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Esterification of polyglycerol with polycondensed ricinoleic acid catalysed by immobilized *Rhizopus oryzae* lipase

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ABSTRACT.

The enzymatic method for synthesising polyglycerol polyricinoleate (PGPR), a food additive named E-476, was successfully carried out in the presence of immobilized *Rhizopus oryzae* lipase in a solvent-free medium. The great advantage of using the commercial preparation of *Rhizopus oryzae* lipase is that it is 10 times cheaper than the commercial preparation of *Rhizopus arrhizus* lipase, the biocatalyst used in previous studies. The reaction, which is really a reversal of hydrolysis, takes place in the presence of a very limited amount of aqueous phase. Immobilization of the lipase by physical adsorption onto an anion exchange resin provided good results in terms of activity, enzyme stability and reuse of the immobilized derivative. It has been established that the adsorption of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64 follows a pseudo-second order kinetics, which means that immobilization is a process of chemisorption, while the equilibrium adsorption follows a Langmuir isotherm. The use of this immobilized derivative as catalyst for obtaining PGPR under a controlled atmosphere in a vacuum reactor, with a dry nitrogen flow intake, allowed the synthesis of a product with an acid value lower than 6 mg KOH/g, which complies with the value established by the European Commission Directive. This product also fulfils the European specifications regarding the hydroxyl value and refractive index given for this food additive, one of whose benefits, as proved in our experiments, is that it causes a drastic decrease in the viscosity (by 50%) and yield stress (by 82%) of chocolate, comparable to the impact of customary synthesised PGPR.

Keywords: Biocatalysis, lipase immobilization, *Rhizopus oryzae* lipase, esterification, vacuum reactor, rheology.

1 INTRODUCTION.

Polyglycerol polyricinoleate (PGPR) is used in the food industry as an emulsifier (E-476). The main application of PGPR is in the chocolate industry, where, besides acting as an emulsifier, it has important properties as a viscosity modifier, improving the moulding properties of the molten chocolate [1]. An additional property of PGPR in chocolate is its ability to limit fat bloom [2-4].

PGPR is prepared using chemical methods which involve the autocatalytic condensation of ricinoleic acid and an alkali-catalysed reaction between the condensed ricinoleic acid and polyglycerol. However, these procedures have the disadvantage of requiring lengthy reaction times and consequently, a large outlay and high energy costs. This fact, together with the high operating temperatures, adversely affect the quality of the final product which may present problems of coloration and odours that make it unsuitable for use in the food industry [5].

As an alternative, the biotechnological production of PGPR with lipases leads to a more appropriate product for use as a food additive since lipases act in mild operation conditions. The benefits of enzymes - such as extremely high specificity and activity under moderate conditions - are prominent characteristics that are increasingly appreciated by different production sectors, among which are the pharmaceutical and fine-chemical industry, together with the more traditional sectors of food and detergents. As a result of recent advances in genomics, proteomics and pathway engineering, biocatalysis is becoming a potential alternative to chemical synthesis [6-8]. The large number of studies published in the field of biocatalysis reflects the growing interest in its applications, e.g., the use of free and immobilized enzymes in the production of bulk and fine chemicals [9-12], the use of organic and green solvents [13-15] or solvent-free reactions [16, 17] and different reactor configurations [18-22].

The enzymatic procedure developed by the present authors to obtain PGPR consists of two steps. Firstly, the ricinoleic acid is polymerized to obtain the estolide [23-25] and then, the polyricinoleic acid is esterified with polyglycerol [26, 27]. The lipase of *Rhizopus oryzae* was reported to have 1(3)-positional specificity, therefore; only primary alcohols are esterified, yielding a mixture of mono- and di- esters. Figure 1 shows both reactions of the process. The first step of the synthesis is catalysed by *Candida rugosa* lipase and the second one can be successfully catalysed by lipases from different sources including *Mucor javanicus, Rhizopus arrhizus* and *Rhizopus oryzae* [26]. The best results, in terms of the characteristics of the final product and reaction time were obtained when commercial preparation of *Rhizopus arrhizus* and *Rhizopus oryzae* lipases were used.

In a previous work [27], a complete study of the biocatalytic synthesis of PGPR with immobilized *Rhizopus arrhizus* lipase was carried out however, the lower price of the commercial preparation of lipase from *Rhizopus oryzae* [26] pointed to the convenience of developing an alternative procedure for the synthesis of polyglycerol polyricinoleate using this enzyme. In order to achieve this, the following objectives were formulated:

- To carry out a thorough study of the process of immobilization of *Rhizopus oryzae* lipase on the support.
- To study the behaviour of the immobilized enzyme in terms of PGPR production, including the reuse of the immobilized derivative.
- To develop the enzymatic reaction in a vacuum reactor in order to shift the chemical equilibrium towards ester synthesis, to obtain PGPR with an appropriate degree of esterification that complies with the Commission European Directive specifications [28] as well as the recommendations given in the Food Chemical Codex [29].
- To check the effects of biocatalytic PGPR on the flow properties of melted chocolate.

2 MATERIALS AND METHODS.

2.1 Enzyme and substrates.

Commercial preparation of lipase (E.C. 3.1.1.3) from *Rhizopus oryzae* (55.7 U/mg solid) was purchased from Fluka. One unit corresponds to the amount of enzyme which releases 1 µmol of oleic acid per minute at pH 8.0 and 40ºC using triolein as substrate. According to the protein analysis carried out in our laboratories following Lowry's procedure modified by Hartree [30], this enzyme contains an average protein in the range of 16 - 26%.

The ricinoleic acid estolide (with an acid value (AV) ≤50 mg KOH/g, which corresponds to an average degree of polymerization of around 3.7, and has a molecular weight (M_W) ≥1838 g/mol), also called polyricinoleic acid (PR), was synthesised in our laboratory by polymerization of ricinoleic acid as described previously [23-25]. Polyglycerol-3 (PG-3), kindly provided by Solvay, is a glycerol oligomer based on an average of three glycerol groups (average MW= 250 g/mol). It is characterized by a very narrow oligomer distribution. It contains minimum 85% diglycerol, triglycerol and tetraglycerol with only trace amounts of glycerol. More information about polyglycerol-3 can be found in its product data sheet [31].

2.2 Immobilization support and reagents.

The anionic exchange resin Lewatit MonoPlus MP 64 (supplied by Fluka) was used as immobilization carrier. The soybean lecithin used as support activator was of commercial grade from Santiveri S.A., Spain. Other chemicals were of analytical grade and were used without further purification.

2.3 Immobilization by physical adsorption.

Five grams of support, Lewatit MonoPlus MP 64, were mixed with 50 ml of a soybean lecithin aqueous suspension (20 mg/ml) in an Erlenmeyer flask and placed in an orbital shaker overnight at room temperature. The activated support was washed with 50 ml of distilled water and then transferred to a jacketed column reactor (2.5 cm i.d. and 30 cm length) equipped with a sinterised glass plate placed 5 cm from the bottom. Unless otherwise stated, the enzyme solution (25 ml, 10 mg/ml in acetate buffer 0.1 M, pH 5) was then added to the reactor and recycled (20 ml/min) for 2 days at 4ºC. The immobilized derivative was washed twice with 25 ml of the same buffer and stored at

4ºC. The amount of protein initially offered and in the wash-liquid after immobilization was determined by Lowry's procedure modified by Hartree [30], using bovine serum albumin as standard. The amount of coupled lipase expressed as protein was taken as the difference between the amount of the initial protein added and the amount of protein in the supernatant and in the wash liquid.
To study the influence of the pH value on the immobilization, different buffers were used for the enzyme solution: 0.1 M sodium acetate buffer to adjust pH values to 4.0, 4.5, 5.0 and 5.5; 0.1 M sodium phosphate buffer to adjust the pH to 5.5, 6.0, 6.5 and 7.0; and 0.1 M sodium citrate buffer for pH values of 5.5 and 6.0. In addition, 0.01 M sodium acetate buffer, pH 5 was used to study the influence of the ionic strength on the immobilization and on the activity of the immobilized derivative obtained.
The kinetic adsorption onto the support for three different concentrations (10, 50 and 150 mg E/ml) of *Rhizopus oryzae* lipase in acetate buffer pH=5 was also evaluated for the pseudo-first order and the pseudo-second order kinetics by analysing the protein content in the supernatant during the immobilization process described above. Finally, thirteen experiments with enzyme concentrations between 5 and 400 mg E/ml were carried out to discern the mechanism of lipase adsorption onto the support.

2.4 Atmospheric reactor assays.

The enzymatic reaction was carried out in an open-air glass-jacketed batch reactor (5.5 cm i.d. and 10 cm length, 250 ml total volume), maintaining the reaction temperature constant at 40ºC. Complete mixing was achieved by means of a four-bladed propeller (diameter: 4 cm) at 450 rpm. The reaction mixture contained 30 g of polyricinoleic acid (PR) and 2 g of polyglycerol-3 (PG). Five grams of immobilized derivative were used and the only water in the reaction system was that soaked into the support (0.6 ml/g approximately).

2.5 Vacuum reactor assays.

For the vacuum reaction, a Parr 5100 series low pressure reactor was used. A round-bottom glass-reaction vessel (6.5 cm i.d. and 4 cm length, 100 ml total volume) was equipped with a circulating water jacket to heat the vessel. The reactor head was of stainless steel and accommodated the reactor controls and instrumentation. A magnetic drive internal stirrer acted as a turbine type impeller (diameter: 3.5 cm). The reactor top also included a vacuum meter, an internal thermocouple, an internal cooling loop, a rupture disk, a liquid sample valve, a gas inlet valve and a gas release valve. Temperature, stirring speed and positive pressure were managed by a controller. The amount of ricinoleic acid, immobilized lipase and water in the reactor at the beginning of the reaction were the same as that reported for the open air jacketed batch reactor. The experiment was carried out at 40ºC, and the stirring rate was kept constant at 450 rpm. The pressure was set at 160 mmHg and 90 l/h of dry nitrogen was passed through the reaction mixture to facilitate water removal (the nitrogen flow was dried by passing through a silica gel column, so that its relative humidity was near to zero).

2.6 Determination of the reaction extension.

The acid value (AV) [29], which represents milligrams of potassium hydroxide needed to neutralise the free acids present in 1 g of sample, was used to follow the extent of the reaction. The acid value corresponds to the free carboxyl group concentration in the

reaction mixture, which decreases due to the esterification of polyricinoleic acid (AV≤50 mg KOH/g) with polyglycerol-3.

2.7 Measurement of the hydroxyl value.

The hydroxyl value is expressed as milligrams of potassium hydroxide required to neutralize acetic acid coupled with the hydroxyl group of the substance when 1 g of sample is acetylated [29].

2.8 Measurement of the iodine value.

The iodine value (IV) represents the number of grams of iodine reacted, under the analysis conditions, with the double bonds in 100 g of substance, and is an indicator of the unsaturation degree of the sample [29].

2.9 Refractive index.

The refractive index of the obtained PGPR was obtained with a ABBE refractometer (2WAJ model, Optika, equipped with water recirculation and temperature control), at 65ºC according to the legal specification of the refractive index for this food additive [28].

2.10 Water content.

Water content of reactor samples was measured with a Karl-Fischer automatic titrator (701 KF, Metrohm), using Hydranal® composite 5, from Riedel-De-Häen.

2.11 Gel permeation chromatographic analysis.

Gel permeation chromatographic (GPC) analysis was carried out to determine the average molecular weight of products using a modular system from Waters, with automatic injector model 717 PLUS and quaternary-gradient pump 600 E. The system was equipped with refractive index detector (model 2414 7.8 mm id x 300 mm GPC Styragel® HR 1 THF column from Waters). The analysis was performed at 35ºC using THF as solvent at a flow rate of 1 ml/min. A calibration curve (*logMW* vs *retention time*) which is used to estimate the molecular weight of samples, was conducted using the retention times of the resolved peaks for monomer, dimer, trimer, and tetramer of ricinoleic and polyricinoleic acid. The number- and weight- averaged molecular weight (Mn and Mw, respectively) were calculated by dividing the chromatogram area into trapezoids and using the formulae:

$$M_n = \frac{\sum A_i M_i}{\sum A_i} \quad (1)$$

$$M_w = \frac{\sum A_i M_i^2}{\sum A_i M_i} \quad (2)$$

where the subscript i refers to the ith trapezoid of A_i area and M_i to the average molecular weight of this trapezoid. The ratio of the weight average to the number average is the polydispersity index, PDI.

$$r = M_w / M_n \quad (3)$$

2.12 Rotational rheometry.

The influence of PGPR on the flow properties of melted chocolate (34% total fat) was characterized with a MCR 300 rheometer (Anton Paar GmbH, Ostfildern, Germany), equipped with a CC27 concentric cylinder system at 40ºC. Flow curves were determined as a function of shear stress (from 900 to 0.1 Pa) and were fitted to two rheological models commonly used in the chocolate industry: the Casson model and the Herschel Bulkley model. However, due to the variation in the shape of the flow curves, which depended on the PGPR concentration added to the chocolate mass, calculation of equilibrium viscosity and yield stress from the resulting equation coefficients could lead to inaccurate results. Therefore, the average viscosity within one minute of shearing at a constant shear rate of 40 s^{-1} was used as a direct measure of apparent viscosity, and the yield stress was determined from the average shear stress measured after applying a shear rate of 5 s^{-1} for 1 min.

3 RESULTS AND DISCUSSION.

Previous work carried out by the authors [26] showed that both immobilised derivatives obtained using two different commercial preparations (*Rhizopus arrhizus* lipase and *Rhizopus oryzae* lipase) were suitable for catalysing the biocatalytic synthesis of PGPR, while the product obtained had a final acid number value below the limit established by the Directive of the European Commission [28]. The main difference between these two preparations lay in the reaction time required to reach the desired conversion values; whereas for *Rhizopus arrhizus* lipase about 100 hours was sufficient, *Rhizopus oryzae* lipase required more than 200 hours. The enzymatic synthesis of PGPR with *Rhizopus arrhizus* lipase has been studied previously and promising results have been obtained [27]. However, as discussed previously [26], *Rhizopus arrhizus* lipase costs about 10 times more than *Rhizopus oryzae* lipase. Based on these results it is considered that the cost associated with the biocatalyst (10-fold higher) in the case of *Rhizopus arrhizus* lipase would have a greater impact on the total cost of the process than the longer time required by *Rhizopus oryzae* lipase (2-fold higher). For this reason, we continued research with this last commercial preparation.

3.1 Optimization of immobilization process.

Effect of pH.

In order to determine the influence of pH on the adsorption process of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64, immobilization assays were carried out using different lipase solutions with a concentration of 10 mg E/ml in the three different buffers with pH values ranging between 5 and 7.

Table 1 shows the results obtained for each of the assays in terms of immobilization yield and amount of protein adsorbed. As can be seen, the pH did not significantly influence the adsorption process of the *Rhizopus oryzae* lipase on the ion exchange resin, at least in the range studied. This would be due to the fact that the isoelectric point of *Rhizopus oryzae* lipase is 6.85 [32], so that, except for phosphate buffer pH = 7, it has positive net charge in all cases.

In contrast, it is shown that the process is highly dependent on the buffer composition. In fact, comparing the results obtained for pH = 5.5, which is the value at which all three buffer solutions converge, it can be seen that the best immobilization yields were achieved with the acetate buffer, while the worst results were obtained with the citrate buffer. That could be explained by the respective dissociation constant (K_a) of the acid containing each solution [33]. Hence, while the acetate buffer and phosphate buffer are mostly in the form of monovalent anions across the pH range used, the citrate buffer is mainly a divalent anion. Since the ionic strength of the medium depends on the molar concentration of ions as well as on the charge of every ion, the ionic strength of the medium is greater in case of the citrate buffer even if all the solutions used had the same concentration, which would explain the unfavourable results obtained with this buffer. The influence of ionic strength on ion protein adsorption has been described elsewhere [34]. The effects attributed to this influence are mainly: contraction of the resin structure, a decrease in porosity of the same (with a corresponding decrease in the available binding sites), as well as the change of the configuration of the enzyme and its interaction with the resin matrix. In addition, the decrease in immobilization yield obtained using phosphate buffer might be due to a higher affinity for the support on the part of the corresponding anion, which would hinder binding of the enzyme with the binding sites of the resin, as well as to the different sizes of the anions.
In order to evaluate the pH effect on the lipase activity, each immobilized derivative obtained was used as catalyst for PGPR production. As can be seen in Figure 2, despite the different loading of the immobilized derivatives no significant differences in the course of the esterification of the polyricinoleic acid with the polyglycerol were obtained, and the results for each experiment were quite similar. It is for this reason that the choice of an appropriate immobilization buffer is complicated. However, since the process of obtaining PGPR has two steps and since the buffer used for the immobilization of *Candida rugosa* lipase, catalyst of the first stage, was sodium acetate pH = 5 [24], it was decided to use the same buffer for subsequent studies of the second stage. Indeed for industrial application, the use of only one buffer for the whole process would facilitate the production process, both operationally and economically.

Influence of ionic strength.

As described in the previous section, the ionic strength of the medium exerts a certain effect on the adsorption of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64. A further study of this influence was carried out by varying the concentration of the buffer to change the ionic strength. The immobilization yields obtained (Table 1) showed that a decrease in the concentration of the buffer led to an increase in the amount of immobilized enzyme. The ionic strength can affect the interactions between ions and protein molecules and between ions and support, which influences the electrostatic and Van der Waals interactions than occur between proteins and the ion exchange resin.
Having seen the importance of ionic strength for protein adsorption, we proceeded to study the influence of ionic strength on the activity of the lipase. To do that we used the two immobilized derivatives described above. Additionally, another derivative was prepared by immobilizing with 0.1 M acetate buffer, pH = 5, but carrying out the last wash with distilled water in order to eliminate the remaining ions on the support. The aim of this experiment was to avoid the potential denaturation of the lipase due to the increase the ion concentration as long as the moisture decreases during the reaction. The results are shown in Figure 3, where it can be seen that, despite its influence on the immobilization yield, the variation of ionic strength had no effect on the activity of *Rhizopus oryzae* lipase. Therefore, for subsequent studies 0.1 M sodium acetate buffer

pH = 5 was used as immobilization buffer meaning that, as explained above, the same buffer is used in both steps of the process.

3.2 Study of the adsorption process.

Adsorption kinetic model.

In order to predict the behaviour that governs the adsorption kinetics of the lipase on the ion exchange resin we established the kinetic parameters were stablished using the pseudo-first order and pseudo-second order Lagergren equations [35, 36], since they are the most widely used equations for the adsorption of an adsorbate from an aqueous solution, and so for the adsorption of enzymes on supports [37-39]. The integrated kinetic equations representing both models are:

Pseudo-first order:

$$\log(q_e - q_t) = \log q_e - \left(\frac{k_1}{2.303}\right)\cdot t \tag{4}$$

Pseudo-second order:

$$\frac{t}{q_t} = \frac{1}{k_2 \cdot q_e^2} + \frac{1}{q_e}\cdot t \tag{5}$$

where q_e and q_t (mg/g) are the amounts of immobilized enzyme per gram of support in the equilibrium and at time t (h), respectively, k_1 (h^{-1}) is the adsorption rate constant of the first order and k_2 the adsorption rate constant of pseudo second order (g/mg h).

Immobilizations were carried out with three enzyme solutions of different concentrations, and the results were fitted to both models, Table 2 shows the values obtained for the corresponding kinetic parameters. As can be seen the adsorption of *Rhizopus oryzae* lipase on the ion exchange resin Lewatit MonoPlus MP 64 follows a pseudo-second order kinetics, a fact evidenced not only by the coefficient of determination (R^2) obtained, but also by the similarity between the q_e values predicted by the model and those obtained experimentally (11.75 mg/g for C_{li} of 10 mg/ml, 49.34 mg/g for C_{li} of 50 mg/ml and 110.15 for C_{li} of 150 mg/ml).

Figure 4 shows the concentration-time curves obtained from the pseudo-second order model together with the experimental values. As can be seen, the greater the enzyme concentration of the solution, the longer the time required to reach equilibrium. Hence, while for the initial concentrations of 10 and 50 mg/ml equilibrium was reached within 24 hours, for the concentration of 150 mg/ml 48 hours were necessary.

Adsorption isotherm.

An adsorption isotherm describes the relationship between the amount of enzyme adsorbed and the enzyme concentration remaining in solution. There are many equations for analysing experimental adsorption equilibrium data. The equation parameters and the underlying thermodynamic assumptions of these equilibrium models often provide some insight into both the adsorption mechanism and the surface properties and affinity of the sorbent.

The Langmuir and the Freundlich models [40, 41] are generally used to describe adsorption processes of proteins [42-44].

The linearized form of the Langmuir isotherm is given by:

$$\frac{1}{q_e} = \frac{1}{q_m} + \frac{1}{q_m \cdot K_L \cdot C_e} \tag{6}$$

and the Freundlich equation in linearized form is:

$$\log q_e = \log K_F + n \cdot \log C_e \tag{7}$$

where q_e is the equilibrium concentration on the support (mg/g), C_e is the equilibrium concentration in the solution (mg/ml), q_m is the maximum capacity of the support (mg/g), K_L indicates a binding constant or Langmuir constant, which is related to the heat of adsorption and is specific to the test conditions and the adsorbent type, K_F is a Freundlich constant and is both a function of the energy of adsorption and temperature and a measure of adsorptive capacity, and 1/n is the Freundlich constant that determines the intensity of adsorption.

Plots of $1/q_e$ *versus* $1/C_e$ and log q_e *versus* log C_e were made to test the Langmuir and Freundlich adsorption models, respectively. In both cases, the respective constants determined are shown on Table 3 with the coefficients of determination. The graphical representations of both theoretical models and the experimental data are shown in Figure 5. These results show that the adsorption process is better described with the Langmuir isotherm; the experimental equilibrium data clearly fitted the equation, with a high correlation coefficient value. The agreement of the experimental data (especially with the Langmuir isotherm) suggests that the enzyme is adsorbed in a mono layer on the anionic resin, that all surface sites are energetically equivalent and that the surface is homogeneous. The maximum capacity of the support is 80 mg prot/g, which can be obtained from a solution containing an initial lipase concentration of 175 mg E/ml.

3.3 PGPR synthesis using immobilized *Rhizopus oryzae* lipase.

Influence of the amount of immobilized derivative in open-air reactor.

To determine the optimal amount of immobilized derivative for the esterification of polyricinoleic acid with polyglycerol three experiments were performed with 2.5, 5 and 10 g of derivative. The results (Figure 6) show the anomalous behaviour observed in the experiment with 10 g of immobilized derivative, since despite of being the highest amount of biocatalyst used in the reaction medium, it did not lead to the greatest reduction in acid number. During the first hours of the reaction the greatest decrease in the acid number was indeed obtained with the highest amount of biocatalyst, but, after 63 hours a slight change in trend resulted in a higher acid number at the end of the experiment, due to the higher amount of water in the medium resulting from the higher amount of resin. Both this type of substrate and product are hydrophobic, but the resin is able to retain water, so the greater the amount of resin in the medium, the higher the water content, and since this is a product of the reaction, this has a negative effect on its progress. For this reason, this effect is more noticeable when the reaction is closer to equilibrium, pointing to the convenience of using smaller amounts of support with a higher enzyme loading.

Effect of enzyme loading of the immobilized derivative in open-air reactor.

Given the results obtained for the adsorption isotherms, enzyme solutions with high concentrations were used to obtain immobilized derivatives with high enzyme loadings which were then used to catalyse the synthesis of PGPR. The time course of the acid value is represented in Figure 7. As can be seen, despite the wide variation in the amount of catalyst, no appreciable differences were observed in the reaction course, which can be attributed to the influence of room humidity on the equilibrium since the reactions were carried out in open air reactor. Hence, the next step was the synthesis of PGPR in a high performance reactor suitable for operating under vacuum conditions and for controlling the water content in the reaction medium.

Synthesis of PGPR in a vacuum reactor.

In addition to the problem caused by the influence of humidity on the reactions studied in this work, legislative specifications require that the product have a high degree of condensation (acid number less than 6 mg KOH/g). The only possibility to attain this is to force the equilibrium towards esterification by removing water from the reaction medium. Such a process was studied in a high performance reactor in vacuum conditions which allowed the water content in the reaction medium to be controlled.

The results obtained in different experiments (data not shown) pointed to the advisability of conducting a continuous flow of dry N_2 through the reactor, so that, after 24 hours of operation, the water content of the medium was in a range between 1500 and 2000 ppm. In this way, the results shown in Figure 8 were obtained. It can be seen that in the new operation conditions established, immobilized *Rhizopus oryzae* lipase catalysed the esterification reaction to obtain PGPR with an acid value that fulfilled EC requirements (6 mg KOH/g). This figure also shows that an increase in the amount of biocatalyst leads to a significant reduction in reaction time necessary to reach a suitable final acid value. Comparing these results with those obtained previously with the commercial preparation of *Rhizopus arrhizus* lipase [27] we found out that we obtained similar trends acid value vs time consuming a higher amount of biocatalyst (5-fold higher) when using the commercial preparation of *Rhizopus oryzae* lipase, but as the commercial preparation of *Rhizopus arrhizus* lipase is 10-fold more expensive the final biocatalyst cost is lower. It should be considered that when scaling the process for industrial application, both the operating time and the consumption of biocatalyst should be monitored to obtain a balance that is economically as favourable as possible.

3.4 Characterization of PGPR obtained.

The main objective of this research was to develop a catalytic process for the enzymatic production of polyglycerol polyricinoleate that may be a valid and better alternative to the current chemical processes used, while ensuring that the final product is of high purity and quality by limiting the occurrence of secondary products. The mild temperature conditions used in the enzymatic reactions described led to this being achieved.

Polyglycerol polyricinoleate as a food additive has to comply with a series of purity specifications set by European Commission Directive [28], which are listed in Table 4. The same table shows the acid number, hydroxyl value and refractive index at 65°C of the PGPR obtained in this work. As can be seen, the PGPR obtained meets all of the legal specifications.

In addition to the specifications set by the European Community for this food additive, the European Society of Manufacturers of Emulsifiers has additional recommendations for attaining different parameters of purity. These recommendations (among them the iodine value) are included in the Food Chemicals Codex (FCC) of 2004 [29]. This parameter indicates the degree of unsaturation of the final product and it is connected with its appropriate properties as emulsifier. The iodine value has no reason to change during the reaction to obtain PGPR because the double bonds in the fatty acid chain should not be affected during polyricinoleic acid production nor during its esterification with polyglycerol. The PGPR produced in this work, as can be seen in Table 4, fulfils the recommendations given for the iodine value so, it is evident that, under the reaction conditions of the developed enzymatic process, double bonds of the polyricinoleic acid were not altered. Therefore, there were no side reactions such as cyclization reactions or

oxidation of the double bond, which would produce secondary products, giving undesirable organoleptic characteristics to the final product.

3.5 Gel permeation chromatography. Average molecular weight determination.

Gel permeation chromatography (GPC) was used to determine the number average molecular weight, weight average molecular weight and polydispersity index of several reaction samples during PGPR production.
Figure 9 shows the superimposed chromatograms of three samples taken as the reaction progression. The first peak from the right corresponds to polyricinoleic acid and the first peak from the left is final PGPR. Chromatographic analyses indicated that the final product has a number average molecular weight of 2983 g/mol, a weight average molecular weight of 3539 g/mol. From the values of weight averaged molecular weight, and taking into account the molecular weight of polyricinoleic acid, it can be established that the final product is a mixture of mono- and di- esters in which polyricinoleic acid is attached to primary hydroxyl groups of polyglycerol, due to the selectivity of the lipase used. The value of the polydispersity index (1.9) along with the shape of the curves, confirmed the low level of low molecular weight compounds and the high purity of the product.
Analysis of the information provided by the chromatograms presented an insight into the substance evolution during the course of the reaction. The results are shown in Figure 10 where the acid value and average molecular weight are represented *vs.* reaction time. The excellent correlation between the increase in the average molecular weight and the decrease in the acid value is one more proof that esterification was the main reaction in the process. The same correlation means the possibility of cyclic side reactions can also be discarded.

3.6 Influence of polyglycerol polyricinoleate on chocolate rheology.

The influence of biocatalytic PGPR on the rheology of dark chocolate was obvious in both apparent viscosity and yield stress. The constant shear rate values indicated a decrease in the rheological parameters as the concentration of emulsifier increased. In samples containing 1% PGPR, a reduction of 82% in yield stress and 50% in apparent viscosity, with respect to measurements of chocolate samples with no added emulsifier, were achieved.
From the comparison of Casson and Herschel Bulkley models we conclude that the latter model best fitted the flow curves of the analysed samples of chocolate containing different concentrations of PGPR, although the Casson model is the most widely used in the characterisation of chocolate rheology. The Casson model became less accurate as PGPR concentration increased. According to the model-estimated parameters, biocatalytic PGPR caused a drastic decrease in the yield stress of chocolate, reaching values below 1 Pa.
Unlike the effect of lecithin, previously investigated [45], which resulted in a yield stress reduction at low concentrations but in a subsequent increase at higher concentrations, PGPR causes a progressive decrease of rheological parameters with increasing concentration. Therefore, PGPR reduction in yield stress is much more pronounced than that obtained using lecithin as emulsifier and would greatly facilitate the handling of chocolate in the process of moulding, extrusion and coating.

4 CONCLUSIONS.

A biocatalytic procedure for the esterification of polyglycerol and polyricinoleic acid to obtain PGPR has been studied. The enzymatic reaction is catalysed by an immobilized derivative of *Rhizopus oryzae* lipase, which is a cheaper commercial preparation than *Rhizopus arrhizus* lipase, which has been used previously. The support used for immobilization is an anionic exchange resin (Lewatit MonoPlus MP 64) and a deep study of the immobilization process has been made.

Optimization of the immobilization showed that the pH value does not have any influence on the immobilization yield, and greater differences are arise from the nature of the buffer, which are attributable both to the ionic strength of the medium and the interaction between different ions and support. Furthermore, a decrease in ionic strength of the medium favours immobilization of the lipase, but has no effect on the activity of the immobilized derivative. In addition, the adsorption process of *Rhizopus oryzae* lipase on the support follows a pseudo-second order kinetics, while the adsorption equilibrium obeys a Langmuir isotherm.

As regards the reaction, the optimum amount of immobilized derivative for the reaction was 5 g, larger quantities leading to into a lower conversion rates due to increased water retention. To reach an adequate acid value, strong anhydrous conditions are needed, so continuous drying is required; under such conditions *Rhizopus oryzae* lipase is able to maintain its catalytic activity. Finally, the reaction time needed can be reduced by increasing the enzyme loading, while the biocatalyst cost is still lower than when using the commercial preparation of *Rhizopus arrhizus* lipase.

The PGPR obtained by this procedure complies with the legal specifications concerning acid number, hydroxyl value and refractive index for this food additive referred to in Directive 2008/84/EC of the Commission of 27 August 2008, which lays down specific purity criteria for food additives other than colours and sweeteners. The resulting product also fulfils the recommendations contained in the Food Chemicals Codex.

Chromatographic analysis of the PGPR obtained showed high number average and weight average molecular weights, without the presence of low molecular weight compounds, leading to a polydispersity index close to unity.

Rheological studies demonstrated the high capability of PGPR obtained by this new enzymatic process to significantly reduce apparent viscosity and yield stress in chocolate.

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Table 1 pH-dependence of immobilisation yield and enzyme loading in the adsorption of *Rhizopus oryzae* on Lewatit MonoPlus MP 64

pH	*Buffer*	*Initial protein amount in solution (mg prot.)*	*Immobilisation yield (%)*	*Enzyme loading (mg prot/g sup)*
5.0	Acetate	46.62	72.46	6.76
5.5	Acetate	47.69	73.25	6.99
5.5	Citrate	54.03	37.24	4.02
6.0	Citrate	50.13	36.35	3.64
5.5	Phosphate	39.84	59.90	4.90
6.0	Phosphate	44.53	66.81	5.69
6.5	Phosphate	47.85	61.50	5.73
7.0	Phosphate	47.67	63.93	6.37

Table 2 Immobilisation yield and enzyme loading in the adsorption of *Rhizopus oryzae* on Lewatit MonoPlus MP 64 with buffers of different concentrations

Buffer	*Initial protein amount in solution (mg prot.)*	*Immobilisation yield (%)*	*Enzyme loading (mg prot/g sup)*
Acetate 0.1 M	64.49	68.93	8.98
Acetate 0.01 M	64.62	75.67	9.78

Table 3 Parameters calculated from the pseudo-first order and the pseudo-second order adsorption kinetics for different concentrations of *Rhizopus oryzae* lipase solution on Lewatit MonoPlus MP 64

C_{li} *(mg/ml)*	*Pseudo-first order kinetics*			*Pseudo-second order kinetics*		
	K_1 (h^{-1})	q_e (mg/g)	R^2	K_2 (g/mg h)	q_e (mg/g)	R^2
10	0.2130	3.35	0.8770	0.2858	11.81	0.9999
50	0.2020	22.49	0.9322	0.0318	50.00	0.9997
150	0.0887	60.65	0.9387	0.0071	111.11	0.9995

Table 4 Equilibrium parameters calculated from the Langmuir and Freundlich isotherms for immobilisation of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64

Langmuir isotherm			*Freundlich isotherm*		
K_L *(ml/mg)*	q_m *(mg/g)*	R^2	K_F $(ml^n \cdot mg^{1-n}/g)$	*n*	R^2
4.4322	84.7458	0.9976	13.9605	0.5628	0.9295

Table 5 Legal purity values for PGPR as a food additive established by the European Commission and the results obtained for the biocatalytic PGPR

	Values for the biocatalytic PGPR	*Legal values*
Acid Value (mg KOH/g)	5.31	< 6
Hydroxyl Value (mg KOH/g)	89.16	80 – 100
n_{65}^{D}	1.4655	1.4630 – 1.4665

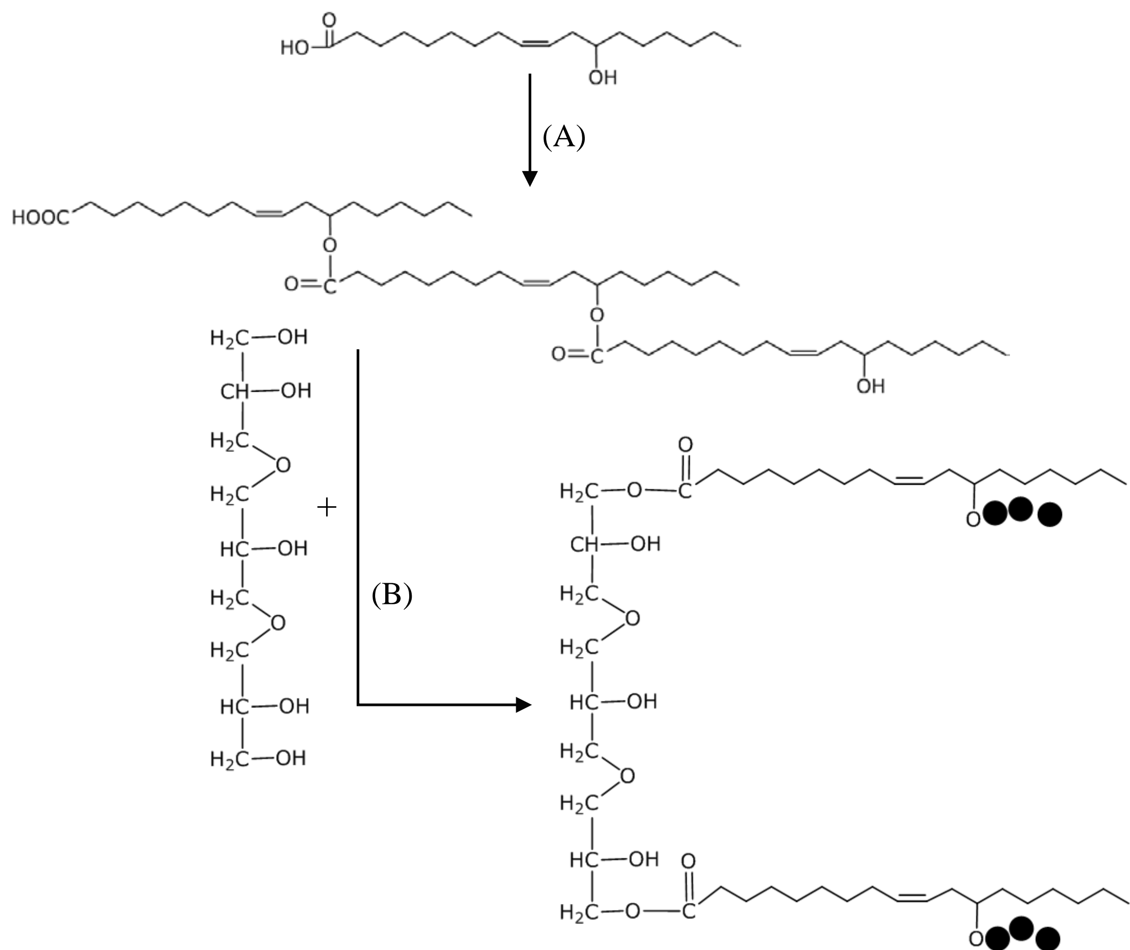

Fig. 1 Scheme of reaction for obtaining of polyricinoleic acid (A) and esterification of polyglycerol with polyricinoleic acid to produce PGPR (B) – black dots represent ricinoleyl chains.

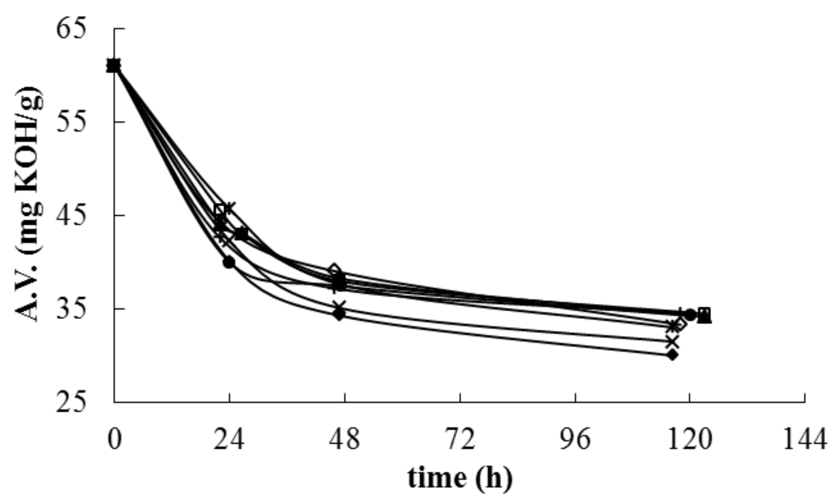

Fig. 2 Effect of the pH value of the buffer used to obtain the immobilised derivative on the esterification course in open air reactors: Acetate pH 5.0 (×), Acetate pH 5.5 (♦), Citrate pH 5.5 (□), Citrate pH 6.0 (▲), Phosphate pH 5.5 (✳), Phosphate pH 6.0 (◊), Phosphate pH 6.5 (+), Phosphate pH 7.0 (●). Reaction conditions: 30 g of PR, 2 g of PG, 5 g of immobilized derivative, 40ºC.

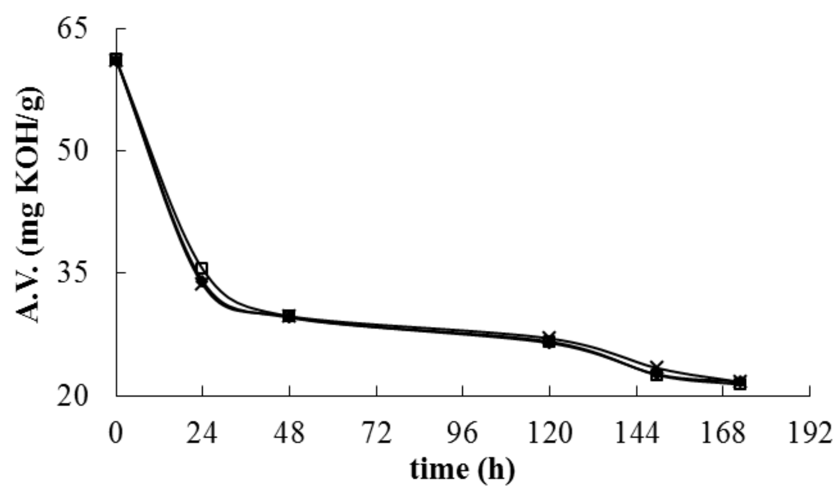

Fig. 3 Effect of the ionic strength on the esterification course depending on the solution used to wash the immobilised derivative: Water (×), Acetate buffer 0.01 M (♦), Acetate buffer 0.1 M (□). Reaction conditions in open air reactors: 30 g of PR, 2 g of PG, 5 g of immobilized derivative, 40ºC.

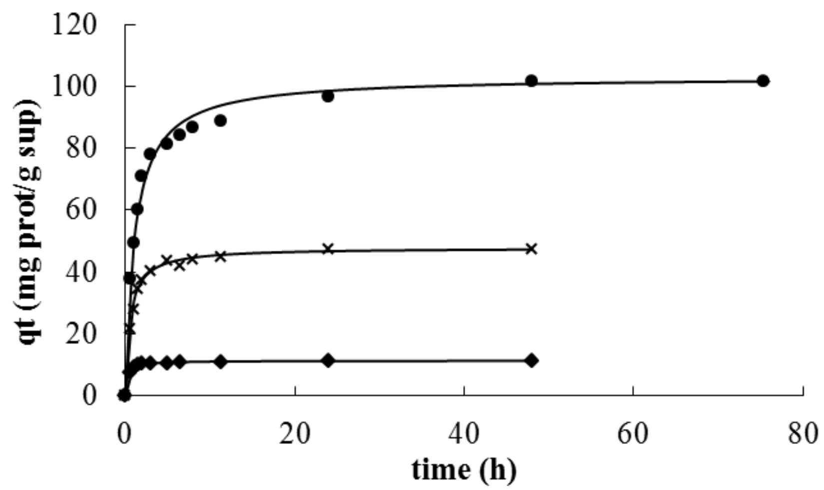

Fig. 4 Time course of adsorption of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64 for three different concentrations of the initial enzyme solution: 10 mg/ml (♦), 50 mg/ml (×), 150 mg/ml (●). Continuous lines correspond to data obtained from the pseudo-second order kinetic model.

¡OJO! Poner en ordenadas (mg/g)

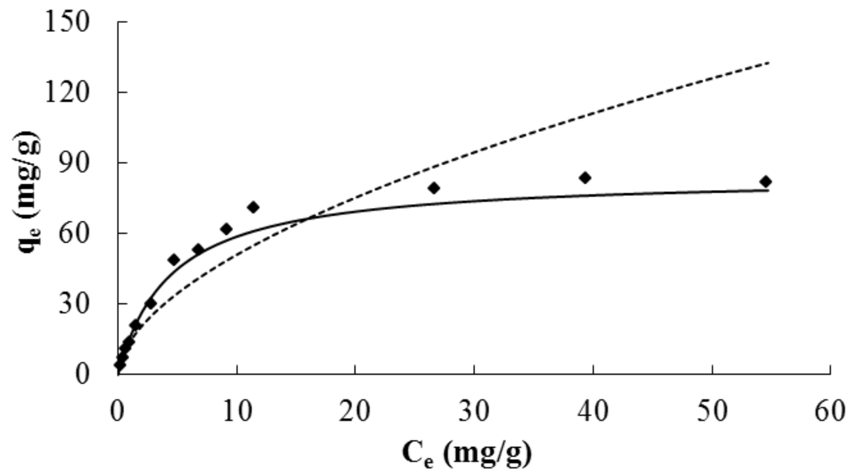

Fig. 5 Experimental data of adsorption of *Rhizopus oryzae* lipase on Lewatit MonoPlus MP 64 (♦) fitted with the Langmuir equation (continuous line) and the Freundlich equation (dotted line).

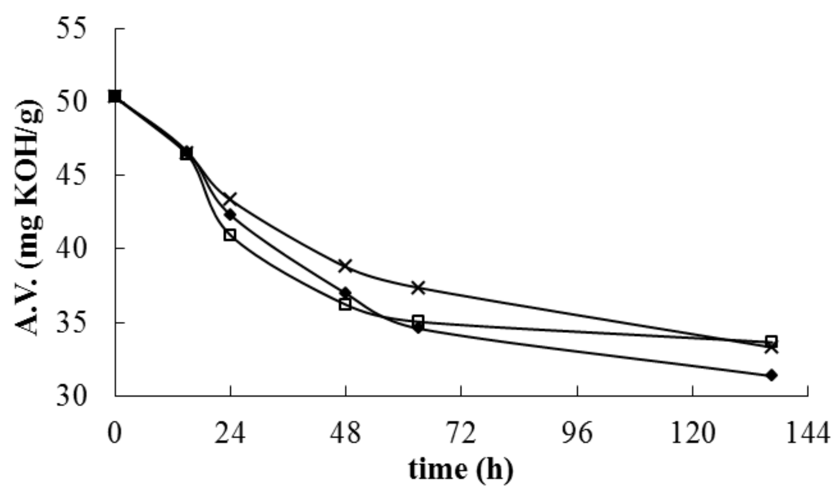

Fig. 6 Reaction course in open air reactors depending on the amount of immobilised derivative: 2.5 g (×), 5 g (♦), 10 g (□). Reaction conditions: 30 g of PR, 2 g of PG, 40ºC.

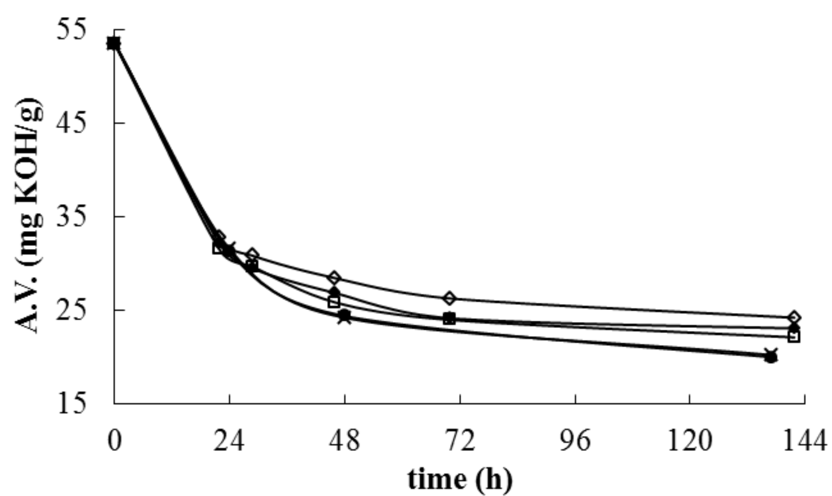

Fig. 7 Reaction course in open air reactors depending on the loading of the immobilised derivative: 7.49 mg protein/g (◊), 34.32 mg protein/g (♦), 72.62 mg protein/g (□), 82.13 mg protein/g (×), 83.29 mg protein/g (●). Reaction conditions: 30 g of PR, 2 g of PG, 5 g of immobilized derivative, 40ºC.

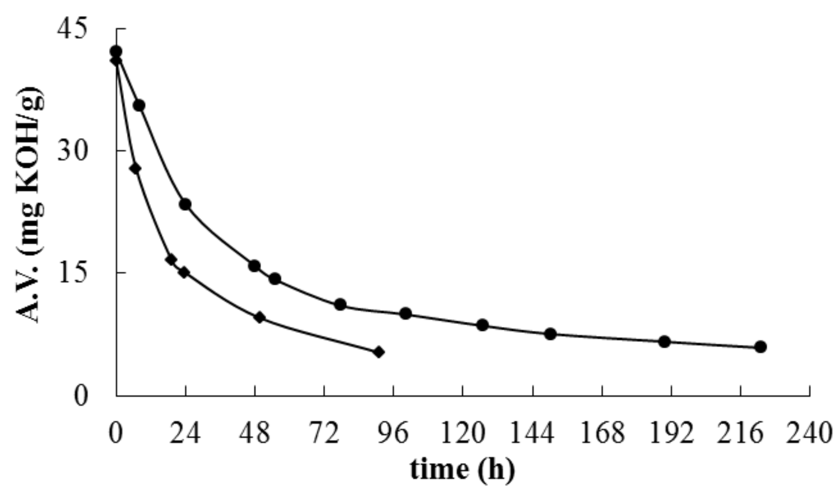

Fig. 8 Reaction course in the vacuum reactor depending on the loading of the immobilised derivative: 16.36 mg protein/g (●), 39.40 mg protein/g (♦). Reaction conditions: 30 g of PR, 2 g of PG, 5 g of immobilized derivative, 40ºC, 160 mmHg, 90 l/h N_2, 450 rpm.

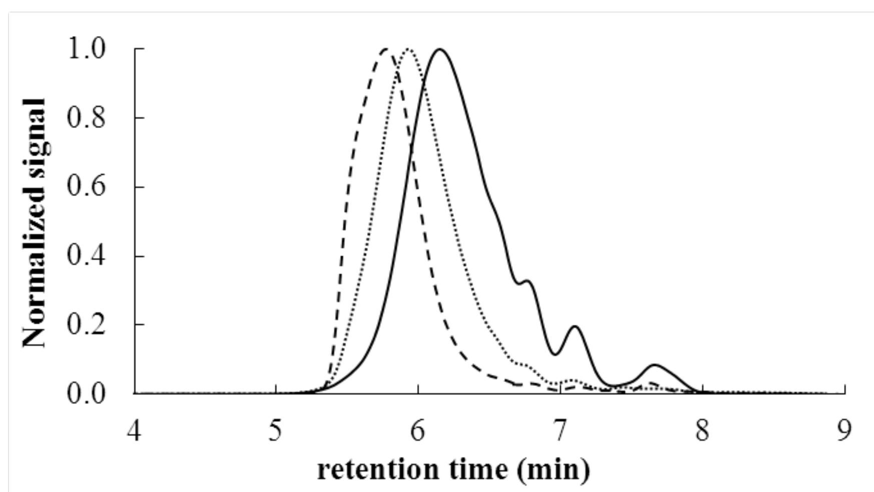

Fig. 9 Superimposed chromatograms of three reaction samples for the reaction carried out in a vacuum reactor (30 g of PR, 2 g of PG, 5 g of immobilized derivative with 16.36 mg protein/g, 40ºC, 160 mmHg, 90 l/h N_2, 450 rpm). Continuous line corresponds to polyricinoleic acid, dotted line to a sample taken after 77.5 h and dashed line to the final PGPR (after 223 h).

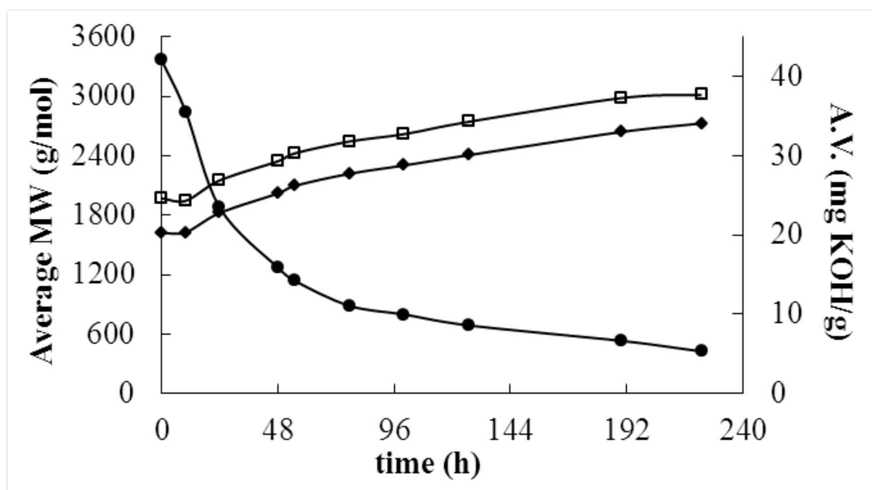

Fig. 10 Comparison of the decrease in the acid value (●) and the increase in the number average molecular weight (♦) and in the weight average molecular weight (□) along the reaction (conditions given in Fig 9).