Enzymatic Reaction – Fractionation Process under SC-CO_2 as a way to Concentrate DHA from Hake Skin Oil

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ABSTRACT

The production of omega-3 concentrates as acylglycerides, especially DHA (docosahexaenoic acid), has acquired a great importance in the last years. In this work, a continuous enzymatic hydrolysis – fractionation process under SC-CO_2 was proposed as a method to concentrate DHA, as acylglyceride, from hake oil. Firstly, some hydrolysis experiments were carried out with a sn-1,3 specific lipase from Mucor miehi immobilized on a macroporous ion-exchange resin at 313 K, at different pressures (18 MPa - 25 MPa) and different CO_2 flow rates (0.5 – 1.9 g/min) in order to evaluate the reaction kinetics. Fixing the reaction conditions at 313 K, 25 MPa and 0.5 g CO_2/min, a subsequent fractionation in two separators was studied changing pressure (8.3 – 25 MPa) and temperature (308 – 333 K) in the first one. It was found that the best separation factors between acylglyceride and FFA, and DHA and palmitic acid respectively, were obtained working in separator 1 at a density close to the critical density of CO_2. In this case, DHA was slightly concentrated as acylglyceride in separator 1.

Keywords: omega-3 concentrates, enzymatic reaction, fractionation, supercritical CO2

INTRODUCTION

The production of polyunsaturated fatty acids, as omega-3 concentrates, especially DHA (docosahexaenoic acid), has acquired a great importance in the last years, both in the food and pharmaceutical industries, due to their benefits in human health. Nowadays, these omega-3 concentrates are mostly commercialised as esters, which are commonly obtained from fish oil and concentrated trough urea complexation or molecular distillation procedures. However, the production of omega-3 concentrates as 2-acylglycerides (2-AG) has become more interesting since this lipid form is more stable against the oxidation [1; 2] and easier to metabolise by humans than the corresponding esters [3].

Taking into account that, in most fish oils, DHA is bounded preferentially to the sn-2 position of triacylglycerides, hydrolysis or ethanolysis reactions catalysed by sn-1,3 specific lipases are a promising way to obtain DHA concentrates as 2-AG. On the other hand, the use of SC-CO2 as reaction media has an enormous attractive not only for its properties (no toxicity, low cost, good mass transport media, etc.), but also because it permits to couple enzymatic reactions with other processes such supercritical extraction, fractionation, chromatography or microencapsulation, which are very interesting for the production of microencapsulated

omega-3 concentrates directly from raw material, minimising the oil manipulation and preventing its oxidation.

The aim of this work was to study a continuous enzymatic hydrolysis – fractionation under SC-CO_2 in order to obtain DHA concentrates as 2-AG from hake oil, which was previously extracted by SC-CO_2 from hake by-products.

MATERIALS AND METHODS

Fish oil was extracted from fish by-products with SC-CO_2 in a semi-pilot plant whose P&I diagram has been presented elsewhere [4]. These fish by-products were mainly the skin offcuts from hakes (*Merluccius capensis – Merluccius paradoxus*) captured in Namibia and provided by Pescanova, a Spanish food company located in Pontevedra (Spain). The offcuts, that consisted mainly of skin with some stuck muscle, were obtained by peeling hakes with a TRIO™ peeler in open seas and then frozen in the fishing boats at -20 ºC. Previous to supercritical fluid extraction (SFE), frozen by-products were cut into small pieces (1-10 mm equivalent diameter, De) with a cutter (CT25, Talleres Cato S.A. Spain) in order to decrease the internal mass transfer resistance during extraction, and freeze-dried (FreeZone 12 Liter Console Freeze Dry System with drying chamber, Labconco) to a moisture content below 20 % (w/w). SFE was carried out at 25 MPa, 313 K and 10 kg CO_2/h, which were reported as optimum conditions in a previous study [5].

Enzymatic hydrolysis - fractionation experiments were carried out in the Institute of Chemical Processes Fundamentals AS CR of Prague (Czech Republic). Enzyme Lipozyme 62 U/g, lipase from *Mucor miehei* immobilised on macroporous anionic exchange resin, was supplied by Novo Nordisk (BioChem Inc.). Carbon dioxide (99.9%) was purchased from Linde Technoplyn (Prague, CR). The solvents used for analysis were classified as analytical grade solvents.

A schematic diagram of the experimental apparatus used in this work is shown in Figure 1. It is composed of a syringe pump (ISCO Inc., Lincoln, NE) for pumping liquid CO_2 and a series of four stainless steel columns (24 cm × 8 mm I.D., 24 cm × 8 mm I.D., and 16 cm × 8 mm I.D.) equipped with sintered metal filters at both ends. The void fraction for all the columns was close to 0.5. The first two columns served as saturators. They were filled with layers of glass beads (2 mm O.D.) and glass wool and wetted with 1–2 ml of distilled water in the first column and fish oil in the second column. In all the experiments, the water saturator column was kept at room temperature, whereas the oil saturation column was immersed in the first water bath, which temperature was maintained within ± 0.1 ºC. The third column, immersed also in the first water bath, was the reactor. It was filled with immobilised enzyme particles mixed with glass beads and distributed in the whole reactor. The last column acts as a first separator (S1) and it was immersed in a second water bath in order to allow a working temperature different from the reactor. This column was also filled with glass beads and glass wool to achieve a better distribution of the non-soluble lipid fraction and avoid an accumulation at the bottom.

During operation, SC-CO_2 carried the dissolved substrates, water and oil, through the reactor filled with enzyme. Two sets of experiments were carried out in order to study both the kinetics of the enzymatic hydrolysis and the fractionation of the reaction products. In the first set, all the reaction products were recovered directly in the second separator, which was maintained at ambient conditions, and the variables studied were the reaction pressure and flow rate. In the second set of experiments, the enzymatic reaction was carried out at fixed

conditions, and the reaction products were fractionated in the two consecutive separators, being the pressure and temperature in separator 1 the studied parameters which influence the separation efficiency.

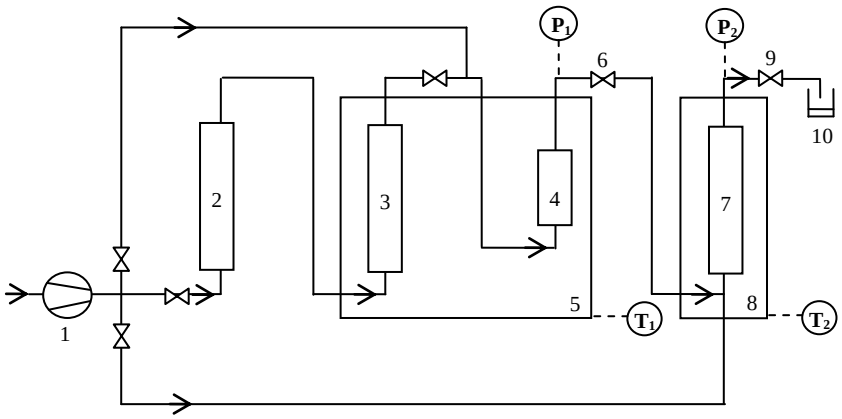

Figure 1. Flowsheet of the experimental equipment used in this work: (1) pump, (2) water saturator, (3) oil saturator, (4) reactor, (5) & (8) water baths, (6) & (9) heated micrometer valves, (7) separator 1, (10) separator 2.

Both enzymatic reaction and fractionation efficiency were evaluated by determining the total free fatty acid amount N_{FFA} (mol), the neutral lipid composition and the fatty acid profile in each sample obtained.

The total amount of N_{FFA} was determined by the colorimetric method proposed by Kwon and Rhee [6] using blue cupric acetate–pyridine reagent. The absorbance was measured at 714 nm.

Neutral lipid composition was determined by liquid chromatography in a HPLC system (Agilent 1200) equipped with a quaternary pump and an auto-injector. The separations were carried out at room temperature in a column (Lichrospher Diol 5mm, 4x 250 mm) and the detection was performed in an Evaporative Light Scattering Detector (ELSD) (Agilent 1200 series) at 45 ºC and 3.5 bar. The mobile phase consisted of (A) hexane / acetic acid (99.5 / 0.5 by volume) and (B) hexane / 1-propanol / acetic acid / water (85 / 14.4 / 0.5 / 0.1 by volume). The linear gradient used is presented in Table 1. The calibration was carried out using standards of palmityl palmitate (99 %), tripalmitin (> 99 %), dipalmitin (99 %), monopalmitin (99 %) and palmitic acid (99 %) in hexane, which showed a good correlation according to the exponential relationship described for an evaporative light scattering detector [7].

The fatty acids profile was determined by GC-FID in samples previously methylated according to the AOAC method [8]. Both equipment and method have been described in a previous work [5].

Table1. Linear gradient for neutral lipid analysis of fish oil by HPLC-ELSD

Time (min)	Flow rate (mL/min)	Mobile phase composition	
		A (%)	B (%)
0	0.8	100	0
20	0.8	80	20
20.5	1.5	80	20
30	1.5	60	40
35	0.8	0	100
40	0.8	0	100

RESULTS

Enzymatic reaction

A set of experiments was carried out in order to study the effect of pressure and flow rate on the reaction rate. The experimental variables, mass of enzyme, M_e; pressure, P; temperature, T; flow rate, Q; superficial velocity in the reactor, u; and residence time, t_s, are reported in Table 2. In all cases, the reaction temperature was fixed at 313 K in order to prevent the thermal oxidation of the omega-3 fatty acids.

Table 2. Experimental conditions of the enzymatic hydrolysis of hake oil under SC-CO_2

Serie	Run	M_e x 10^5 (kg)	P (MPa)	Q x 10^5 (kg CO_2 s^{-1})	u x 10^3 (m s^{-1})	t_s (s)
A	1	48	18	14.7	35.6	2340
	2	51	18	15.0	36.4	6840
	3	47	18	16.9	40.9	10800
B	4	48	20	14.7	34.7	2400
	5	51	20	14.7	34.7	4380
	6	47	20	17.0	40.2	7980
C	7	48	25	14.7	33.2	2400
	8	51	25	13.6	30.7	3180
	9	47	25	17.9	40.5	5220
D	10	48	25	8.8	19.9	4050
	11	51	25	8.8	19.9	5400
	12	47	25	9.6	21.7	11400
E	13	48	25	30.7	69.4	1320
	14	51	25	33.0	74.7	1920
	15	47	25	31.2	70.6	3000

Figure 2 shows the amount of free fatty acids per kg of enzyme found in the reaction product obtained at various experimental conditions and recovered at different sampling times. It is observed that, at the same experimental conditions, the amount of FFA generated per kg of enzyme increases proportionally with the sampling time, which proves that the steady state has been reached in the continuous packed bed reactor. On the other hand, it is observed that the reaction rate increases when both, pressure and flow rate, are increased.

The effect of pressure may be explained considering the amount of reactants (water and oil) at the reactor inlet, which is higher the higher the pressure, due to their solubility increase in SC-CO_2. An increase in the flow rate, also increases the amount of reactants entering the reactor per unit time, and therefore, the reaction rate; however, some improvement of the external mass transfer may also be occurring, as it was observed in previous studies [9].

The experimental data, average reaction rate, V, amount of substrate at reactor inlet, c_o, and superficial velocity in the reactor, u, were simulated using the kinetic model proposed by Sovova et al. [9] for a packed bed reactor with a first order Michaelis-Menten kinetic controlled by the external mass transfer. The fitting parameters, Michaelis-Menten constants, V_{max} and K_m, and the mass transfer constant, K_u, were close to those obtained in previous studies (Table 3).

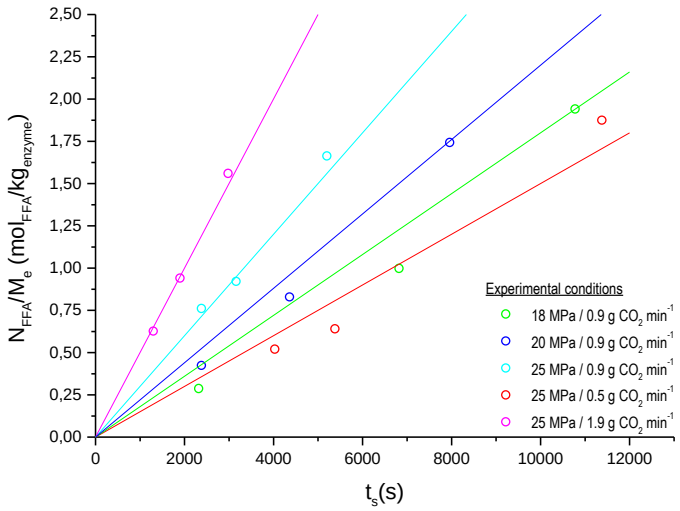

Figure 2. Amount of free fatty acids produced per kg of mass enzyme along the time in the packed bed enzymatic reactor at different reaction conditions.

Table 3. Fitting parameters found in the hake oil enzymatic hydrolysis according to the model described by Sovova et al. [9]

Raw material	K_m (mol m^{-3})	V_{max} x 10^3 (mol s^{-1} kg^{-1})	K_u ($m^{2.17}s^{-0.17}kg^{-1}$)
Hake oil	0,050	0,75	0,10
Blackcurrant oil [9]	0,070	1,7 – 4,9	0,23 – 0,72

Enzymatic hydrolysis – fractionation

After the preliminary study of the enzymatic oil hydrolysis, a set of experiments was performed focusing on the fractionation step. In all cases, the extraction conditions were fixed at 25 MPa, 313 K and 0.9 g CO_2/min. The experimental variables, pressure, P and temperature, T, in separator 1, as well as the SC-CO_2 density, $\rho_{SC\text{-}CO2}$, are summarized in Table 4.

Table 4. Experimental conditions used in separator 1 for studying the enzymatic hydrolysis – fractionation of hake oil.

Run	P (MPa)	T (K)	$\rho_{SC\text{-}CO2}$ (kg / m^3)
1	8.3 ± 0.3	313 ± 1	334 ± 59
2	10.2 ± 0.4	313 ± 1	643 ± 27
3	15.5 ± 0.8	313 ± 1	788 ± 14
4	8.3 ± 0.4	308 ± 1	523 ± 104
5	10.2 ± 0.4	308 ± 1	720 ± 12
6	10.0 ± 0.1	333 ± 1	287 ± 4
7	25.0 ± 0.1	333 ± 1	787.2 ± 0.1

Figure 3 shows that, at constant temperature, the lipid fraction (%wt.) recovered in separator 1 is lower when pressure in this separator is increased. This was expected since the solubility of reaction products in CO_2 increases with increasing pressure, thus, they do not precipitate in separator 1 but reach separator 2 still solubilised in CO_2.

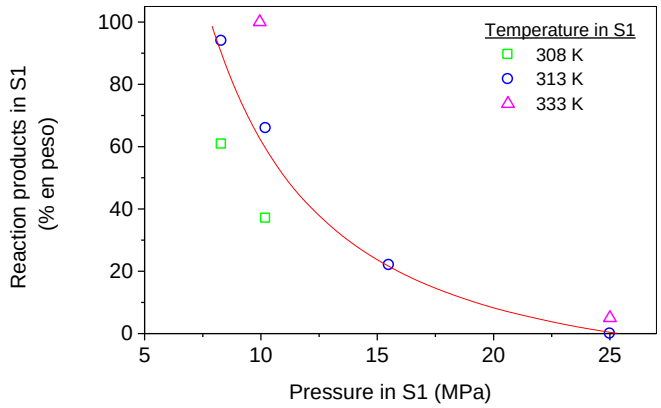

Figure 3. Mass fraction of the reaction products recovered in separator 1 at different experimental conditions.

The amount of product collected in separator 1 ranges from near null, when pressure and temperature in S1 are close to those in the reactor, to 100 % when pressure and temperature in S1, although higher than those at critical point of CO_2, lead to a CO_2 density lower than its critical density.

The relative separation efficiency or selectivity, β, among free fatty acids and other neutral lipids was determined according to the ratio of distribution coefficients (Eq. 1)

$$\beta_{i,FFA} = \frac{k_i}{k_{FFA}} = \frac{w_{i,S2}/w_{i,S1}}{w_{FFA,S2}/w_{FFA,S1}} = \frac{\left(w_i/w_{FFA}\right)_{S2}}{\left(w_i/w_{FFA}\right)_{S1}} \quad (1)$$

where *i* indicates a glyceride compound (TAG, DAG and MAG) and *w* the mass fraction of each component in S_1 and S_2, respectively.

In addition, the relative separation efficiency or selectivity, β, among DHA and palmitic acid (PA), selected as representative of low saturated fatty acids, ($\beta_{DHA,PA}$) was determined according to Eq. 2

$$\beta_{DHA,PA} = \frac{k_{DHA}}{k_{PA}} = \frac{w_{DHA,S1}/w_{DHA,S2}}{w_{PA,S1}/w_{PA,S2}} = \frac{\left(w_{DHA}/w_{PA}\right)_{S1}}{\left(w_{DHA}/w_{PA}\right)_{S2}} \quad (2)$$

Figure 4a shows that the best selectivities were found among TAG and FFA, $\beta_{TAG,FFA}$, and DAG and FFA, $\beta_{DGA,FFA}$, respectively, when the SC-CO_2 density in separator 1 was slightly above its critical density (468 kg/m^3). This behaviour agrees with the experimental results reported in the literature for other separation processes using SC-CO_2 such batch-fractionation of hake oil ethyl esters [10] or countercurrent extraction of tocopherols [11].

The separation factor between MAG and FFA was close to one at all the pressure and temperature conditions studied in S1, indicating a poor separation efficiency of these type of compounds.

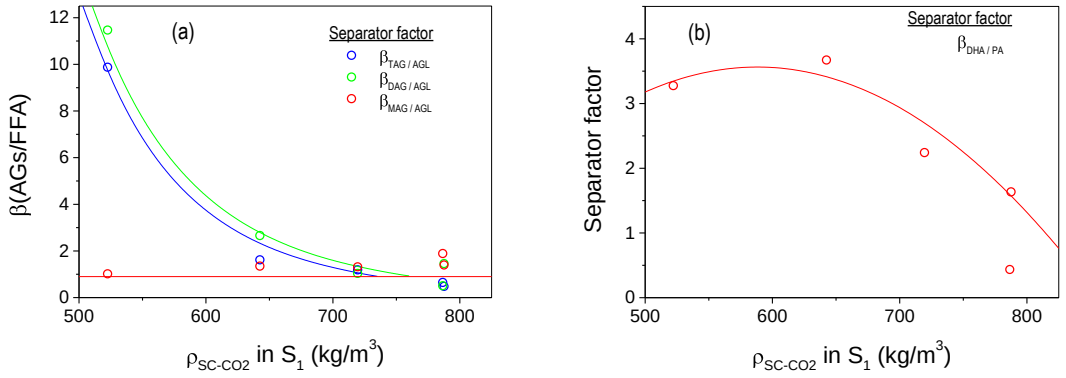

Figure 4. Influence of SC-CO_2 density in separator 1 on the separator factors (a) between AGs and FFA (b) and DHA and PA, respectively.

Figure 4b shows the separation factor between PA and DHA, $\beta_{PA,DHA}$, as a function of the density in S1. Separation factors up to four were observed, indicating good possibilities for separation of this type of fatty acids. These separation factors may be indicating that DHA is mostly in the acylglyceride form (DAG and TAG) and low saturated fatty acids, as PA, are mostly as free fatty acids.

Finally, Figure 5 shows the fatty acid profile of the original hake oil and the two fractions collected in S1 and S2 after the enzymatic hydrolysis – fractionation process. It can be observed that the fraction recovered in separator 1 is slightly enriched not only in DHA ($C_{22:6n-3}$) but also in DPA ($C_{22:5n-3}$), another important omega-3 acid, which increase by around 35 % and 29 % compared to the original hake oil. On the contrary, both palmitic acid ($C_{16:1}$) and oleic acid ($C_{18:1n-9}$) decreased in this fraction by approx. 22 % and 13 % compared to the original hake oil.

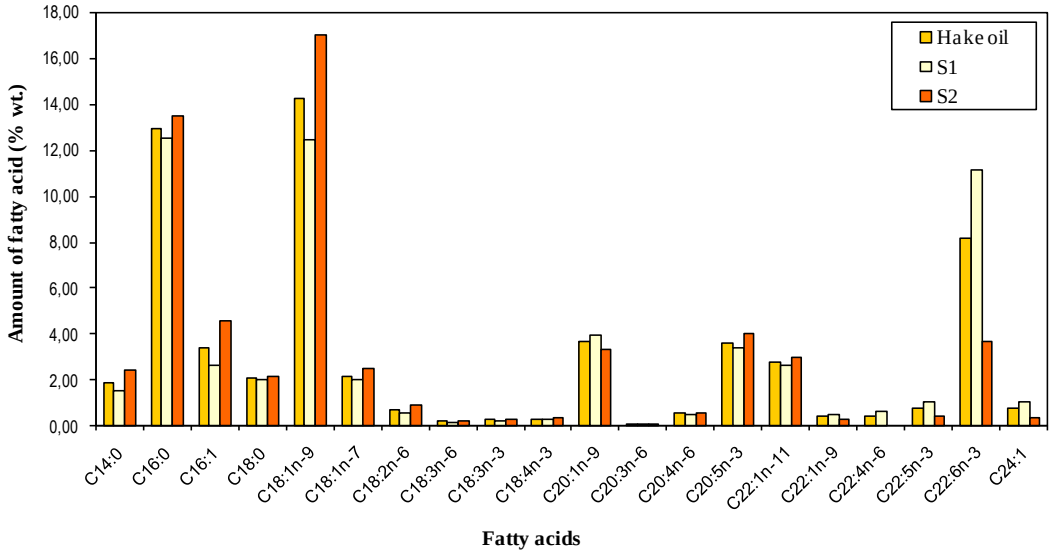

Figure 5. Comparison among the fatty acid profile of the original hake oil and the two fractions obtained after the enzymatic hydrolysis – fractionation process. (Enzymatic hydrolysis conditions: at 25 MPa, 313 K and 0.9 g CO_2/min. Experimental conditions in separator 1: 8.3 MPa, 308 K).

CONCLUSIONS

A continuous enzymatic hydrolysis – fractionation process in a SC-CO_2 media is proposed as a good procedure to produce DHA, concentrated in the acylglyceride form, from hake oil previously extracted by SC-CO_2 (25 MPa and 313 K) from hake by-products. A preliminary study about the kinetics of the enzymatic hydrolysis was carried out by using an immobilized enzyme, sn-1,3 specific, from Mucor miehei at 313 K, different pressures (18 – 25 MPa) and flow rates (0.5 – 1.9 g CO_2/min). It was observed that the reaction rate increased noticeably with pressure, which indicates that the process is controlled by the amount of reactants at

reactor inlet; and also increased with the CO_2 flow rate, which besides increasing the amount of reactants entering the reactor per unit time may be improving the external mass transfer.

Fixing the reaction conditions (313 K, 25 MPa, 0.9 g CO_2 /min), a subsequent fractionation step was studied in two consecutive separators by changing pressure (8.3 – 25 MPa) and temperature (308 – 333 K) in the first one (S1). It was observed that the best separator factor was obtained between TAG and FFA, and DGA and FFA respectively, when the density in separator 1 was slightly above the critical density of CO_2. Moreover, the best separation factor between DHA and PA was also found under these conditions, which may indicate that DHA in the form of tri- or diacylglycerides is slightly enriched in the first separator.

In any case, it must be taken into account that, due to its simplicity, this procedure is not profitable to produce DHA concentrates at commercial scale. Alternative processes, such as this enzymatic reaction performed in an expanded CO_2 media, instead of in a SC-CO_2 media, may improve substantially the reaction rate and would be worth to be explored. Furthermore, the study of the enzymatic oil ethanolysis, as alternative to the enzymatic hydrolysis, is also of great interest in order to improve the fractionation step, since the solubility difference between acylglycerides and fatty acid esters in SC-CO_2 is much higher that the solubility difference between acylglycerides and free fatty acids [12].

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