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Article *in* Food Research International · August 2003

DOI: 10.1016/S0963-9969(03)00052-8

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Concentration of eicosapentaenoic acid and docosahexaenoic acid from fish oil by hydrolysis and urea complexation

N. Gámez-Meza[a,b,*], J.A. Noriega-Rodríguez[a], L.A. Medina-Juárez[a,b], J. Ortega-García[a], J. Monroy-Rivera[b], F.J. Toro-Vázquez[c], H.S. García[b], O. Angulo-Guerrero[b]

[a] *Departamento de Investigaciones Científicas y Tecnológicas de la Universidad de Sonora, Apartado Postal 1819, Hermosillo, Sonora, Mexico*
[b] *Unidad de Investigación y Desarrollo en Alimentos, Instituto Tecnológico de Veracruz, Veracruz, Mexico*
[c] *Facultad de Ciencias Químicas, Universidad Autónoma de San Luis Potosí, San Luis Potosí, Mexico*

Received 2 July 2002; accepted 4 March 2003

Abstract

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) derived from chemically or enzymatically hydrolyzed from sardine oil were concentrated by urea complexation. The enzymatic hydrolysis was effected in aqueous emulsion using five commercial lipases from *Pseudomonas*, three immobilized (PS-CI, PS-CII and PS-DI) and two soluble lipases (AK-20 and PS-30). EPA and DHA were determined by gas–liquid chromatography as methyl esters. Results showed that an immobilized lipase preparation (PS-CI) produced the highest degree of hydrolysis for EPA and DHA (81.5 and 72.3% from their initial content in the oil) after 24 h. After complexation of saturated and less unsaturated free fatty acids, the highest concentration of EPA (46.2%) and DHA (40.3%) was obtained using an ethanolic solution with 20% (w/w) urea and 12% (w/w) PS-CI hydrolyzed with a 78% yield. Combination of enzymatic or chemical hydrolysis with urea complexation is a promising method to obtain highly concentrated n-3 PUFA from sardine oil.

Keywords: DHA; EPA; Hydrolysis; n-3 fatty acids; *Pseudomonas* sp. Lipases; Urea complexation; Fish oil

1. Introduction

The n-3 long-chain polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), have biochemical effects in the prevention or treatment of several human diseases (Harris, 1989; Haumann, 1997). EPA is the precursor of prostaglandins, tromboxanes and leukotrienes, which are effective anti-aggregatory substances. DHA is a component of membrane phospholipids of brain and retina cells, consequently is essential for the human health (Simopoulos, 1996). Therefore, consumption of appropriate amounts of n-3 fatty acids must be considered.

Marine oils are considered the major commercial source of EPA and DHA (Gámez, Higuera, Calderón, Vázquez, Noriega, & Angulo, 1999; Stansby, 1981). However, fish oils are unattractive because of their content of substantial amounts of undesirable fatty acids and cholesterol (Ackman, Ratnayake, & Olson, 1988; Wanasundara & Shahidi, 1999). For this reason, fish oil is preferentially used as raw material to prepare n-3 PUFA concentrates. Unfortunately, it is difficult to purify PUFA if the oil is fractionated as triglycerides. Additionally, it was reported that n-3 PUFA were moderately absorbed by the intestine as triglycerides and most promptly absorbed when free fatty acids (FFA) was given orally (Bottino, Vanderburg, & Reiser, 1967; Henderson, Burkow, & Millar, 1993; Lawson & Hughes, 1988). Therefore, it is convenient to prepare n-3 concentrates as FFA after chemical or enzymatic hydrolysis of marine oils, such as sardine oil.

* Corresponding author. Tel.: +52-662-259-21-69; fax: +52-662-259-21-97.
E-mail address: ngamez@guayacan.uson.mx (N. Gámez-Meza).

0963-9969/03/$ - see front matter © 2003 Elsevier Science Ltd. All rights reserved.
doi:10.1016/S0963-9969(03)00052-8

Chemical hydrolysis may partially destroy the natural all-*cis* n-3 PUFA if the process is inadequately effected, due to the high temperatures involved. The mild conditions used in enzymatic reactions offer a promising alternative to avoid the oxidation and *cis–trans* isomerization.

Lipases (EC 3.1.1.3) are glycerol ester hydrolases that catalyze the hydrolysis of triacylglycerols into fatty acids, partial acylglycerols, and glycerol. Considerable attention has been given to microbial lipases to prepare n-3 fatty acid concentrates as acylglycerols, because it has been presumed that PUFA, mainly EPA and DHA, are resistant to hydrolysis by lipases. However, there are some reports in which lipases from *Chromobacterium viscosum* and *Pseudomonas* sp. released both fatty acids (Garcia, Arcos, Ward, & Hill, 2000; Hoshino, Yamane, & Shimizu, 1990; Tanaka, Hirano, & Funada, 1992, 1994). Within this framework, in this study five commercial lipase preparations from *Pseudomonas* sp. were tested for their enzymatic ability to hydrolyze EPA and DHA from sardine (*Sardinops sagax caeruleus*) oil. Additionally, urea complexation was used in combination with enzymatic and chemical hydrolysis to evaluate the efficiency of the combined process in the preparation of a free n-3 PUFA concentrated.

2. Materials and methods

2.1. Materials

Fresh crude oil from whole sardine (*Sardinops sagax caeruleus*) was obtained from a fishmeal plant located at the Gulf of California, Mexico. Refining (R), bleaching (B), and deodorizing (D) of the sardine oil was carried out according to recommended procedures for fish oil (Bimbo, 1998; Young, 1986). RBD oil was stored under nitrogen at −20 °C, in 4-l sealed dark amber glass containers until used. The reagents (analytical grade) and solvents (chromatography grade) were purchased from Sigma (St. Louis, MO).

The characteristics of the enzymes used and their activities are shown in Table 1. Two lipases were in free form, one from *Pseudomonas fluorescens* and the other from *P. cepacia* (AK-20 and PS-30, respectively). Three were in immobilized form from *Pseudomonas* sp., two lipases supported on chemically modified ceramic, with a crude protein content of 10 and 2% (PS-CI and PS-CII, respectively), and other immobilized on diatomite (PS-DI) with a 3% of crude protein. Amano Pharmaceutical Co. (Nagoya, Japan) kindly provided all lipases (Amano, 1998).

Table 1
Characteristics of the microbial *Pseudomonas* lipases used

Source	Commercial name[a]	Presentation	Protein content (%)	Activity (U/g)
P. cepacia	PS-C I	Immobilized on CMC[c]	10	1671[b]
P. cepacia	PS-C II	Immobilized on CMC[c]	2	1566[b]
P. cepacia	PS-D I	Immobilized on diatomite	3	621[b]
P. fluorescens	AK-20	Soluble	–[d]	2000[e]
P. cepacia	PS-30	Soluble	–[d]	3000[e]

[a] All enzymes were kindly provided by Amano Pharmaceutical (Nagoya, Japan).
[b] Enzyme activity reported by Amano using 1-phenethyl alcohol as substrate.
[c] CMC, chemically modified ceramic.
[d] Not reported.
[e] Enzyme activity reported by Amano using olive oil as substrate.

2.2. Enzymatic hydrolysis

Sardine oil (12 ml, treated with 0.05% w/w *ter*-butylhydroquinone, TBHQ), and phosphate buffer (18 ml of a 0.1 M solution, pH 7.0 and 0.7% v/v Triton X-100) were placed into 50-ml glass bottles. To start the reaction, the different immobilized lipase preparations were added at 0.25, 0.50 or 0.75% (w/w oil basis). Lipase powder was dissolved in 3 ml of 0.1 M phosphate buffer (pH 7.0), before mixing with the emulsion. The bottles were flushed with nitrogen, sealed with rubber caps and parafilm, and placed into an incubator at 40±1 °C with magnetic stirring. Reaction mixture samples (0.5 ml) were withdrawn periodically. Glycerides were extracted with 10 ml of *n*-hexane after adding 3.5 ml of 0.5 N KOH (30% ethanol solution) to neutralize the fatty acid released during hydrolysis. FFA in the water layer were extracted with 10 ml *n*-hexane after returning to acidic pH 1.0 with 4 N HCl as described previously (Shimada, Fukushima, Fujita, Honda, Sugihara, & Tominaga, 1998). FFA were methylated according to the official method for fatty acids Ce 2-66 (AOCS, 1998). The fatty acid methyl esters were analyzed by gas chromatography. The percent hydrolysis was computed by the ratio of EPA and DHA released from their original content in the oil. Large scale hydrolysis was carried out mixing sardine oil (120 ml, containing 0.05% w/w TBHQ), and phosphate buffer (180 ml of a 0.1 M solution, pH 7.0 and 0.7% v/v Triton X-100) in a 500-ml glass bottle at 40±1 °C, 0.5% w/w of PS-CI lipase and magnetic stirring. After 24 h of hydrolysis the mixture was transferred to a separatory funnel and thoroughly extracted with three 80-ml portions of hexane. The lipid fraction was recovered following hexane removal at 40 °C using a rotary evaporator. This fraction was kept under nitrogen at −30 °C until used in the urea complexation.

2.3. Chemical hydrolysis

Two-hundred grams of RBD sardine oil (containing 0.02% TBHQ) were saponified using 400 ml of 7N KOH

in 70% ethanol by refluxing at 90 °C for 1 h. Distilled water (480 ml) was added to the saponified mixture and the unsaponifiable matter was extracted into hexane (2×700 ml) and discarded. The aqueous layer containing saponified matter was acidified to pH = 1.0 with 3 N HCl. The mixture was transferred into a separatory funnel, and the released fatty acids were extracted into hexane (400 ml). The hexane layer containing free fatty acids was then dried over anhydrous sodium sulfate and the solvent removed in a rotatory evaporator at 40 °C under vacuum. Free fatty acids were maintained under nitrogen at −30 °C until used in the urea complexation.

2.4. Urea complexation

FFA (15 g) were mixed with urea (25 g) in 95% aqueous ethanol (100 ml) and heated with stirring until the whole mixture turned into a clear homogeneous solution (Wanasundara & Shahidi, 1999). This solution was transferred to centrifugal tubes and rapidly cooled by immersion in cold water, then kept refrigerated at 5 °C

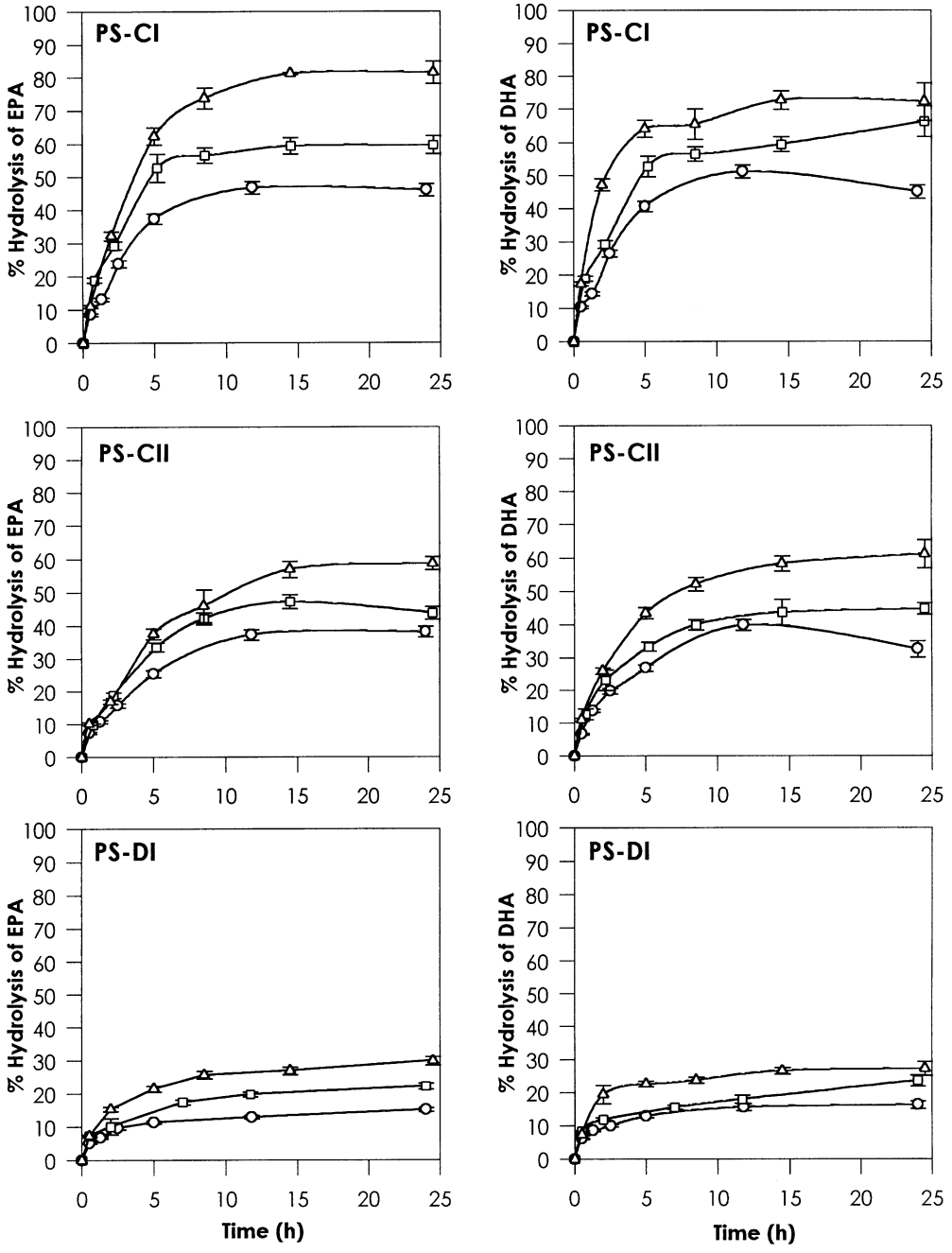

Fig. 1. Enzymatic hydrolysis of EPA and DHA of the sardine oil by different *Pseudomonas* immobilized lipases at 40 °C and pH 7.0. Amount of enzyme (% w/w oil): (○), 0.25; (□), 0.50; (△), 0.75. Values represent means of duplicated±standard deviation.

for 8 h. Crystals were removed by centrifugation (6000×*g*) for 20 min at 5 °C. The supernatant was kept at −30 °C for 12 h then centrifuged again (6000×*g*) at −30 °C for 20 min. Non-complexing supernatant (containing the PUFA) was acidified at pH 4.0 and equal volumes of warm (65 °C) water and hexane were added. After stirred thoroughly for 30 min, separation of the phases and evaporation of solvent, the mass of FFA was determined gravimetrically and the yield was calculated from initials n-3 PUFA.

2.5. Gas chromatography (GC) analysis

FFA were transformed into the corresponding methyl esters with 12% borontrifluoride in methanol (Ce 2-66 AOCS, 1998). A Varian 3400 gas chromatograph, equipped with a flame-ionization detector analyzed the composition of FFA. The column used was Omegawax 250 (30 m×0.25 mm i.d., 0.25 μm film thickness; Supelco, Inc., Bellefonte, PA). The oven temperature was held at 205 °C for 5 min, then increased to 240 °C at 4 °C/min and held at 240 °C for 8 min. The injector and detector were held at 250 and 260 °C, respectively. Nitrogen was used as carrier gas at 20 cm/s flow rate. Identification of the fatty acids was based on a menhaden oil fish standard obtained from Supelco (4-7116). Heptadecanoic acid (C17:0) was used as internal standard (Vazhappilly & Chen, 1998).

2.6. Statistical analysis

Data were expressed as mean ± standard deviation of two different experiments and subjected to one-way analysis of variance and Tukey's test analysis ($P < 0.05$). The computational program Statgraphics Plus 2.0 (Statistical Graphics Corp.) was used to perform statistical analysis.

3. Results and discussion

3.1. Enzymatic hydrolysis

Figs. 1 and 2 show percentages of hydrolysis for EPA and DHA for the *Pseudomonas* lipases investigated as a function of time and the different enzyme concentrations used. In general, immobilized enzymes produced greater EPA and DHA hydrolysis than soluble enzymes, with the exception of PS-DI, which showed low hydrolytic activity for EPA and DHA (29.9 and 27.2%,

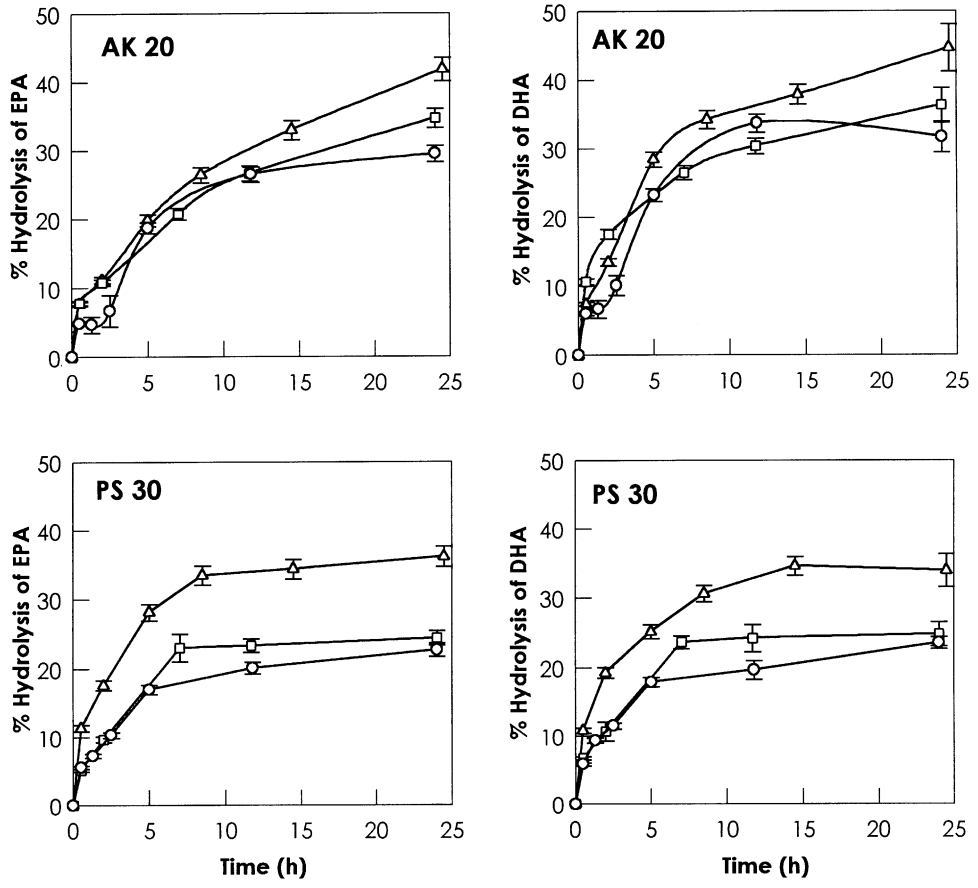

Fig. 2. Enzymatic hydrolysis of EPA and DHA of the sardine oil by different *Pseudomonas* soluble lipases at 40 °C and pH 7.0. Amount of enzyme (% w/w oil): (○), 0.25; (□), 0.50; (△), 0.75. Values represent means of duplicated ± standard deviation.

Table 2
Catalytic efficiency[a] after 24 h hydrolysis of sardine oil

Enzyme amount[b]	PS-CI	PS-CII	PS-DI	AK-20	PS-30
Eicosapentaenoic acid (EPA)					
0.25	0.237±0.008a	0.196±0.007b	0.062±0.002c	0.132±0.004d	0.090±0.003e
0.50	0.133±0.004a	0.097±0.002b	0.048±0.002c	0.070±0.003d	0.057±0.002e
0.75	0.139±0.005a	0.088±0.003b	0.048±0.001c	0.064±0.002d	0.056±0.002d
Docosahexaenoic acid (DHA)					
0.25	0.233±0.019a	0.168±0.016b	0.067±0.005c	0.142±0.012b	0.093±0.006c
0.50	0.148±0.010a	0.103±0.007b	0.045±0.003c	0.074±0.007d	0.058±0.004e
0.75	0.111±0.008a	0.091±0.006b	0.046±0.002c	0.068±0.004d	0.052±0.003c

Values represent means of duplicated±standard deviation. Values in the rows with different letters (a–e) are significantly different.
[a] Catalytic efficiency was determined as mg of hydrolyzed fatty acid/mg enzyme used.
[b] % (w/w oil basis).

respectively). Low activity of PS-DI could be attributed to the hydrophilic nature of the support (Malcata, Reyes, Garcia, & Hill, 1990). PS-CI produced the greatest hydrolysis of EPA and DHA (Fig. 1), probably due to the protein content of the enzyme preparation (Table 1). In the case of free lipases (Fig. 2) the one from *P. fluorescences* (AK-20) at 0.25 and 0.50% (w/w oil) of enzyme was more effective on EPA and DHA hydrolysis than *P. cepacia* lipase (PS-30). Seemingly every lipase investigated showed different hydrolysis rates as a function of the concentration used. Thus, at the lowest enzyme concentration evaluated (0.25% w/w oil basis), PS-CI hydrolyzed ca. 46.0% of EPA and 52.6% of DHA from the original fish oil after 24 h. In contrast, at the highest concentration (0.75% w/w), the same enzyme released 81.5 and 72.3% of EPA and DHA, respectively in the same time period (Fig. 1). Immobilized lipases time course plots showed that the greatest hydrolysis of EPA and DHA was attained after 8 h of reaction. After this time there were no considerable changes on EPA and DHA released. In the case of free enzymes (Fig. 2), there was a continuous increase in the hydrolysis activity. However, the hydrolysis rate decreased as reaction time increased beyond 5 h.

Table 2 shows the catalytic efficiency per mg enzyme for all enzymes and concentrations tested, after 24 h of hydrolysis. Lipase PS-CI showed the highest efficiency ($P<0.05$) on EPA and DHA esters hydrolysis (0.237 and 0.233 mg/mg enzyme, respectively). It can be noted that the degree of hydrolysis did not increase proportionally with the amount of enzyme used, thereby reducing the catalytic efficiency values. Soluble enzyme AK20 at 0.25% showed similar catalytic efficiency to that of immobilized lipase PS-CI at 0.5%.

Table 3 shows the fatty acids composition of the original and hydrolyzed sardine oil by the different

Table 3
Composition of the original and hydrolyzed sardine oil after 24 h of hydrolysis by different lipases[a]

Fatty acid	Total fatty acids (wt.%)					
	Sardine oil	PS-CI	PS-CII	PS-DI	PS-30	AK-20
C14:0	9.84±1.51a	6.20±0.53b,c	6.29±0.24b,c	6.75±0.21b	5.77±0.68c	6.14±0.29b,c
C16:0	20.53±1.39a	11.04±0.78b	11.96±1.16b	11.98±1.34b	10.24±1.36b	11.03±0.99b
C16:1	10.37±1.07a	9.22±0.76a	10.08±1.42a	10.42±054a	9.02±1.40a	10.38±0.54a
C18:0	3.81±0.36a	2.24±0.24b	2.60±0.37b,c	2.95±0.42b,c	3.01±0.30c	3.20±0.35a,c
C18:1	12.48±2.00a	10.56±0.29b	11.80±0.52a,b	12.99±0.70a,c	13.29±1.19a,c	13.44±1.29a,c
C18:2 n-6	2.13±0.44a	2.94±0.24b,c	3.27±0.24c	3.18±0.44b,c	2.52±0.22a,b,c	2.46±0.72a,b
C18:3 n-3	1.08±0.23a,b	0.86±0.16a	0.97±0.11a,b	1.06±0.13a,b	1.17±0.13b	1.02±0.22a,b
C18:4 n-3	2.13±0.18a	1.21±0.31b	1.11±0.14b	0.62±0.01c	0.80±0.25b,c	0.74±0.08c
C20:0	1.57±0.36a	1.92±0.38a,b	2.16±0.16b	2.31±0.35b	2.21±0.12b	2.05±0.30a,b
C20:1	0.48±0.04a,b	0.48±0.11a,b	0.80±0.18b	0.52±0.05a,b	0.45±0.13a	0.57±0.20a,b
C20:4 n-6	0.87±0.19a	0.81±0.21a	0.67±0.14a,b	0.43±0.17b	0.45±0.20b	0.31±0.09b
C20:5 n-3	14.51±1.33a	11.82±1.40b	6.68±0.67c	4.34±0.28d	5.25±0.69c,d	5.37±0.21c,d
C22:5 n-3	1.28±0.39a,b	1.68±0.25a	1.52±0.12a	0.85±0.28c	0.96±0.08b,c	0.84±0.08c
C22:6 n-3	12.55±2.37a	9.07±0.88b	7.36±0.94c	3.42±0.49d	4.85±0.85d	4.52±0.55d

Values represent means of duplicated±standard deviation. These values do not total 100% because minor fatty acids are not reported. Values in the rows with different letters (a–d) are significantly different.
[a] 0.75% (w/w oil basis) enzyme concentration, 40 °C, pH 7 and continuous stirring by 24 h.

enzymes (0.75% w/w) used after 24 h of reaction. We observed that at the beginning of the hydrolysis (3 h) the lipases display a significant preference for saturated fatty acids containing 14–18 carbon atoms (data not showed). However, the resistance to release EPA and DHA was less as the hydrolysis reaction progressed.

Recent studies with *Pseudomonas* lipases have demonstrated the hydrolytic activity for n-3 fatty acids (García et al., 2000; Tanaka, Hirano, & Funada, 1992). In this work, EPA and DHA were hydrolyzed by all lipases tested. PS-DI, PS-30 and AK-20 lipases presented a similar ($P<0.05$) extent. In contrast, significant differences ($P<0.05$) were found for hydrolysis by PS-CI compared with all lipases (Table 3). Results of this study showed that both, the lipase source (*Pseudomonas* species) as well as enzyme preparation (free or immobilized) had different hydrolytic activities on EPA and DHA esters.

3.2. Urea complexation

The fatty acid composition of refined sardine oil and the n-3 PUFA concentrates obtained by urea inclusion are presented in Table 4. Fractionation results show a total reduction in saturated FFA (14:0, 16:0, 18:0, and 20:0) content, large reduction in monounsaturated FFA (16:1 and 18:1) and remarkable increase in n-3 PUFA (18:4, 20:5, and 22:6). The 18:4 n-3 content increased two-fold with PS-CI hydrolyzed and up to 3.5-fold from the original sardine oil, when chemical hydrolyzed was used. Others authors (Ackman et al., 1988; Haagsma, Gent, Luten, Jong, & Doorn, 1982) have reported a significant increase of this fatty acid. A 92.9% of the total fatty acids in the concentrate were n-3. Some studies have reported similar levels of n-3 PUFA in he concentrates using urea-complexation combined with chemical hydrolysis (Ganga, Nieto, Sanhuez, Romo, Speiski, & Valenzuela, 1998; Wanasundara & Shahidi, 1999). EPA was enriched from 14.5 to 46.2% and DHA from 12.5 to 40.3% with a 78.0% yield when PS-CI hydrolyzed was used. When chemical hydrolyzed was used, EPA and DHA were increased at 34.1 and 39.4% with 90.5% yield. In order to achieve a maximum content of total n-3 fatty acids in the concentrate with a reasonable recovery, process variables should be carefully controlled (Wanasundara & Shahidi, 1999).

Our results showed that one immobilized lipase (PS-CI) had the highest lipolytic activity on EPA and DHA esters from sardine oil. Immobilized enzyme preparations offer functionality advantages, such as ease of separation from the products, and the possibility to implement continuous processes via packed-bed reactors. However, reaction times (5–8 h) and costs involved in the enzymatic process could make the chemical hydrolysis preferable at the present.

In conclusion, combination of enzymatic or chemical hydrolysis with urea complexation is a promising method to obtain highly n-3 PUFA concentrated from sardine oil.

Table 4
Fatty acid composition of refined sardine oil and n-3 PUFA concentrate obtained by urea complexation

Fatty acid[a]	Sardine oil	n-3 PUFA Concentrated from Hydrolyzed	
		Chemical[b]	PS-CI[c]
C14:0	8.71±1.51	–[d]	–
C16:0	21.51±1.39	–	–
C16:1	11.12±1.07a	2.95±0.16b	4.50±0.95c
C17:0	1.45±0.33	–	–
C17:1	1.12±0.21	–	–
C18:0	2.07±0.36	–	–
C18:1	13.89±2.00a	4.30±0.66b	2.48±0.82c
C18:2	1.66±0.44	–	–
C18:3 (n-3)	0.98±0.23	–	–
C18:4 (n-3)	2.00±0.18a	7.21±0.68b	4.21±0.88c
C20:0	1.26±0.36	–	–
C20:1	0.48±0.04a	2.52±0.09b	–
C20:4	0.74±0.19a	1.89±0.05b	–
C20:5 (n-3)	14.51±1.33a	34.17±0.68b	46.26±2.71c
C22:5 (n-3)	2.18±0.39a	2.28±0.54a	2.16±0.54a
C22:6 (n-3)	12.55±2.37a	39.47±2.69b	40.32±1.34b
Total	96.11	94.79	99.93
EPA + DHA	27.06	73.64	86.58
Total n-3	32.10	83.13	92.95

Values represent means of duplicated ± standard deviation. Values in the rows with different letters (a–c) are significantly different.

[a] % of total fatty acids.

[b] 90.5% of n-3 PUFA yield.

[c] 78.0% of n-3 PUFA yield.

[d] –, not detected.

Acknowledgements

This work was supported by SIMAC (project no. 980101017) and CONACyT of Mexico. We thank Amano Pharmaceutical for kindly providing the lipases and Refinadora e Hidrogenadora de Guaymas, S.A. de C.V. for providing crude sardine oil.

References

Ackman, R. G., Ratnayake, W. M. N., & Olsson, B. (1988). The "basic" fatty acid composition of Atlantic fish oils: potential similarities useful for enrichment of polyunsaturated fatty acids by urea complexation. *Journal of the American Oil Chemists' Society*, *65*, 136–138.

Amano. (1998) Lipases for Resolution and Asymmetric Synthesis. Amano Pharmaceutical. Co., LTD. Technical Bulletin 961031A.

AOCS. (1998). *Official methods and recommended practices of American Oil Chemists' Society* (5th ed.). Champaign, IL: American Oil Chemists' Society.

Bimbo, A. P. (1998). Guidelines for characterizing food-grade fish oil. *Inform*, *9*, 473–483.

Bottino, N. R., Vanderburg, G. A., & Reiser, R. (1967). Resistance of certain long-chain polyunsaturated fatty acids of marine oils to pancreatic lipase hydrolysis. *Lipids*, *2*, 489–493.

Gámez-Meza, N., Higuera-Ciapara, I., Calderón de la Barca, A. M., Vázquez-Moreno, L., Noriega-Rodríguez, J., & Angulo-Guerrero, O. (1999). Seasonal variation in the fatty acid composition and quality of sardine oil from Sardinops sagax caeruleus of the Gulf of California. *Lipids*, *34*, 639–642.

Ganga, A., Nieto, S., Sanhuez, J., Romo, C., Speiski, H., & Valenzuela, A. (1998). Concentration and stabilization of n-3 polyunsaturated fatty acids from sardine oil. *Journal of the American Oil Chemists' Society*, *75*, 733–736.

Garcia, H. S., Arcos, J. A., Ward, D. J., & Hill, C. G. (2000). Synthesis of glycerides containing n-3 fatty acids and conjugated linoleic acid by solvent-free acidolysis of fish oil. *Biotechnology and Bioengineering*, *70*, 587–591.

Haagsma, N., Gent, C. M., Luten, J. B., Jong, R. W., & Doorn, E. (1982). Preparation of an ω3 fatty acid concentrate from cod liver oil. *Journal of the American Oil Chemists' Society*, *59*, 117–118.

Harris, W. S. (1989). Fish oils and plasma lipid and lipoprotein metabolism in humans: a critical review. *Journal of Lipid Research*, *30*, 785–809.

Haumann, B. F. (1997). Nutritional aspects of n-3 fatty acids. *Inform*, *8*, 428–447.

Henderson, R. J., Burkow, C. I., & Millar, R. M. (1993). Hydrolysis of fish oils containing polymers of triacylglycerols by pancreatic lipase in vitro. *Lipids*, *28*, 313–319.

Hoshino, T., Yamane, T., & Shimizu, S. (1990). Selective hydrolysis of fish oil by lipase to concentrate n-3 polyunsaturated fatty acids. *Agricultural and Biological Chemistry*, *54*, 1459–1467.

Lawson, L. D., & Hughes, B. G. (1988). Human absorption of fish oil fatty acids as triacylclycerols, free acids or ethyl esters. *Biochemistry and Biophysics Research Communications*, *152*, 328–335.

Malcata, F. X., Reyes, H. R., Garcia, H. S., Hill, C. G., & Amundson, C. H. (1990). Immobilized lipase reactors for modification of fats and oils—a review. *Journal of the American Oil Chemists' Society*, *67*, 890–910.

Shimada, Y., Fukushima, N., Fujita, H., Honda, Y., Sugihara, A., & Tominaga, Y. (1998). *Journal of the American Oil Chemists' Society*, *75*, 1581–1586.

Simopoulos, A. P. (1996). Omega-3 Fatty Acids. Part I: Metabolic Effects of Omega-3 Fatty Acids and Essentiality. In G. A. Spiller (Ed.), *Handbook of Lipids in Human Nutrition* (pp. 56–61). Boca Raton, New York: CRC Press.

Stansby, M. E. (1981). Reliability of fatty acid values purporting to represent composition of oil from different species of fish. *Journal of the American Oil Chemists' Society*, *58*, 13–16.

Tanaka, Y., Hirano, J., & Funada, T. (1992). Concentration of docosahexaenoic acid in glyceride by hydrolysis of fish oil with Candida cylindracea lipase. *Journal of the American Oil Chemists' Society*, *69*, 1210–1214.

Tanaka, Y., Hirano, J., & Funada, T. (1994). Synthesis of docosahexaenoic acid-rich triglyceride with immobilized Chromobacterium viscosum lipase. *Journal of the American Oil Chemists' Society*, *71*, 331–334.

Vazhappilly, R., & Chen, F. (1998). Eicosapentaenoic acid and docosahexaenoic acid production potential of microalgae and their heterotrophic growth. *Journal of the American Oil Chemists' Society*, *75*, 393–397.

Wanasundara, U. N., & Shahidi, F. (1999). Concentration of omega 3-polyunsaturated fatty acids of seal blubber oil by urea complexation: optimization of reaction conditions. *Food Chemistry*, *65*, 41–49.

Young, F. V. K. (1986). *The refining and hydrogenation of fish oil (Fish Oil Bulletin 17)*. St. Alban's, Hertfordshire, United Kingdom: International Association of Fish Meal Manufacturers.