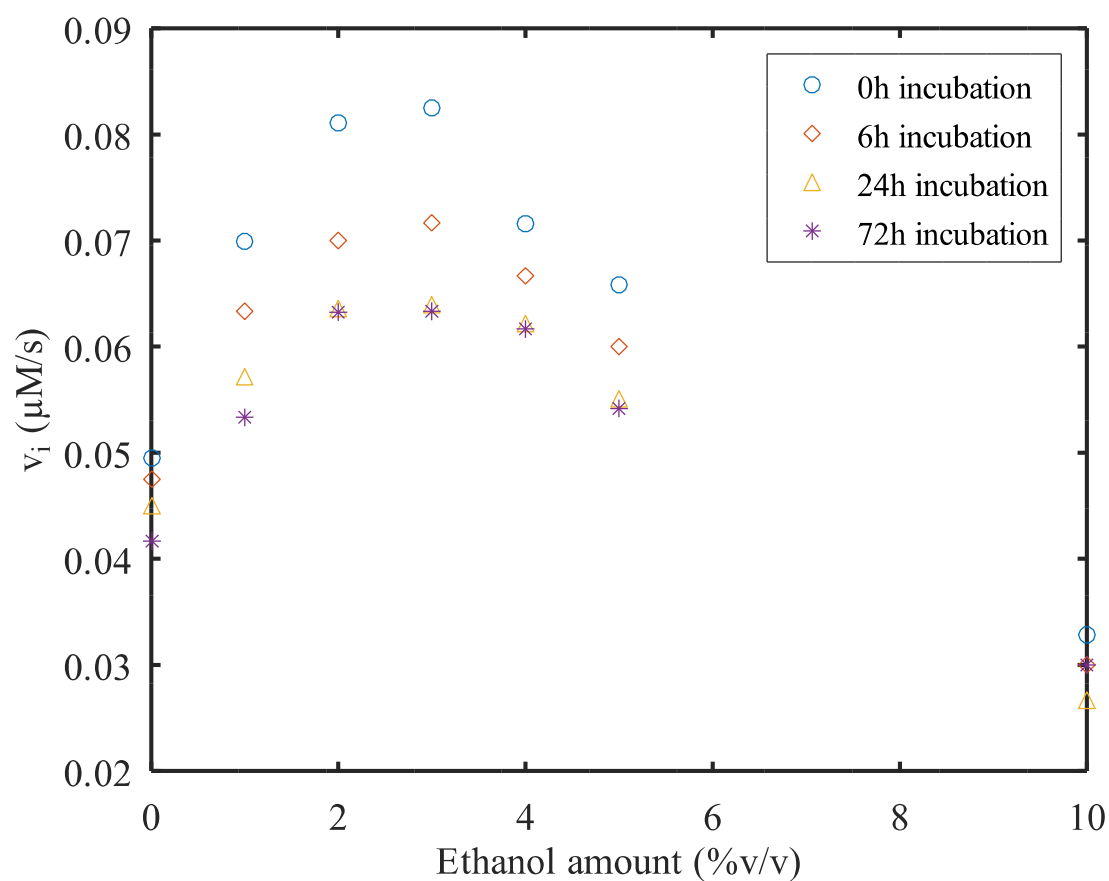


Figure 2 : Effect of ethanol content and incubation time on initial reaction rates of LOX towards LA (initial concentration 50 μM) calculated as HPX production rates

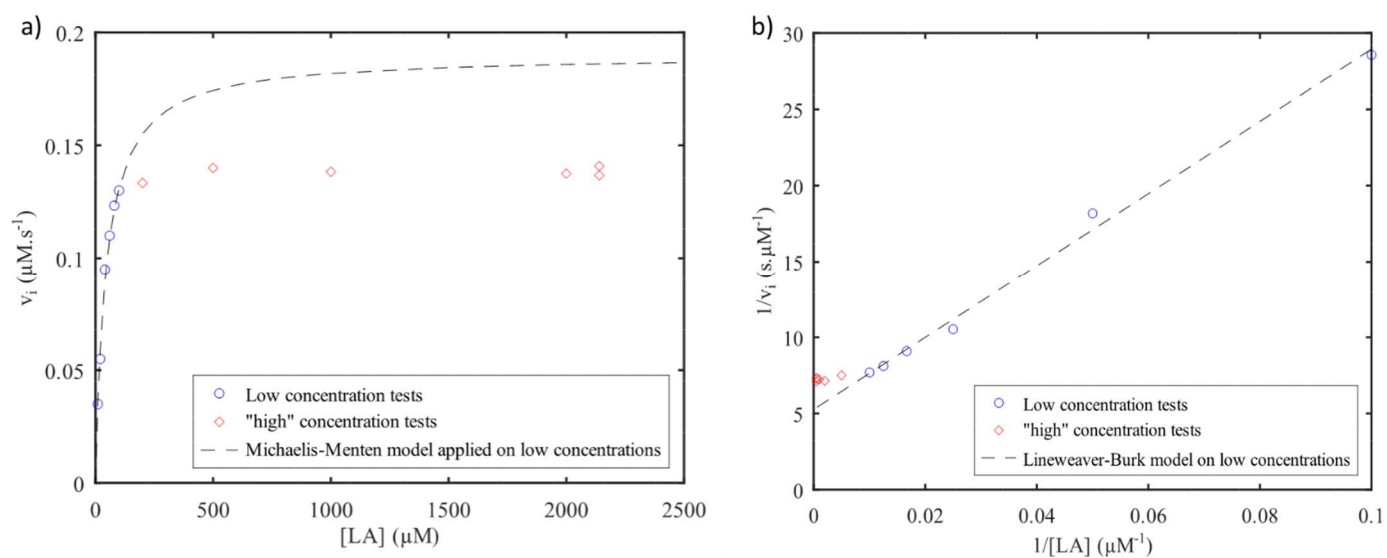


Figure 3 : Michaelis-Menten (a) and Lineweaver-Burk (b) curves for SBLOX-1 conversion of LA into HPX in borate buffer pH 9 (2% (v/v) ethanol)

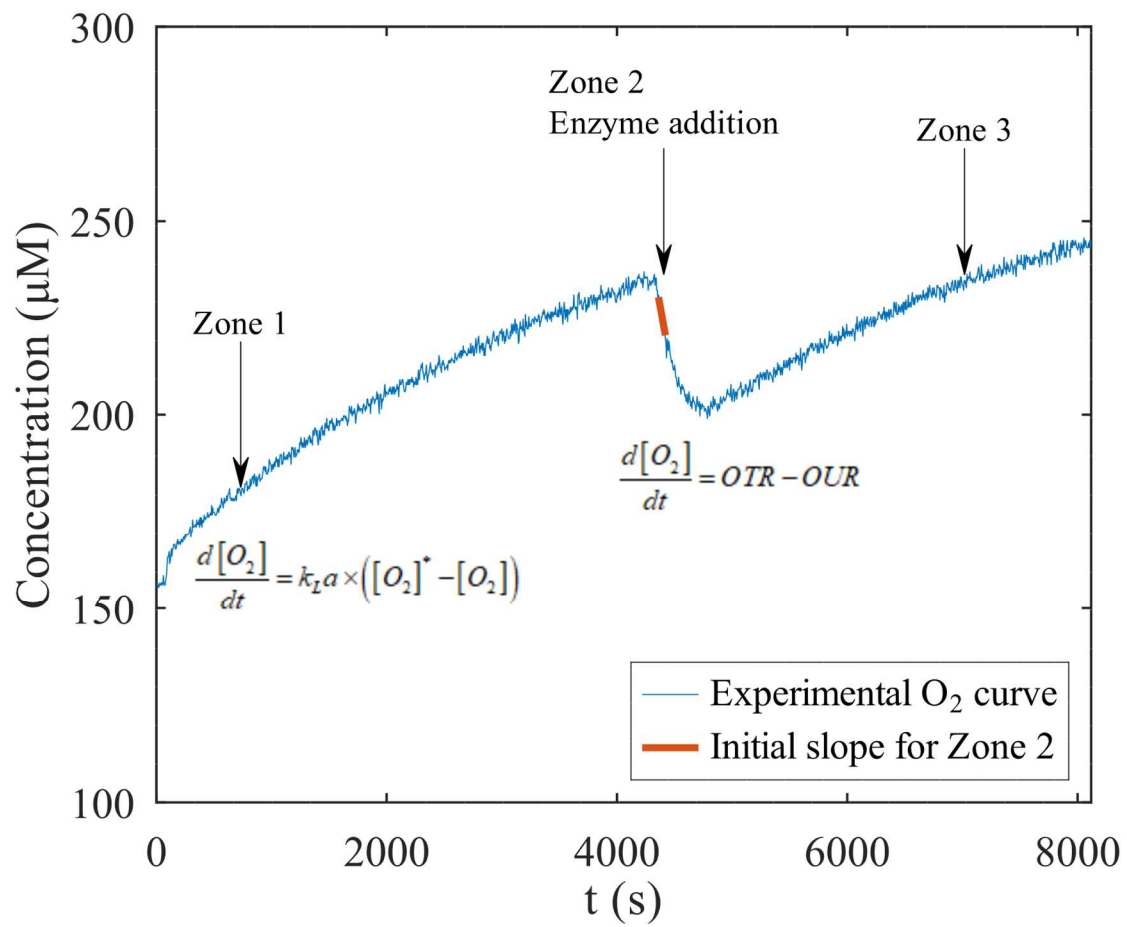


Figure 4 : Illustration of the different steps (« zones ») followed to determine $k_L a$ and calculate initial enzymatic reaction rate.

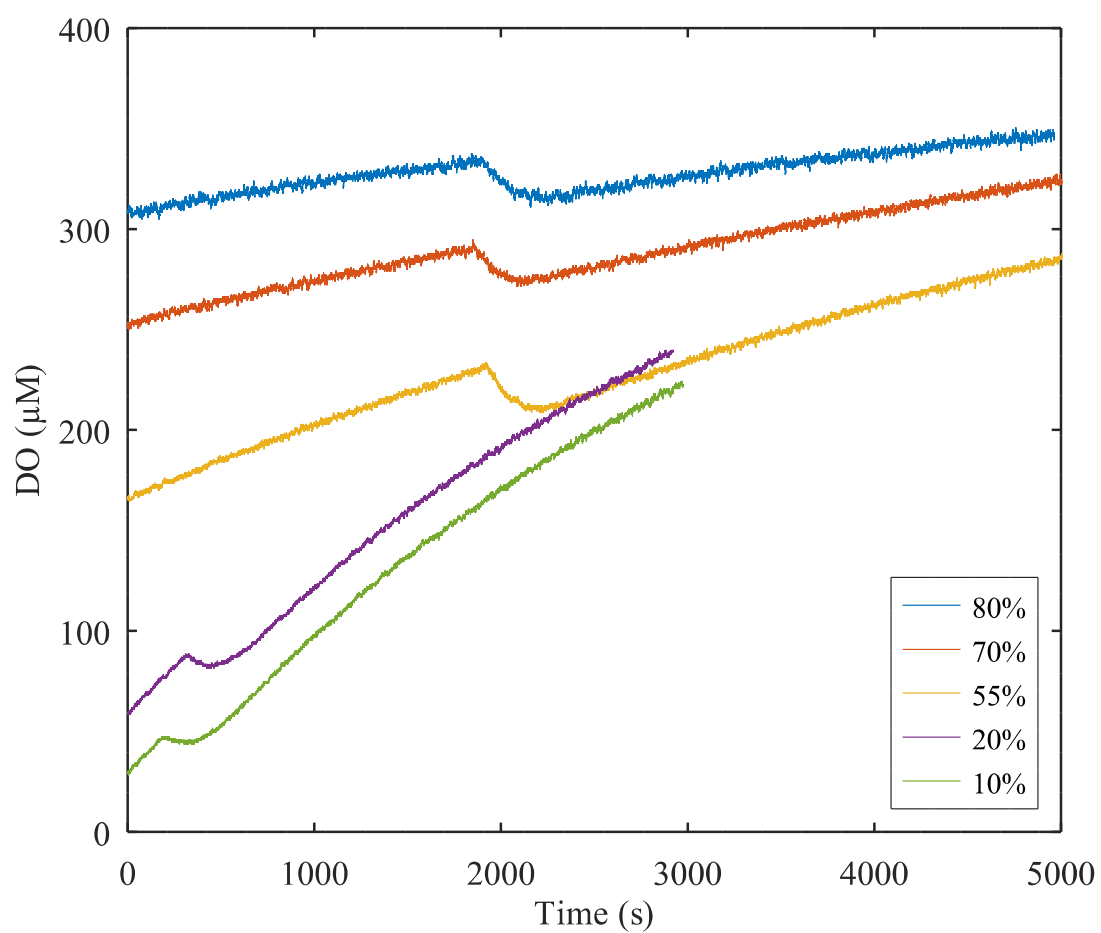


Figure 5 : Modification of dissolved oxygen concentration during enzymatic reaction with 20 μM of initial LA concentration and various initial DO amounts. Zones 1 to 3 are represented. The sudden slope change corresponds to the enzyme addition for each curve.

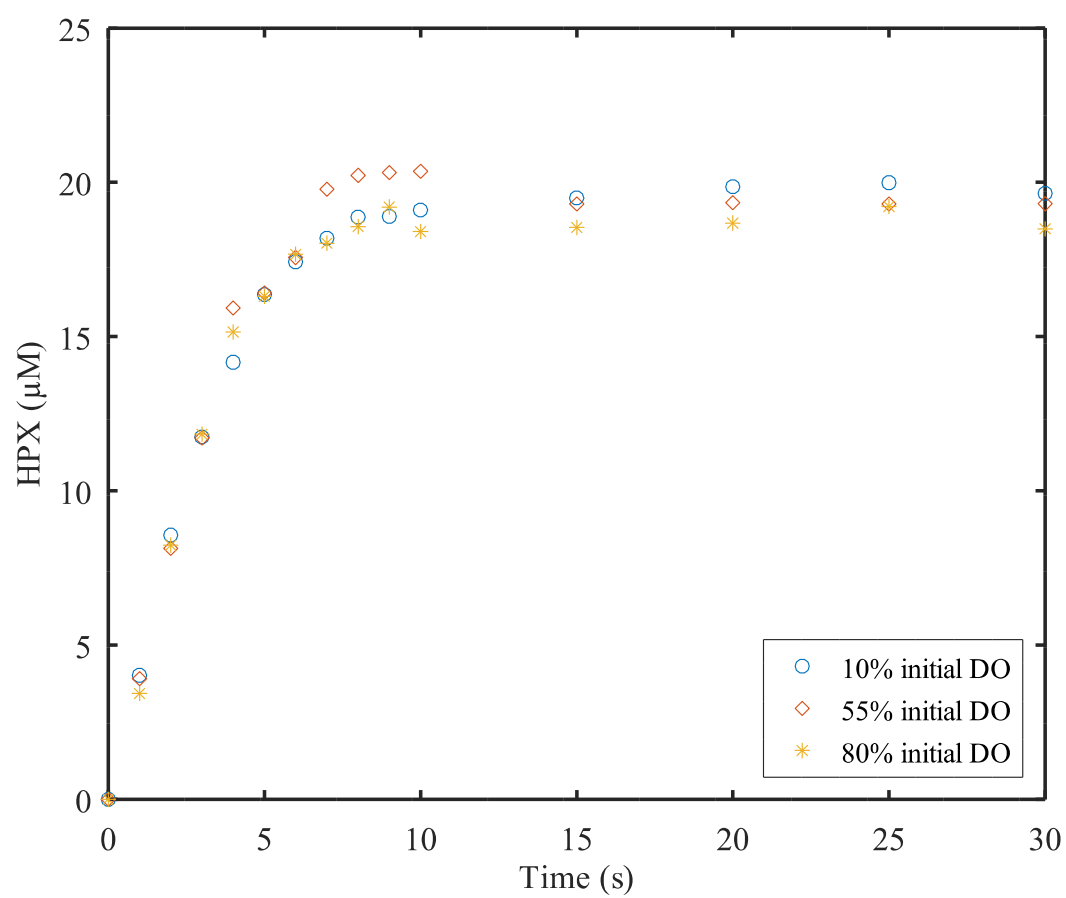


Figure 6 : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm) in reaction media containing 20 μM of initial LA and different initial DO amounts.

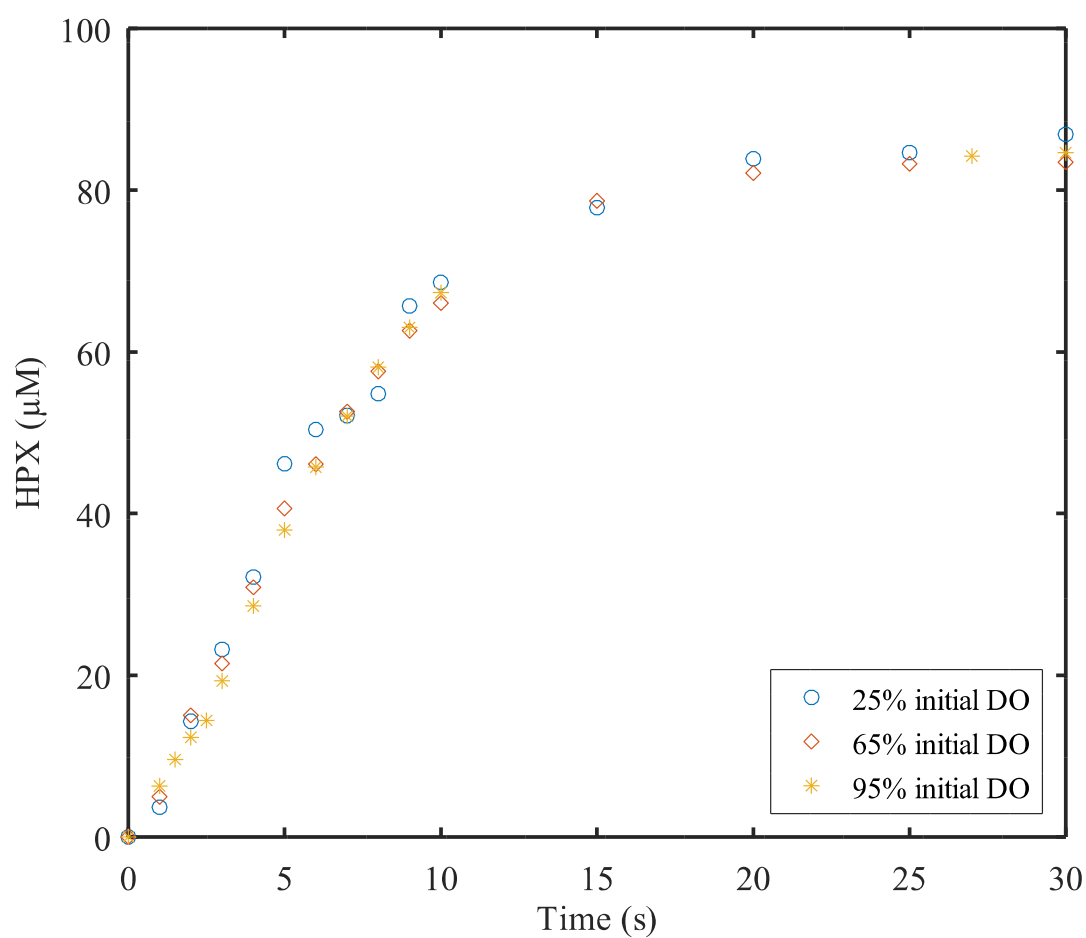


Figure 7 : HPX Production kinetics by SBLOX-1 (followed by UV absorbance measurements at 234 nm) in reaction media containing 90 μM of initial LA and different initial DO amounts.

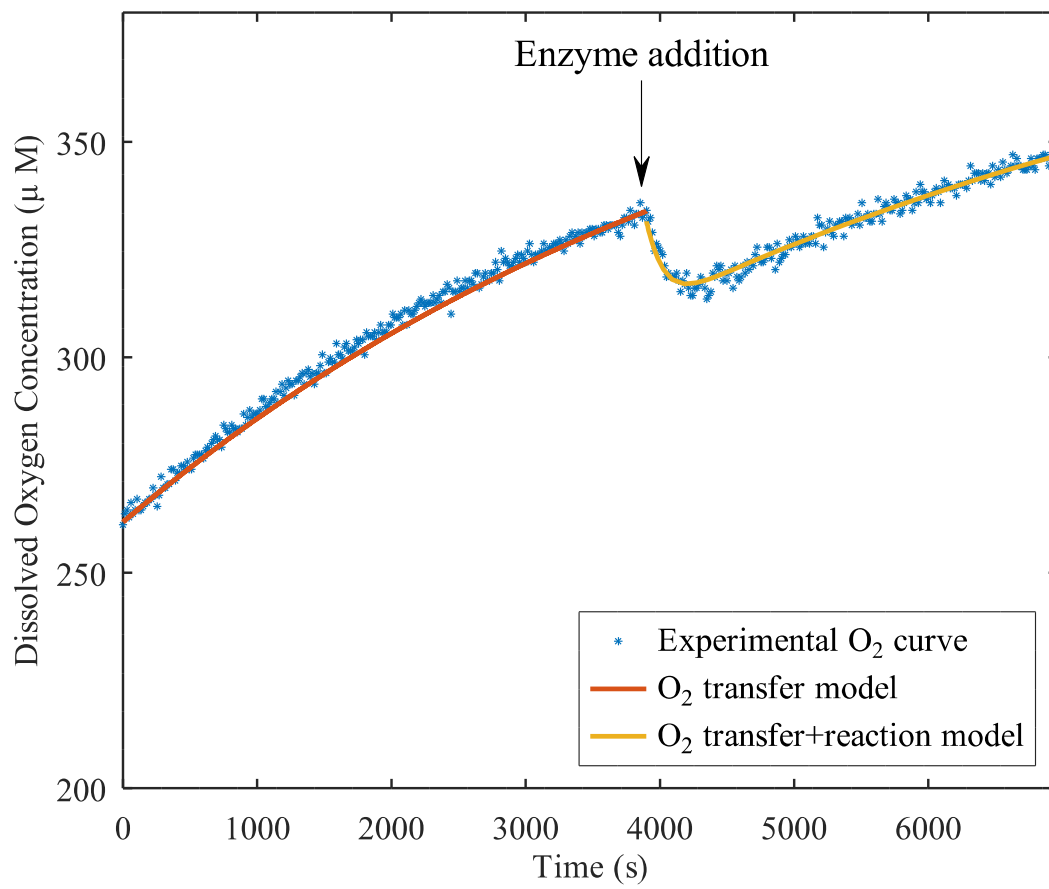


Figure 8: Example of experimental and modeled DO curves for the successive steps of the proposed methodology (zones 1, 2 and 3) taking into account all transfer and reaction constants

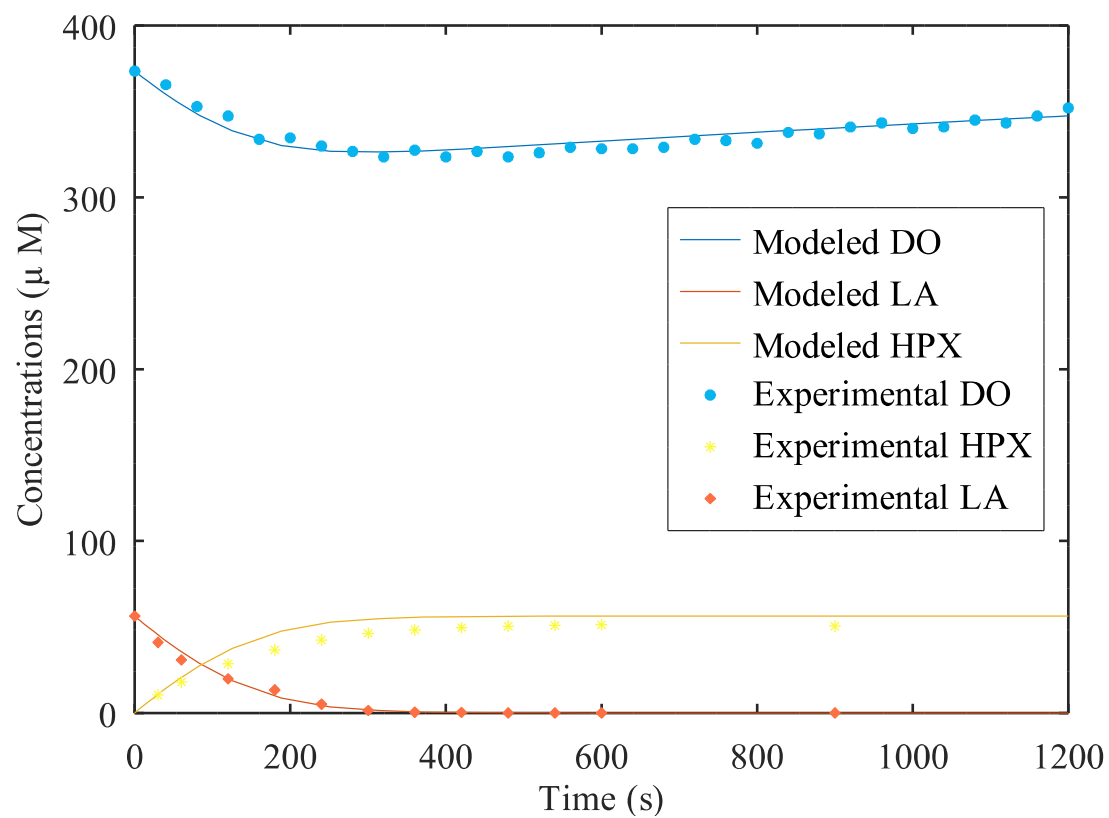


Figure 9 : Example of experimental and modeled data (equations 4). DO are monitored with Clark electrode, HPX and LA are quantified by HPLC-DAD.

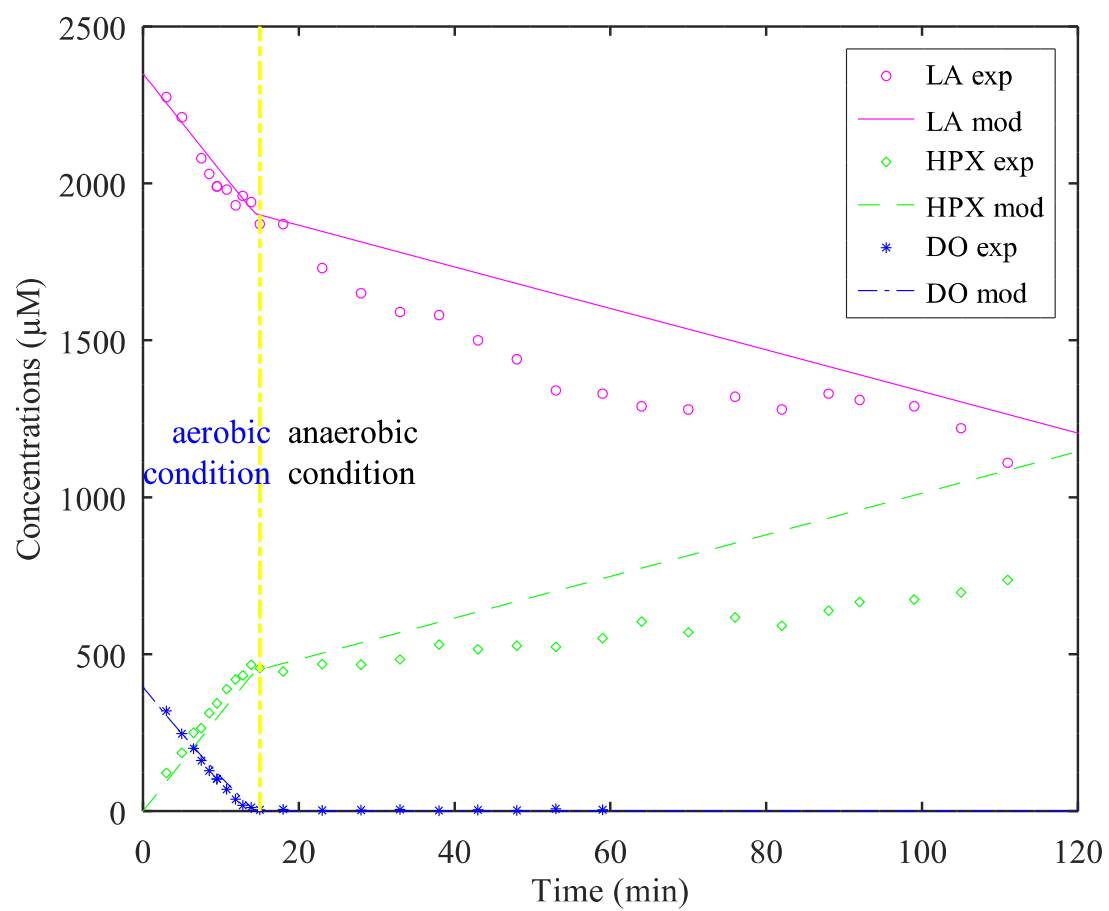


Figure 10 : DO, LA and HPX kinetics for $k_{\text{La}}=1.1 \text{ h}^{-1}$

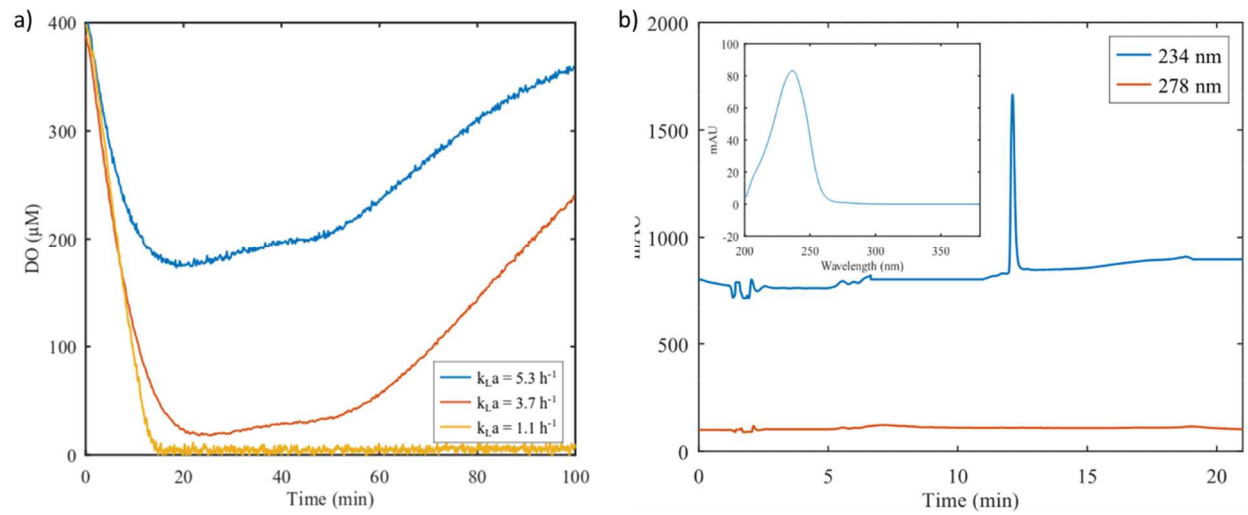


Figure 11 : a) DO concentration curves for the production tests at three different $k_L a$ values. Only zones 2 and 3 are present b) Chromatogram at 234 nm of the final product obtained with production test at $k_L a \ 3.7 \text{ h}^{-1}$. Included curve represents the UV spectrum of the HPX peak.

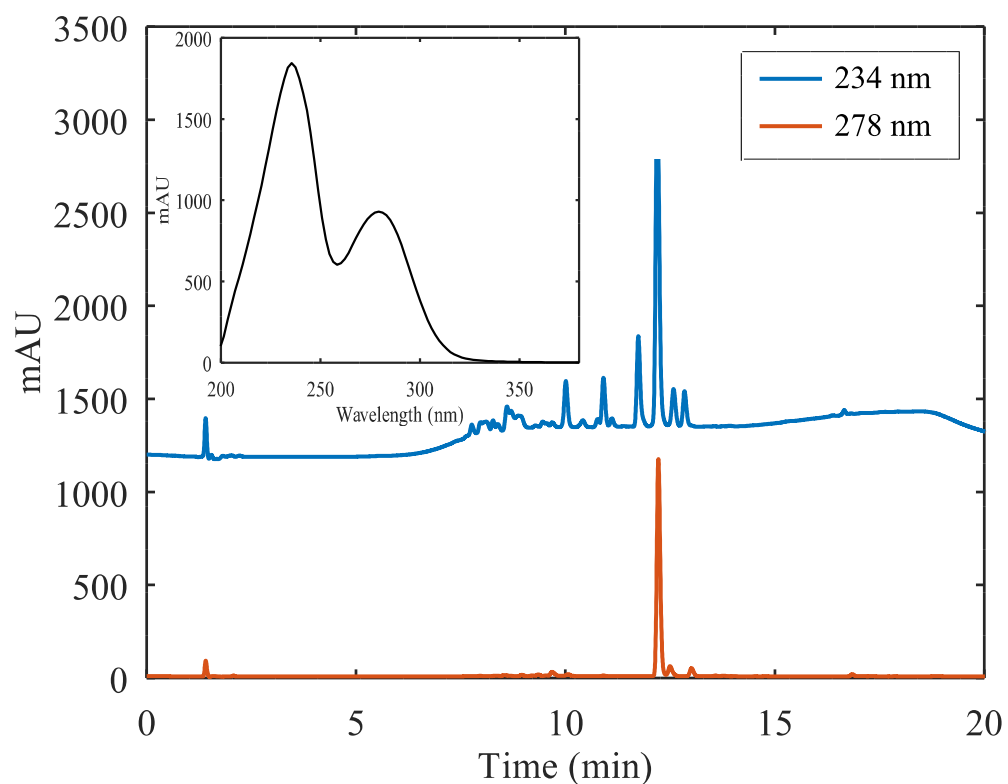


Figure 12 : Chromatograms of fresh commercial linoleic acid 13-hydroperoxide at 234 nm and 278 nm.

Included curve represents the UV spectrum of the main peak. 234 nm is the maximum absorbance wavelength for HPX and 278 nm the maximum absorbance wavelength for oxodienes.

Table 1 : List of chemicals

Chemicals	Reference or CAS number	Quality/purity/activity	Supplier
NaOH	480507	98%	CARLO ERBA
Ethanol	1.11727.1000	96%	MERCK
Borax	1330-43-4	99%	SIGMA
Boric acid	B-6768	99%	SIGMA
Linoleic acid	10-1802-13	99%	LARODAN
13-HPX 9-HPX	laboratory produced + commercial*		
SBLOX-1	9029-60-1	7,9 U/mg	FLUKA
Acetonitrile	75-05-8	HPLC PLUS gradient grade	CARLO ERBA
Formic acid	64-18-6	98-100% for analysis	SIGMA

* Supplier is not indicated as the product purity is criticized in the present work.

Table 2 : SBLOX-1 $K_M(LA)$ values reported in literature

Source	Reaction conditions	$K_M(LA)$ μM	Analytical method
Allen <i>et al.</i> [28]	Tris buffer pH 9 ; 25 °C	24	UV 234 nm
Berry <i>et al.</i> [27]	Phosphate buffer pH 9; 25 °C	62 ± 15	Dissolved oxygen
Galpin <i>et al.</i> [29]	0.1 M borate buffer pH 9 25°C	15	UV 234 nm
Glikmann <i>et al.</i> [7]	0.1 M borate buffer pH 9 25°C	22-27	UV 234 nm and dissolved oxygen
Karadag <i>et al.</i> [30]	0.1 M borate buffer pH 9 25°C	12.5	UV
Karout <i>et al.</i> [31]	0.05 M tris-HCl buffer pH 9 room temperature	67 ± 8	UV
Knapp <i>et al.</i> [12]	0.1 M borate buffer pH 9 20-21 °C	18 ± 3	Dissolved oxygen

Graphical abstract

