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ORIGINAL PAPER

Maximization of the docosahexaenoic and eicosapentaenoic acids content in concentrates obtained from a by-product of rainbow trout (*Oncorhynchus mykiss*) processing

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Abstract
This study was focused on a by-product (i.e., belly muscle) resulting from the commercial processing of rainbow trout (*Oncorhynchus mykiss*). In it, n-3 long-chain polyunsaturated fatty acid (LCPUFA) concentrates were obtained from the belly oil by optimization of the urea-complexation process variables. Thus, the effect of urea:fatty acids (FA) ratio (0–6, w/w), crystallization temperature (− 30 to 30 °C), crystallization time (3.0–48.0 h) and stirring speed (0–1000 rpm) on the eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) contents in concentrates was analyzed by response surface methodology. As a result, high values were obtained for total FA yield recovered, and contents on LCPUFA, EPA and DHA in the non-urea complexing fraction, as well as a great retention of saturated and monounsaturated FA in the urea-crystal adducts. After validation of the model obtained, the combination of process variables levels that maximizes the desirability function (0.91 score) for response variables was 4.21, –15 °C, 24 h and 1000 rpm, respectively. In agreement with the great significance and availability of farmed rainbow trout, belly muscle by-product confirmed to be a profitable source of n-3 LCPUFA to be commercialized as an added-value component.

Keywords *Oncorhynchus mykiss* · Belly by-product · EPA and DHA · Concentrates · Urea complexation · Response surface methodology

Introduction

In agreement with pharmaceutical and dietetic purposes, fish oils are attracting a great attention for their high content on n-3 long chain polyunsaturated fatty acids (LCPUFA) [1, 2]. Consequently, a wide range of chemical and enzymatic methods have been developed to produce LCPUFA concentrates from such kinds of oils [3, 4]. One of the simplest and most efficient technologies for the industrial preparation of LCPUFA concentrates is urea complexation. This technology allows handling large quantities of fish material in a simple equipment, is relatively inexpensive and is based on the fact that saturated and monounsaturated fatty acids (FA) can form more stable urea inclusion compounds than PUFA [5–7]. In this method, free FA resulting from a previous oil saponification are made to react with urea, so that saturated and monounsaturated FA are complexed with urea, being satisfactorily removed from the non-complexed fraction where LCPUFA concentrates are obtained [8, 9].

By-products of aquatic species are body parts that are removed before they reach the final consumer to improve their keeping qualities, reduce the shipping weight or increase the value of the main fish product [10, 11]. They can include different kinds of products such as blood, viscera, heads, bellies, bones, skin, trimmings and fins. Thus, relevant quantities of fats, proteins and other constituents can be present in such by-products, which could be used for human nutrition if properly exploited and utilized [12, 13].

Rainbow trout (*Oncorhynchus mykiss*) has received a great attention because of a wide farming production in many countries. Most previous research has shown a high

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yield of n-3 LCPUFA content of this species [14, 15]. Rainbow trout belly is a by-product resulting from the trimming process, which is obtained from the central part of the stomach after a longitudinal cut of the fish, without removing skin, bones and stapes [16]. In a previous study, the lipid oxidation development was analyzed during the preparation of LCPUFA concentrates from this by-product [17]; as a result, values for the different process variables were optimized to attain a minimum oxidation development (i.e., peroxide, anisidine and polyene values) during the urea-complexation process.

The present research was conducted to optimize the yield of LCPUFA concentrates from rainbow trout belly. For it, the urea-complexation method was applied. The different variables of the complexation process (urea:FA contents ratio, crystallization temperature, crystallization time and stirring speed) were optimized by application of the response surface methodology (RSM) to obtain the highest content of n-3 LCPUFA in concentrates.

Materials and methods

Initial fish material and chemicals

Rainbow trout belly was obtained from an aquaculture facility (Salmones Antártica S.A., Aysen, Chile). After being separated from the remaining body, belly samples were frozen and stored at − 70 °C in 900-g portions in sealed plastic bags until used.

Fatty acid methyl esters (FAME) standards were purchased from NU-CHEK PREP, INC (Elysian, MN, USA), these include methyl esters from 52 different FA ranging from C4:0 to C24:1n-9 (GLC Reference standard 463; Lot 021-U). C23:0 methyl ester (2COT N-23M-A29-4 NU-CHECK-PREP-INC) was employed as internal standard for the quantitative analysis during the gas–liquid chromatography (GLC) assessment. All solvents and chemicals used in the study were reagent grade (Merck, Santiago, Chile).

Oil extraction from the rainbow trout belly

Oil extraction was carried out in agreement with Zuta et al. [5] For it, 100 g of belly muscle was homogenized with a 1800-mL mixture of hexane/isopropanol (3/2, v/v) and stirred for 30 s. The homogenate was then filtered through a Whatman No. 1 filter paper, while the homogenizer, funnel and residue were further washed twice with 50-mL portions of hexane/isopropanol mixture. Pooled filtrates were washed by addition of an aqueous solution of sodium sulfate (50 g/750 mL). Then, the organic layer was separated from the aqueous one in a separatory funnel and dried by filtering it through Whatman paper containing anhydrous sulfate salt. Finally, the solvent was partially removed with a rotary evaporator and the resulting rainbow trout belly oil (RTBO) was stored at − 70 °C under nitrogen atmosphere until being employed.

RTBO characterization

Initial RTBO was characterized by means of different physical and chemical analyses. Standard AOCS [18] official method procedure was employed for the following assessments: free fatty acid (FFA) content (method Ca 5a–40:1), peroxide value (PV; method Cd 8b–90:1–2), *p*-anisidine value (AV; method Cd 18–19:1–2), total oxidation (TOTOX) value (method Cg 3–91), insoluble impurities content (method Ca 3a–46:1), unsaponifiable matter (UM) content (method Ca 6b–53:1–2), iodine value (IV; method Cd 1–25:1–4), refractive index (RI; method Cc7–25) and moisture and volatile matter contents (method Ca 2d–25:1).

Conjugated diene (CD) and triene (CT) formation was measured at 233 and 268 nm, respectively [19], being the results expressed in agreement with the following formula: CD (or CT) = $B \times V/w$, where B is the absorbance reading at 233 (or 268) nm, V is the volume (mL) and w is the mass (mg) of oil measured.

Color parameters (L^*, a^* and b^*) were measured by means of the instrumental color analysis (CIE 1976), performed by employing a tristimulus Hunter Labscan 2.0/45 colorimeter [19]; for each sample analysis, color scores were obtained as mean values of four measurements obtained by rotating the measuring head 90° between triplicate measurements per position.

RTBO saponification

RTBO (400 g) was mixed with a saponifying solution comprising KOH (120 g), H_2O (400 mL) and 96% aqueous ethanol (400 mL; v/v) [20]. The saponification was carried out at 60 °C for 1.5 h, with constant stirring under nitrogen stream. After this period, 200 mL distilled water and 130 mL ethanol were added. Unsaponifiables were separated by extraction with 2 L hexane. The aqueous alcohol phase was acidified to pH 1.0 with 6 N HCl and the resulting FA were recovered by extraction with 2 L hexane. This organic phase was then filtered under anhydrous sodium sulfate, the organic solvent partially removed using a rotary evaporator at 40 °C and the remaining FA solution was stored at − 70 °C, after being flashed with nitrogen.

LCPUFA concentrates preparation

Concentrates from RTBO were prepared by the urea-complexation method [5]. For this process, different conditions of urea/FA contents ratio, crystallization temperature, and

time and stirring speed of the urea/FA mixture were taken into account as further described in the experimental design section. For it, 30 g FA resulting from the RTBO saponification were mixed with varying quantities of urea and 95% ethanol. The mixture was then stirred and heated at 60 °C, so that urea was dissolved and a clear homogeneous solution was produced. In a following step, the urea–FA adducts were allowed to crystallize, urea crystals being separated by filtration through a Whatman No.1 paper with a Büchner funnel. On the other side, the non-urea-complexing fraction was diluted with 100 mL of distilled water, acidified to pH 4.5 with 6 N HCl, and extracted twice with 50 mL of hexane. Both hexane extracts were combined and dried over anhydrous sodium sulfate. The solvent was then partially removed using a rotary evaporator at 45 °C. The resulting LCPUFA concentrates were stored at − 70 °C with 100 ppm of α-tocopherol under nitrogen atmosphere until used for further analysis.

FA analysis by GLC

To analyze the FA composition of the initial RTBO and of the different LCPUFA concentrates, transmethylation and methylation processes, respectively, were carried out to obtain the corresponding FAME. Thus, a two-step conversion was carried out, according to previous research [17]. FAME analysis was performed on an HP 5890 series II GLC. A fused silica capillary column (100 m length × 0.25 mm i.d.) coated with SP™-2560 (Supelco, Bellefonte, PA, USA) was employed. GLC setting conditions were as previously mentioned [17].

DataApex Clarity™ software (DataApex Ltd., Prague, Czech Republic) for chromatogram analysis was used. The concentration of the different FAME was determined from the calibration curves by assessment of the peak/area ratio. NU-CHEK GLC463 was used as standard to identify the FA profiles and DataApex Clarity TM program. Quantification of all kinds of FA (g/100 g total FA) was achieved by employing C23:0 methyl ester as internal standard.

Experimental design and optimization of response variables

The study was performed with a central composite rotatable design 2^4 + star of 28 experiments based on the RSM. The following conditions for the independent (i.e., process) variables were considered (Table 1): urea:FA contents ratio (variable *A*; 0, 1.5, 3.0, 4.5 and 6.0, w/w), crystallization temperature (variable *B*; − 30, − 15, 0, 15 and 30 °C), crystallization time (variable *C*; 3.0, 14.3, 25.6, 36.8 and 48.0 h) and stirring speed (variable *D*; 0, 250, 500, 750 and 1,000 rpm). On the basis of the non-urea complexing fraction, the following response variables (*Y* variables) of the experiment design were chosen: total FA yield (variable Y_1; g FA in the non-urea complexing fraction/100 g initial RTBO FA) and contents of EPA (variable Y_2; g/100 g total FA in concentrate) and DHA (variable Y_3; g/100 g total FA in concentrate).

Four replicates were performed at the central point of the experimental design to estimate the experimental error. All experiments were carried out randomly to minimize the effect of unexplained variability in the observed responses due to extraneous factors. Multiple regression equations were fitted to the responses by discarding non-significant terms ($p > 0.05$) to obtain response surfaces. A multiple-response optimization was performed to optimize several responses simultaneously, this maximizing the desirability function that ranged between 0 and 1 scores.

A quadratic polynomial regression model was assumed for predicting individual *Y* variables. The model proposed for each response of *Y* value is expressed in the following equation:

$$Y_i = \beta_0 + \sum_{i=1}^{4} \beta_i X_i + \sum_{i=1}^{4} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} X_i X_j + \varepsilon.$$

In it, β_0, β_i, and β_{ii} are intercept, linear, and quadratic coefficients, respectively; β_{ij} denotes the interaction coefficient term for the interaction of variables *i* and *j*; X_i represents the process variables and ε corresponds to the random error [21].

Statistical analysis

A statistical analytical system was used for multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of ridge maximum of data in the response surface regression (RSREG) procedure. Estimated response surfaces and contours of estimated response surface were developed using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the process variables with the least effect on the response at a constant value and changing the levels of the other two variables. Analyses were performed in triplicates. The 95% confidence intervals of each quality parameter were calculated, taking into account the number of replicates and considering the standard deviation of each sample. The lack-of-fit test was performed by comparing the variability of the current model residuals with the variability between observations at replicate settings of the factors. Statgraphics®Centuriun XVI-2011 software (StatPoint Technologies, Inc., Rockville, USA) was used.

Table 1 Central composite rotatable design 2^4 + star and values obtained for the different response variables (experimental and predicted)

Run	Process variables[a]				Response variables[b]					
					Experimental values			Predicted values		
	A	*B*	*C*	*D*	Y_1	Y_2	Y_3	Y_1'	Y_2'	Y_3'
1	1.5	−15	14.3	250	52.3	17.7	11.5	50.2	19.5	16.8
2	4.5	−15	14.3	250	8.5	25.6	40.0	15.4	26.9	34.7
3	1.5	15	14.3	250	55.4	17.4	12.1	63.9	19.4	14.0
4	4.5	15	14.3	250	32.2	29.9	21.7	29.0	26.8	19.9
5	1.5	−15	36.8	250	49.0	17.8	11.6	40.2	18.7	14.9
6	4.5	−15	36.8	250	13.3	22.3	29.1	15.1	26.1	32.8
7	1.5	15	36.8	250	58.3	10.3	5.9	53.8	18.6	12.1
8	4.5	15	36.8	250	37.0	23.4	16.1	28.7	26.0	18.1
9	1.5	−15	14.3	750	43.5	23.6	17.7	40.3	19.5	16.8
10	4.5	−15	14.3	750	13.0	28.0	39.9	16.3	26.9	34.7
11	1.5	15	14.3	750	54.0	18.7	12.8	53.9	19.5	14.0
12	4.5	15	14.3	750	29.7	31.0	22.5	29.9	26.8	19.9
13	1.5	−15	36.8	750	23.0	22.5	15.1	30.3	18.7	14.9
14	4.5	−15	36.8	750	18.3	27.2	32.8	16.1	26.1	32.8
15	1.5	15	36.8	750	45.7	18.2	12.0	43.9	18.6	14.0
16	4.5	15	36.8	750	24.7	26.3	20.5	29.7	26.0	18.0
17	0	0	25.6	500	71.3	12.2	8.3	73.5	9.7	0.4
18	6	0	25.6	500	26.3	23.1	17.6	24.4	24.6	24.2
19	3	−30	25.6	500	13.0	18.1	31.8	10.1	19.8	35.3
20	3	30	25.6	500	36.6	22.4	16.2	37.3	19.6	17.8
21	3	0	3.0	500	53.4	20.1	13.7	48.0	23.9	18.1
22	3	0	48.0	500	32.1	27.1	20.2	37.7	22.3	14.4
23	3	0	25.6	0	21.7	27.5	29.9	28.2	31.1	26.6
24	3	0	25.6	1000	21.9	28.2	30.2	19.2	31.1	26.6
25	3	0	25.6	500	24.4	32.3	31.4	23.7	31.1	26.6
26	3	0	25.6	500	27.7	31.2	23.5	23.7	31.1	26.6
27	3	0	25.6	500	20.3	31.6	25.1	23.7	31.1	26.6
28	3	0	25.6	500	23.3	37.0	27.0	23.7	31.1	26.6

[a]Process variables: *A* [urea/fatty acids (FA) contents ratio, w/w], *B* (crystallization temperature, °C), *C* (crystallization time, h), and *D* (stirring speed, rpm)

[b]Response variables: *Y*1 (yield; g FA in the non-urea complexing fraction/100 g initial rainbow trout belly oil FA), *Y*2 (EPA content, g/100 g total FA in concentrate), and *Y*3 (DHA content, g/100 g total FA in concentrate). Corresponding predicted response variables: *Y*1′, *Y*2′ and *Y*3′, respectively

Results and discussion

Characterization of initial RTBO

Data concerning the characterization of the initial RTBO are expressed in Tables 2 and 3. Moisture and volatile matter values were greater than those obtained in previous research concerning crude and refined oils from salmon (Table 2) [19]; however, the insoluble impurities content was found lower than that obtained in such salmon oil samples.

Related to the lipid fraction, unsaponifiable matter content showed similar values to those reported for refined salmon oil, but higher than in the case of crude salmon oil [19]. The unsaturation degree, expressed as the IV, was included in the 130–200 range, which is the normally expected score for fish oil [22]. Related to the acidic degree, the FFA content obtained agreed with previous research related to refined fish oils [19, 23]; according to MINSAL [24], values should be lower than 0.25% to be accepted for human consumption.

Concerning the rancidity stability, CD and CT contents were found low, in agreement with previous research on salmon oils [19]. Similarly, values obtained for the PV, AV and TOTOX value (0.85, 1.48 and 3.18, respectively) revealed a low lipid oxidation development which agreed with previous research on different kinds of fish oils, these including RTBO [17, 19, 23]. Consequently, the present RTBO oxidation values are found under the limits recommended by the "International Fish Oil Standards" for the

Table 2 Characterization of the initial rainbow trout (*Oncorhynchus mykiss*) belly oil

Quality parameter	Score
Moisture and volatile matter (g kg^{-1} oil)	26.9 ± 1.8
Insoluble impurities (g kg^{-1} oil)	0.05 ± 0.03
Unsaponifiable matter (g kg^{-1} oil)	16.1 ± 8.3
Iodine value (g iodine/100 g oil)	165.3 ± 20.2
Free fatty acids (g kg^{-1} oil)	1.0 ± 0.2
Conjugated dienes	0.00 ± 0.00
Conjugated trienes	0.04 ± 0.04
Peroxide value (meq active oxygen kg^{-1} oil)	0.85 ± 0.51
p-anisidine value	1.48 ± 0.77
TOTOX value	3.18 ± 1.15
*a** color parameter	6.20 ± 0.70
*b** color parameter	6.59 ± 0.57
*L** color parameter	6.22 ± 0.38
Refractive index (n_D 40 °C)	1.4767 ± 0.0000

Values expressed as mean ($n = 9$) values ± standard deviation

human consumption (15 and 19.5 for AV and TOTOX value, respectively) [25], as well as under the limits (20 and 26, respectively) recommended by the guidelines for Good Manufacturing Practice of Fish Oil (Global Organization for EPA and DHA Omega-3) [26] and the Council for Responsible Nutrition [27].

Concerning physical properties of the RTBO, color analysis revealed mean scores of 6.20 ± 0.70, 6.59 ± 0.57 and 6.22 ± 0.38 for *a**, *b** and *L** parameters, respectively. Pando et al. [19] found similar values for the *b** value when crude and refined salmon oils were tested; however, lower *L** scores and higher *a** values were obtained in the present research.

The RI of the RTBO was 1.4767. Previous research shows that the RI of different fish oils (cod, *Gadus morhua*; herring, *Clupea harengus*; sardine, *Sardinops caerulea*) was included in the 1.4600–1.4810 range [22]. Concerning non-marine oils and fats, RI value for tallow was 1.4580 [28], whereas a 1.4660–1.4700 range was reported for soybean oil [24]. The RI of oils has been reported to be characteristic within certain limits for each kind of oil. The RI value would be related to the degree of saturation but it is also affected by other factors such as FFA content, oxidized degree and heat treatment undergone during its processing.

Results concerning the FA composition of the RTBO are depicted in Table 3. The two most abundant FA were C18:1n-9 and C16:0, followed by C20:5n-3, C16:1n-7 and C22:6n-3. A similar pattern has been described for previous research on RTBO [17] as well as on the muscle tissue of rainbow trout from seawater and freshwater [14]. When the FA groups are considered in the current study, the distribution obtained indicated the following proportions: 28.52% (saturated), 36.23% (monounsaturated), 35.25% (polyunsaturated), and 28.31% (n-3 polyunsaturated).

Production of LCPUFA concentrates

According to the experimental design shown in Table 1, 28 trials were carried out. This table includes the experimental values obtained for the different response variables (Y_1, Y_2 and Y_3), as well as those concerning the predicted values for such corresponding variables when experimental values are replaced by application of the model (Y_1', Y_2' and Y_3' values).

As a result, all the process variables (*A*–*D*) significantly ($p < 0.05$) affected the response of the three variables during the urea-complexation process. Additionally, the enrichment of EPA (Y_2) and DHA (Y_3) in concentrates varied inversely to total FA yield (Y_1), so that correlation coefficient values (r) obtained were −0.7230 and −0.8870 ($p < 0.05$), respectively. A similar relationship among response variables was obtained when predicted values are considered; thus, predicted EPA (Y_2') and DHA (Y_3') contents provided correlation coefficients values (r) of −0.7060 and −0.8996 when compared with the predicted total FA yield (Y_1'), respectively.

In previous research, Liu et al. [9] also proved that the content of EPA and DHA was inversely related to the liquid recovery yield when tuna (*Thunnus albacares*) oil was studied. Related to seal blubber (*Phoca groenlandica*) oil employment, Wanasundara and Shahidi [8] showed that DHA was found almost exclusively in the non-urea complexing fraction whereas a small proportion of EPA was invariably complexed with urea. In agreement with the actual results, several works have proved that EPA has a higher tendency than DHA to form urea adducts, this leading to lower contents in PUFA concentrates for EPA than for DHA [8, 9, 29].

Effect of process variables on response variables: regression coefficients and Pareto charts

The quadratic polynomial equation adjusted for predicted models of total FA yield (Y_1), and contents of EPA (Y_2), DHA (Y_3) and EPA + DHA (Y_4) are described in Table 4. This table shows the regression coefficients of predictive second-order polynomial model for response variables. The results of fitting a multiple regression model describe the effect of the different process variables on the response variables considered for the LCPUFA concentrate preparation by the urea-adduct process. Regression coefficients were removed from Table 4 in cases where p values obtained from the ANOVA analysis were found higher or equal to 0.05, so that they were not considered statistically significant at the 95% or higher confidence level. After the stepwise elimination of the non-significant effects ($p > 0.05$),

Table 3 Composition of fatty acids (FA) and FA groups in the initial rainbow trout belly oil (RTBO) and in the optimized concentrate (g/100 g FA) after validation

Individual fatty acids[a]			RTBO	Optimum concentrate[b]
Trivial name	Systematic name	Abbreviated name		
Lauric	Dodecanoic	12:0	0.05	ND[c]
Myristic	Tetradecanoic	14:0	5.68	0.40
Palmitic	Hexadecanoic	16:0	17.90	0.18
Palmitelaidic	9*t*-hexadecenoic	16:1n-7	0.32	ND
–	7c-hexadecenoic	16:1n-9	0.22	ND
Palmitoleic	9c-hexadecenoic	16:1n-7	8.72	0.64
–	11c-hexadecenoic	16:1n-5	0.23	ND
–	13c-hexadecenoic	16:1n-3	0.46	0.30
–	Heptadecanoic	17:0	1.01	ND
–	10c-heptadecenoic	17:1n-7	0.01	0.80
Stearic	Octodecanoic	18:0	3.76	ND
Oleic	9c-octadecenoic	18:1n-9	19.48	5.95
Cis-vaccenic	11c-octadecenoic	18:1n-7	3.73	ND
Linoleic	9c,12c-octadecadienoic	18:2n-6	4.40	1.10
–	9c,15c-octadecadienoic	18:2n-3	0.65	ND
Gamma Linolenic	6c, 9c, 12c-octadecatrienoic	18:3n-6	0.14	0.82
Arachidic	eicosanoic	20:0	0.12	ND
Alpha linolenic	9*c*,12*c*,15*c*-octadecatrienoic	18:3n-3	0.66	0.28
–	8c-eicosenoic	20:1n-12	0.09	ND
–	11c-eicosenoic	20:1n-9	2.30	ND
Stearidonic	6c,9c,12c,15c-octadecatetraenoic	18:4n-3	1.39	8.31
–	11c,14c-eicosadienoic	20:2n-6	1.66	6.65
Dihomo-gamma linolenic	8c,11c,14c-eicosatrienoic	20:3n-6	0.20	ND
–	11c,14c,17c-eicosatrienoic	20:3n-3	0.45	0.36
–	8c,11c,14c,17c-eicosatetraenoic	20:4n-3	0.52	0.33
Erucic acid	13c-docosenoic	22:1n-9	0.29	0.26
EPA	5c, 8c,11c,14c,17c-eicosapentaenoic	20:5n-3	12.42	20.50
Nervonic	15c-tetracosenoic	24:1n-9	0.38	ND
DPA or clupanodonic	7c,10c,13c,16c,19c-docosapentaenoic	22:5n-3	5.25	2.10
DHA	4c,7c,10c,13c,16c,19c- docosahexaenoic	22:6n-3	7.51	51.02
Fatty acids groups				
Total saturated FA			28.52	0.58
Total monounsaturated FA			36.23	7.95
Total polyunsaturated FA			35.25	91.47
Total n-3 LCPUFA			26.15	74.31
EPA + DHA			19.93	71.52

[a]FA are referred by their trivial, systematic and abbreviated names

[b]Composition of the optimum concentrate obtained after validation with the optimized process factors from Table 5, Parts b and c

[c]ND: Not detected

response-predicting models were obtained (Table 4). Thus, the regression analysis of the results indicated that the coefficients of determination (R^2 parameter) for total FA yield, EPA, DHA and EPA + DHA contents variables were 0.9219, 0.7843, 0.8345 and 0.8427, respectively ($p < 0.05$).

The lack-of-fit test was designed to determine whether the selected models are adequate to describe the observed data, or whether a more complicated model should be used. Since the p values for lack-of-fit cases in the ANOVA assessment were greater or equal to 0.05, all the predicted models appeared to be adequate for describing the results obtained for the response variables at the 95.0% confidence level (Table 4).

Table 4 Regression coefficients and *p* values of predictive second-order polynomial model for the different response variables

Process variables[a]	Response variables							
	Y_1 (fatty acid yield)		Y_2 (EPA content)		Y_3 (DHA content)		Y_4 (EPA + DHA content)	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Constant	130.56		− 2.48		− 12.77		− 15.81	
Linear								
A	− 32.34	0.00	11.82	0.01	13.48	0.00	25.80	0.00
B	0.45	0.00	− 0.00	0.95	0.11	0.01	− 0.30	0.02
C	− 2.60	0.03	0.78	0.51	0.95	0.27	1.81	0.27
D	− 0.03	0.04	0.00	0.09	0.00	0.24	0.01	0.09
Quadratic								
$A \times A$	2.81	0.00	− 1.56	0.01	− 1.58	0.01	− 3.23	0.00
$B \times B$			− 0.01	0.01			− 0.02	0.03
$C \times C$	0.04	0.00	− 0.02	0.03	− 0.02	0.03	− 0.04	0.01
$D \times D$								
Interaction								
$A \times B$					− 0.13	0.04		
$A \times C$	0.14	0.05						
$A \times D$	0.01	0.04						
$B \times C$								
$C \times D$								
Lack of fit		0.16		0.37		0.36		0.31
R^2	0.9219		0.7843		0.8345		0.8427	
Adjusted								
R^2	0.8890		0.7088		0.7765		0.7877	
SE	3.06		2.69		3.42		4.80	
MAE	3.69		2.19		3.04		4.59	
DW value	1.84	0.41	2.00	0.38	2.10	0.54	2.53	0.87
Lag 1 residual autocorrelation		0.075	− 0.075		− 0.071		− 0.310	

*R*2 regression coefficient, *SE* standard error, *MAE* mean absolute error, *DW* Durbin–Watson

[a]Process variables (*A, B, C* and *D*) as expressed in Table 1

Focused on tuna oil, Liu et al. [9] found that the regression models for the total FA yield and the total content of EPA and DHA were highly significant with satisfactory coefficients of determination (0.99 and 0.97, respectively). Concerning seal blubber oil employment, Wanasundara and Shahidi [8] found that the regression models for data on total n-3 FA and DHA were highly significant ($p < 0.01$) with satisfactory R^2 values (0.99 and 0.93, respectively).

In the current study, the total FA yield (Y_1) showed to be significantly affected by the urea-complexation process ($p < 0.01$; Table 4). The lineal terms of urea:FA contents ratio (A), crystallization temperature (B), crystallization time (C), and stirring speed (D), the quadratic terms of urea:FA contents ratio (AA) and crystallization time (CC), the interaction between urea:FA contents ratio and crystallization time (AC) and the interaction between urea:FA contents ratio and stirring speed (AD) showed a significant effect on total FA yield in the urea-complexation process ($p < 0.01$).

In the case of the EPA content (Y_2, Table 4), regression coefficients indicated that the linear term of urea:FA contents ratio (A) and the quadratic terms of urea:FA contents ratio (AA), crystallization temperature (BB) and crystallization time (CC) were significant ($p \leq 0.05$). For DHA content (Y_3, Table 4), regression coefficients indicated that linear terms of urea:FA contents ratio (A) and crystallization temperature (B), the quadratic terms of urea:FA contents ratio (AA) and crystallization time (CC) and the interaction between urea:FA contents ratio and crystallization temperature (AB) were found significant ($p \leq 0.01$), suggesting that they could be determinant for the amount of DHA in the final concentrate.

For EPA + DHA content (Y_4, Table 4), regression coefficients indicated that linear terms for urea:FA contents ratio (A), crystallization temperature (B), stirring speed (D), the quadratic terms of urea:FA contents ratio (AA), crystallization temperature (BB) and crystallization time (CC) were significant in the urea-complexation process ($p \leq 0.01$), suggesting that they could exert a decisive effect on the EPA + DHA content in the final concentrate.

Previous research on tuna oil employment [9] indicated that for the total FA recovery yield, quadratic terms of the urea:FA contents ratio and crystallization temperature were highly significant, but crystallization time was not significant ($p > 0.05$); additionally, when the total content of DHA and EPA was considered, linear and quadratic terms of urea:FA contents ratio and crystallization temperature were found significant, while crystallization time did not show an effect on the complexation process ($p > 0.05$).

Effect of process variables on response variables: analysis by RSM

The linear, quadratic and interaction terms in the second-order polynomial were used to generate a three-dimensional response surface graph. The process variables were subsequently placed in the different axes to analyze their influence on the four response variables (Fig. 1).

In this figure, Panel a shows the response surface for total FA yield as a function of the crystallization temperature and urea:FA contents ratio. It can be observed that the total FA yield in the liquid recovery in the non-urea complexing fraction increased with the crystallization temperature; contrarily, it decreased with the urea:FA contents ratio. Additionally, the total FA yield presented a minimum value in the response surface at high levels of urea:FA contents ratio, at low levels of crystallization temperature and stirring speed, and at intermediate levels of crystallization time. Similar results were found by Liu et al. [9], who showed that the value of the liquid recovery yield presented a minimum in the response surface for the effect of urea:FA contents ratio and crystallization temperature when tuna oil was investigated. In the current study, the analysis of the regression results (adjusted R^2 coefficient) provided a variability value of 88.9% ($p \leq 0.05$) in the experimental design for total FA yield (Table 4).

Figure 1 (Panel b) exhibits the response surface of the urea-complexation process for EPA content in LCPUFA concentrates. It can be observed that the EPA content increased with the urea:FA contents ratio and crystallization temperature, while crystallization time led to a maximum content of EPA at intermediate levels ($p \leq 0.05$); contrarily, the EPA content was not affected by the stirring speed ($p > 0.05$). According to Guil-Guerrero and Hassan [20], the recovery in the urea-inclusion method from cod liver oil was strongly enhanced by application of orbital agitation during the crystallization process, in which EPA yield increased from 60 to 70% without stirring to 90–97% when a 800-rpm stirring speed was applied; meantime, DHA yield was shifted from 53–73% to 85–99%, respectively. In the actual study, the R^2 adjusted coefficient indicated that the fitted model explained 70.9% of the variability of EPA content ($p \leq 0.05$), being the SE score of 2.69 (Table 4). Meantime, a mean absolute error (MAE) value of 2.19 indicated the average value of the residuals. The Durbin–Watson (DW) value was greater than 0.05, so that there was no indication of serial autocorrelation in the residuals ($p > 0.05$).

Figure 1 (Panel c) shows the response surface for DHA content as a function of the crystallization temperature and urea:FA contents ratio. The amount of DHA increased with the urea:FA contents ratio and by reducing the crystallization temperature. This result agrees with the inverse relationship found between the urea:FA contents ratio and

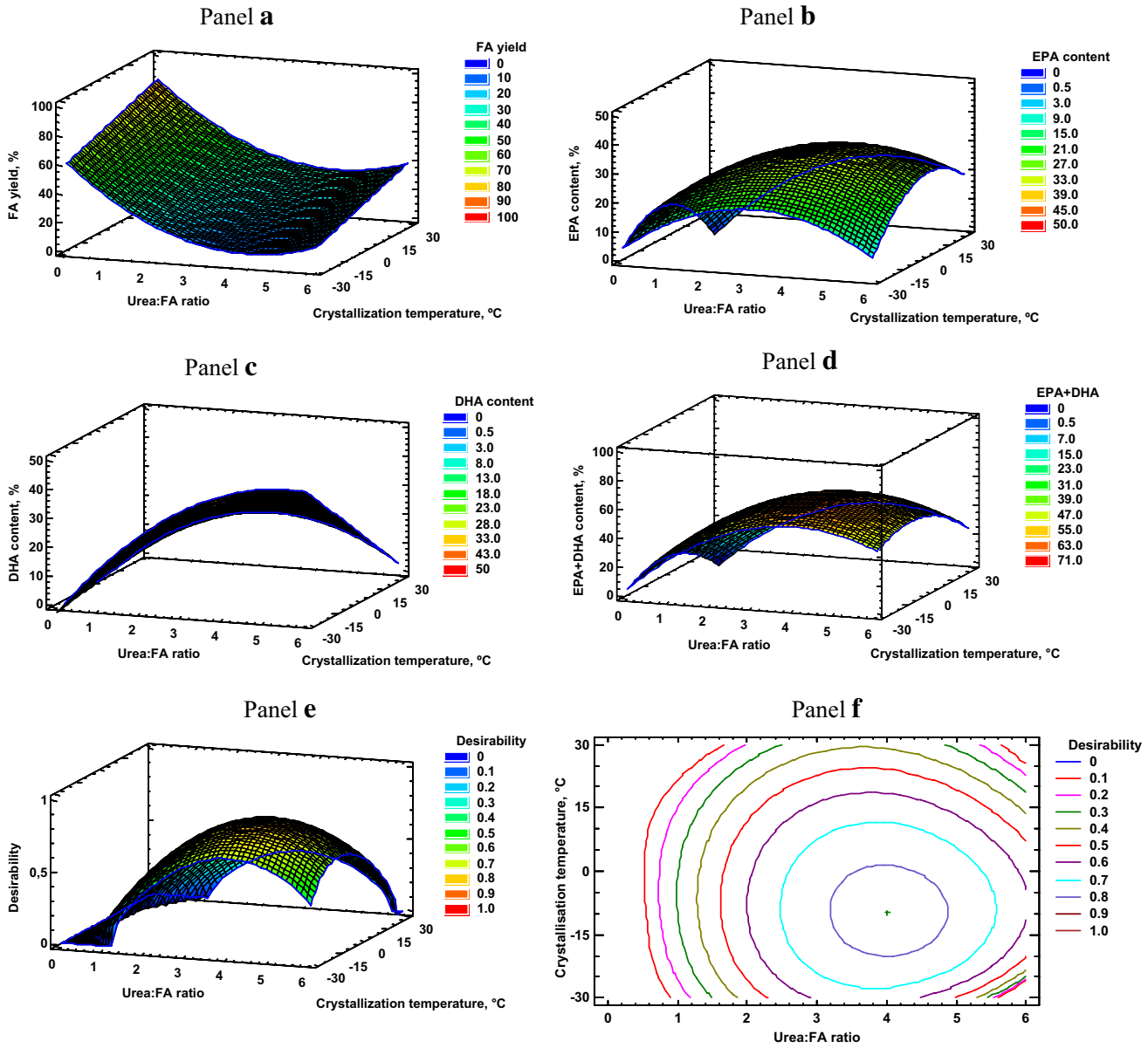

Fig. 1 Effect of urea:fatty acids (FA) contents ratio and crystallization temperature on the: **a** total FA yield; **b** EPA content (g/100 g total FA); **c** DHA content (g/100 g total FA); **d** EPA + DHA content (g/100 g total FA); **e** desirability function; **f** contours of estimated response surface

the crystallization temperature, which has been described by several authors concerning the employment of other marine oils [8, 9]. In the current study, the results suggested that a linear significant ($p \leq 0.05$) effect of a lower crystallization temperature and a linear and quadratic effect of a high urea:FA contents ratio produced a higher DHA content in LCPUFA concentrates during the urea-complexation process. The adjusted R^2 value indicated that the model fitted explained 77.7% of the variability of DHA ($p < 0.05$) (Table 4), while the standard error (SE) showed the standard deviation of the residuals to be 3.42. A MAE score of 3.04 indicated an average low value for the residuals. The DW value was of 2.10, so that there was no indication of serial autocorrelation in the residuals ($p > 0.05$).

Figure 1 (Panel d) shows the response surface for EPA + DHA content as a function of the crystallization temperature and urea:FA contents ratio. It can be observed that both variables led to a maximum content of EPA + DHA at intermediate levels. The adjusted R^2 value indicated that the model fitted explained 78.77% of the variability of the EPA + DHA content ($p \leq 0.05$) (Table 4).

Optimization of the process variables by means of the RSM

Table 5 shows the combination of factor levels which maximizes the response variables in concentrates for the indicated region (Fig. 1, Panels e and f). A ratio of 3.8 for urea:FA contents ratio, a crystallization temperature of 0 °C, a crystallization time of 24.4 h and a stirring speed of 1000 rpm was the combination that maximized the EPA production, thus leading to a maximum predicted value of 34.8% (Table 5, Part a). For DHA, scores combination of 5.50 (urea:FA contents ratio), − 30 °C (crystallization temperature), 23.3 h (crystallization time) and 1000 rpm (stirring speed) led to a maximum predicted value of 47.5% (Table 5, Part a). When both FA are considered (EPA + DHA content), a similar pattern for the process variable values was found necessary to obtain the highest production (68.2%; Table 5, Part a); thus, 4.0, − 9, 24 and 1000 values, respectively, led to such highest stationary point. As previously mentioned, a higher DHA content in concentrates than in EPA can be explained on the basis of its lower tendency to form urea adducts.

These results agree with previous research which has reported that DHA is the most abundant acid in the non-urea complexing fraction during urea-complexation experiments carried out on cod liver oil [29]. Meantime, Wanasundara and Shahidi [8] found that although a major portion of EPA was recovered in the non-urea complexed fraction of seal blubber oil, a small proportion was detected to be invariably complexed with urea, this leading to a lower proportion of EPA than DHA in concentrates. In such experiment, 70.1 and 67.6% scores for the DHA content (predicted and observed, respectively) were obtained, but the content of EPA in the concentrate was predicted to decrease by increasing the DHA content; thus, a 9.36% value in the minimum stationary point was reached for the EPA content but a negligible value was reported to be observed. Concerning the evaluation of the EPA + DHA content, previous research has shown to reach a maximum stationary point of 89.4% [9].

Table 5 (Part b) indicates the combination of variables levels which maximizes the desirability function over the indicated region, so that the optimum situation would be attained. A combined response surface of the optimized response variables was obtained on the basis of the responses obtained for EPA, DHA and EPA + DHA contents (Fig. 1, Panel e). A maximum desirability of 0.91 score (0–1 range) was obtained in the multiple-response optimization of EPA, DHA and EPA + DHA contents (Table 5, Part b). As a result, a maximum EPA + DHA content (67.7/100 g total FA) could be attained, provided the following process conditions were applied: 4.21 (urea:FA contents ratio), − 15.0 °C (crystallization temperature), 24.0 h (crystallization time) and 1000 rpm (stirring speed). The predicted values for the maximum stationary points for EPA and DHA contents were 32.5 and 37.0%, respectively. Figure 1 (Panel f) shows the contours of the estimated response surface of urea/FA contents ratio and crystallization temperature. It can be concluded that the most convenient conditions to be employed to reach high EPA and DHA contents should include high scores of urea:FA contents ratio, crystallization time and stirring speed, but low crystallization temperature values.

Results in the present study suggest that a linear and significant ($p \leq 0.05$) effect of stirring speed on the total FA yield in the urea-complexation process was attained. Previous research concerning the effect of stirring speed as a process variable in the urea-complexation process can be considered scarce. Thus, Guil-Guerrero and Hassan [20] checked it during complexation of cod liver oil, although its effect on the recovery of EPA and DHA was only studied at temperatures above 16 °C; in such study, a significant

Table 5 Process variables optimization and multiple-response optimization of the response variables

Response variable	Process variable				Stationary point	Optimum value[a]
	A	*B*	*C*	*D*		
Part a: optimization of the process variables						
EPA	3.80	0	24.4	1000	Maximum	34.8
DHA	5.50	− 30	23.3	1000	Maximum	47.5
EPA + DHA	4.00	− 9	24.0	1000	Maximum	68.2
Part b: multiple response optimization of the response variables						
EPA						32.5
DHA	4.21	− 15	24.0	1000	Maximum	37.0
EPA + DHA						67.7
Maximum desirability						0.91
Part c: experimental validation of the multiple response optimization of the response variables						
EPA + DHA	4.21	− 15	24.0	1000	Maximum	71.52

Process variables (*A, B, C* and *D*) as expressed in Table 1

[a]Values expressed as g/100 g total fatty acids

recovery increase of both acids in PUFA concentrates was concluded by means of a stirring speed increase, in agreement with the present results. It has to be pointed out that the present research takes into account lower complexation temperatures, so that lipid oxidation development during PUFA concentrates preparation can be minimized [15, 17].

Previous research has focused on the rancidity stability of LCPUFA concentrates obtained from RTBO by urea-complexation process [17]. Taking into account the model proposed in such study, the current combination of the process variables at which the optimum EPA + DHA content was attained, would lead to scores of 7.53, 2.79 and 17.85 for PV, AV and TV, respectively. Such values would be found under the limits recommended by the "International Fish Oil Standards" for the human consumption (15 and 19.5 for AV and TOTOX value, respectively) [25], as well as under the limits (20 and 26 for AV and TOTOX value, respectively) recommended by the guidelines for Good Manufacturing Practice of Fish Oil [26] and the Council for Responsible Nutrition [27]. Additionally, before considering the current concentrates for common and commercial consumption, safety international requirements ought to be addressed and fulfilled.

FAME analysis of optimized PUFA concentrates and validation of the optimized process

The FA composition of the optimized LCPUFA concentrates is given in Table 3. The validation of the optimized process was carried out by combination of the factors at which the optimum EPA + DHA content was reached (Table 5, Part c). According to the RSM analysis, the amount of total EPA + DHA content was increased 3.6 times from the initial belly oil value (19.93%) to the LCPUFA concentrate content (71.52%) after being validated with the optimized factors of the process.

Contrary to the FA composition of the initial RTBO (Table 3), the most abundant FA found in the optimum concentrate were (g/100 g total FA): C22:6n-3 (DHA) (51.02%), C20:5n-3 (EPA) (20.50%), C18:4n-3 (8.31%), and C20:2n-6 (6.65%); additionally, a marked content decrease could be obtained after urea complexation in saturated (C14:0, C16:0 and C18:0) and monounsaturated (C16:1n-7, C18:1n-9 and C20:1n-9) FA. When the FA groups are considered, initial RTBO and optimized LCPUFA concentrates provided marked differences in saturated (28.52 vs. 0.58%), monounsaturated (36.23 vs. 7.95%), polyunsaturated (35.25 vs. 91.47%), n-3 long chain polyunsaturated (26.15 vs. 74.31%) FA, and in DHA + EPA content (19.93 vs. 71.52%). Previous studies have obtained 70–90% scores for n-3 PUFA concentrates from cod liver oil [29] and from mackerel processing waste [5], and maximum stationary points of 89.38% for EPA + DHA content from tuna oil [9] and 70% for DHA content in seal blubber oil [8].

Conclusions

Optimization of the LCPUFA concentrates yield from the RTBO was carried out. Thus, maximization of the EPA and DHA contents in concentrates was obtained by employment of RSM. After validation of the model obtained, the combination of process variable levels which maximizes the desirability function (0.91 score) for response variables was 4.21 (urea:FA contents ratio), − 15 °C (crystallization temperature), 24 h (crystallization time) and 1000 rpm (stirring speed); such combination led to a 71.52 (g/100 g total FA) value for the EPA + DHA content.

On the basis of the great industrial significance and availability of rainbow trout in many countries, its belly muscle by-product confirmed to be a profitable source of n-3 LCPUFA to be further commercialized as a highly healthy product and to be used as an adding-value component. Before considering the current concentrates for common and commercial consumption, safety international requirements ought to be addressed and fulfilled.

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Compliance with ethical standards

Conflict of interest All Authors declares that they have no conflict of interest.

Human and animal rights statement This article does not contain any studies with human or animal subjects.

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