Optimisation of rancidity stability in long-chain PUFA concentrates obtained from a rainbow trout \((Oncorhynchus mykiss)\) by-product

Mª Macarena Berríos\(^1\), Alicia Rodriguez\(^1\), Matías Rivera\(^1\), Mª Elsa Pando\(^1\), Mª Antonieta Valenzuela\(^2\), and Santiago P. Aubourg\(^3,\ast\)

\(^1\) Department of Food Science and Chemical Technology. Faculty of Chemical and Pharmaceutical Sciences. University of Chile, Santiago, Chile.

\(^2\) Department of Biochemistry and Molecular Biology. Faculty of Chemical and Pharmaceutical Sciences. University of Chile, Santiago, Chile.

\(^3\) Department of Food Technology, Marine Research Institute (CSIC), Vigo, Spain.

\ast\ Correspondent: saubourg@iim.csic.es
This research was focused on the production of polyunsaturated fatty acid concentrates from a farmed rainbow trout (*Oncorhynchus mykiss*) by-product (i.e., belly muscle). The effect of different process variables (urea/fatty acids (FA) contents ratio, crystallisation time and temperature and stirring speed of the urea/FA mixture) on the lipid oxidation development during the urea complexation process was investigated. For this purpose, an experimental design (26 runs) following the response-surface methodology was developed. As a result, peroxide value and TOTOX index showed to be dependent on the crystallisation time and temperature and the urea/FA ratio, while no influence of the crystallisation stirring speed was detected on both indices; additionally, polyene index was affected by the urea/FA ratio and its interaction with the crystallisation time. An optimised desirability score near 1.0 was attained provided values of 2.8°C, 3.05h and 3.57 were applied for crystallisation temperature, crystallisation time and urea/FA ratio, respectively.

**Running title:** Rancidity stability of PUFA concentrates  
**Keywords:** *Oncorhynchus mykiss*; by-products; belly muscle; urea complexation; PUFA concentrates; response-surface methodology; rancidity
Aquatic lipids provide high contents of $n$-3 long-chain polyunsaturated fatty acids (LC-PUFA) (namely, eicosapentaenoic and docosahexaenoic acids, EPA and DHA, respectively), which have been reported to be responsible for beneficial effects on human health related to cardiovascular system, foetus and neonate brain development, dementia and cognitive function (Komprda, 2012). Nutritional research has recommended a healthy intake of essential fatty acids (FA) equivalent to five parts of $n$-6 FA per one part of $n$-3 FA, although current Western diets are known to deliver a higher $n$-6/$n$-3 ratio (15-16.7/1) (Simopoulos, 2002). In spite of such advantages, aquatic lipids are known to easily deteriorate (i.e., hydrolysis and oxidation development) as a result of extraction, processing or storage, these leading to the formation of primary and secondary lipid oxidation products. Consequently, marked loss of essential nutrients, detrimental changes in sensory quality and shelf life may occur in aquatic foods (Howell, 1994; Rodríguez et al., 2010; Undeland, 2016).

For analytical purposes, oil extraction with organic solvents has widely been used (Aubourg, 2010). However, when the industrial preparation of LC-PUFA concentrates is concerned, different kinds of chemical and enzymatic treatments have been developed (Gbogouri et al., 2006; Nolsøe & Undeland, 2009). Among methods without prior hydrolysis, several procedures can be outlined such as solvent fractionation, vacuum or molecular distillation, supercritical fluid extraction, silver ion complexation or enzymatic treatment (Liu et al., 2006; Rubio-Rodríguez et al., 2010). One of the simplest and efficient technologies is urea complexation, which allows handling large quantities of fish material in a simple equipment and is relatively inexpensive (Wanasundara & Shahidi, 1999; Gámez-Meza et al., 2003; Zuta et al., 2003). In this method, saturated and
monounsaturated FA are easily complexed with urea and can be subsequently removed by filtration, being the non-complexed fraction enriched with LC-PUFA. Although a wide range of studies have been addressed to the preparation of PUFA concentrates by applying the urea complexation process, scarce attention has been accorded to the lipid oxidation development under such process.

By-products of aquatic species are body parts that are removed before they reach the final consumer in order to improve their keeping qualities, reduce the shipping weight or increase the value of the main product (Kolakowska et al., 2006; Falch et al., 2006; Encinas-Arzate et al., 2014). Worldwide, fish processing discards include blood, viscera, heads, bellies, bones, skin, trimmings and fins. Along with reduction of waste production, discards have been used as sources of valuable bio-ingredients such as proteins, minerals and lipids that could be used for human nutrition if properly exploited (Aidos et al., 2001; Skåra et al., 2004; Linder et al., 2005).

Rainbow trout (Oncorhynchus mykiss) has received a great attention because of a wide farming production (FAO, 2007). Most previous research has shown high and profitable n-3 LC-PUFA content for this species (Haliloğlu et al., 2004; Aubourg et al., 2009). Rainbow trout belly is a by-product resulting from the trimming process. It is obtained from the central part of the abdomen after a longitudinal cut of the fish, without removing skin, bones and stapes (Mørkøre et al., 2002; Sone & Nortvedt, 2009); this by-product is highly appreciated in countries like Japan, being called “Harasu”. In the present research, the urea-complexation method was used to obtain LC-PUFA concentrates from the rainbow trout belly. Optimising by response-surface methodology (RSM) of different variables of the complexation process (urea/FA contents ratio; crystallisation temperature;
crystallisation time; stirring speed of the urea/FA mixture) was achieved to minimise the rancidity development during the PUFA concentrates preparation.

**EXPERIMENTAL PROCEDURES**

**Starting fish by-product and chemicals**

Belly from rainbow trout was obtained from Salones Antárctica S. A. facility (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), and included methyl esters from 52 different FA ranging from C 4:0 to C 24:1n-9 (GLC Reference standard 463; Lot 021-U). C 23:0 (2COT N-23M-A29-4 NU-CHECK-PREP-INC) was employed as internal standard for the quantitative analysis during the gas-liquid chromatography (GLC) analysis.

All chemicals and reagents used were reagent grade purchased from either Mundolab S. A. (Santiago, Chile) or Merck S. A. (Santiago, Chile).

**Extraction of rainbow trout belly oil (RTBO)**

The oil from rainbow trout belly was obtained according to the Zuta *et al.* (2003) procedure. For it, 20 g of belly tissue were homogenised with a 360-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 homogeniser, funnel and residue were further washed twice with 40-mL portions of hexane/isopropanol (3/2) mixture. Pooled organic filtrates were washed by addition of 150 mL of an aqueous solution of sodium sulphate (1 g/15 mL distilled water). Then, the organic layer was separated from the
aqueous one in a separatory funnel and dried by filtering it through a Whatman paper containing anhydrous sulphate salt. Finally, the solvent was removed with a rotary evaporator (Heidolph VV2011, Schwabach, Germany) and the resulting RTBO was stored at −70 °C.

Analyses on the starting RTBO were performed on 9 independent samples (n=9), and expressed as mean ± standard deviation.

RTBO saponification
RTBO (400 g) was mixed with a saponifying solution comprising KOH (120 g), H₂O (400 mL) and 96 % aqueous ethanol (400 mL; v/v) (Guil-Guerrero & Hassan, 2001). 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. Unsaponifiabales 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 through anhydrous Na₂SO₄, 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.

Urea complexation process
The method of Zuta et al. (2003) with some modifications was employed. For this process, different conditions of urea/FA contents ratio, crystallisation temperature and time and stirring speed of the urea/FA mixture were taken into account as described in the experimental design section. Thus, 30 g FA resulting from the RTBO saponification were mixed with varying quantities of urea and dissolved in 95 % ethanol. The mixture was then
stirred and heated at 70 °C, so that urea was dissolved and a clear homogeneous solution was produced. In a following step, the urea-FA adducts were allowed to crystallise. Later on, urea crystals were separated by filtration through a Whatman No.1 paper with a Büchner funnel, and 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. Hexane extracts were combined and dried over anhydrous sodium sulphate. The solvent was then partially removed using a rotary evaporator at 45 °C. PUFA concentrates were weighed to determine their yield with respect to the initial amount of saponified FA, flushed with nitrogen, and stored at –70 °C with 100 ppm of α-tocopherol under nitrogen atmosphere.

**Analysis of the fatty acid composition**

In order to analyse the FA composition of the starting RTBO and of the different PUFA concentrates obtained, transmethylation and methylation processes, respectively, were carried out to obtain the corresponding FAME. Thus, a two-step conversion was realised, according to previous research (IUPAC, 1987; Pando et al., 2014). For this purpose, a mixture of 100 mg of RTBO or PUFA concentrate and 10 mL of 0.2 N sodium methylate in methanol with two glass beads (4 mm) was placed into a 50-mL-ground-necked volumetric flask, heated at reflux (95±1 °C) for 10 min and allowed to cool to 50 °C. Then, two drops of 1 % phenolphthalein in 95 % aq. ethanol and 4 % sulphuric acid in methanol were added until the disappearance of the pink colour, so that an acid medium was obtained. The mixture was heated again (95±1 °C) at reflux for 30 min, being then allowed to cool. After adding 2.5 mL of hexane, the mixture was washed with a saturated NaCl solution, dried over anhydrous sodium sulphate and stored for 30 min at 4 °C. The FAME contained in the hexane phase (upper phase) were taken for further GLC analysis.
A HP 5890 gas-liquid chromatograph (Hewlett-Packard, Palo Alto, CA, USA) with a fused silica capillary column 100 m x 0.25 mm i.d., coated with SPTM-2560 (Supelco, Bellefonte, PA, USA) was employed. GLC setting conditions were as follows: injection temperature 250 °C; flame ionisation detector (FID) temperature 250 °C; flow rate of carrier gas (N₂) 1.2 mL min⁻¹; oven temperature range 160-220 °C with an increasing rate of 2 °C min⁻¹. DataApexClarity™ 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 to identify the FA profiles. Quantification of all individual FA (g/100 g total FA) was achieved by employing C 23:0 as internal standard.

**Lipid damage assessment**

The peroxide value (PV) was determined by the acetic acid-isoctane method (AOCS, 1993) with some modifications. Thus, 1 g of oil was dissolved in 10 mL of a mixture comprising acetic acid and chloroform (3/2, v/v). Then, 0.1 mL of a solution of potassium iodide was added and the mixture was allowed to stand for 1 min in the dark. Then, 6 mL of distilled water and 1 mL of 1 % aq. starch indicator were added, and titration was carried out with 0.1 N sodium thiosulphate. A blank determination was performed in the absence of lipid matter. Results were expressed as meq. of active oxygen kg⁻¹ oil.

The anisidine value (AV) of the oil samples was determined according to the AOCS (1993) procedure. This method is based on the reaction of \(p\)-anisidine diluted in acetic acid with the \(\alpha\)- and \(\beta\)-unsaturated aldehydes (primary 2-alkenals) present in the oil. Results were expressed as 100 times the absorbance increase (350 nm; ATI Unicam UV-spectrophotometer, Cambridge, UK) produced by the reaction of an aliquot of the oil with...
an anisidine solution (0.25 % in acetic acid, w/v) after shaking and standing for 10
minutes.

The TOTOX (total oxidation) index (TI) was also analysed. It is a comprehensive
oxidation parameter calculated from a weighed sum of PV and AV by applying the
following equation: TI = 2 x PV + AV.

On the basis of the results obtained from the FAME analysis by GLC, the polyene
index (PI) was calculated as the following FA contents ratio: (C 20:5n-3 + C 22:6n-3)/C
16:0.

**Experimental design and optimisation of response variables**

The study was performed with a central composite rotatable design $2^4 + star$ of twenty-six
runs based on the RSM (Table 1). The following coded values for the independent variables
were considered: urea/FA contents ratio (0, 1.5, 3.0, 4.5 and 6.0, w/w; variable A),
crystallisation temperature (−30, −15, 0, 15 and 30 ºC; variable B), crystallisation time
(3.05, 14.30, 25.55, 36.80 and 48.05 h; variable C), and stirring speed of the urea/FA
mixture (0, 250, 500, 750 and 1,000 rpm; variable D). Each experimental condition was
carried out in triplicate (n=3). Four replications were performed at the central point of the
experimental design to estimate the experimental error.

The RSM was used to optimise the rancidity stability of PUFA concentrates
preparation. On the basis of the non-urea complexing fraction, the following response
variables of the experiment design were chosen: PV, AV, TI and PI. All experiments were
carried out randomly to minimise the effect of unexplained variability in the observed
responses due to extraneous factors (Table 1) (Liu *et al.*, 2006; Wanasundara & Shahudi,
1999). Multiple regression equations were fitted to the responses by discarding non-
significant terms \((p>0.05)\) in order to obtain response surfaces. A multiple-response optimisation was performed to optimise several responses simultaneously, this Maximising the desirability function that ranged between 0 and 1 scores.

A quadratic polynomial regression model was assumed for predicting individual \(Y\) variables (i.e., response variables). The model proposed for each response of \(Y\) value was:

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

where \(\beta_0\), \(\beta_i\), and \(\beta_{ii}\), are intercept, linear, and quadratic coefficients, respectively; \(\beta_{ij}\) denotes the interaction coefficient term for the interaction of variables \(i\) and \(j\); \(X_i\) represents the independent variables; and \(\varepsilon\) corresponds to the random error (Myers et al., 1995; Keskin et al., 2012).

**Statistical analysis**

A statistical analytical system was used for multiple regression analysis, analysis of variance (ANOVA), canonical analysis and analysis of maximum ridge of data in the response-surface regression (RSREG) procedure. Estimated response surface and contours of estimated response surface were developed using the fitted quadratic polynomial equations obtained from RSREG analysis and holding the independent variables with the
least effect on the response at a constant value and changing the levels of the other two
variables (Liu et al., 2006; Derringer & Suich, 1980).

Analyses were performed in triplicate. The 95% confidence intervals of each lipid
quality parameter were calculated, taking into account the number of replications and
considering the standard deviation of each sample. Results obtained were analysed by a
multifactorial analysis of variance (ANOVA). 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 Plus® 5.1 software
(Manugistics Inc., Rockville, MD, USA) was used.

**RESULTS AND DISCUSSION**

**Characterisation of the starting RTBO**

Characterisation of the initial RTBO employed in the present work was achieved by means
of the different lipid oxidation indices, as well as by the FA composition assessment.

Values obtained for the PV, AV and TI were 0.87±0.60 meq. active oxygen kg⁻¹ oil,
1.45±0.81 and 3.19±1.20, respectively. Such values can be considered low and similar to
those observed in salmon (*Salmo salar*) oil obtained from filleting by-products (Skåra et
al., 2004). Additionally, actual values are placed under the acceptability limits established
by different regulations and institutions such as the Codex Alimentarius Commission
(2013) and the European Food Safety Authority (EFSA, 2010).

On the contrary, previous research employed starting by-products showing higher
lipid oxidation development. This accounts for raw and refined commercial Coho salmon
(*Oncorhynchus kisutch*) oils (Pando et al., 2014), skin, viscera and muscle tissue by-
products from mackerel (Zuta et al., 2003) and maatjes herring (Clupea harengus) oil (Aidos et al., 2001).

Concerning the FA composition, C 18:1n-9 and C 16:0 (20.6 % and 18.2 %, respectively; g/100g total FA) were found the most abundant. Other FA present were C 20:5n-3 (11.1 %), C 16:1n-7 (8.5 %), C 22:6n-3 (6.9 %), C 14:0 (5.7 %), C 22:5n-3 (5.1 %) and C 18:2n-6 (4.8 %). When FA groups are considered, scores of 28.43 %, 38.35 % and 33.76 % were obtained for saturated (SFA), monounsaturated (MUFA) and PUFA.

Additionally, a 0.99 score for the PI was obtained.

Previous research accounts for different FA compositions in oils employed for further preparation of PUFA concentrates. Such differences can be explained on the basis of the by-products chosen, the fish species and the diet composition in farmed fish. Thus, Kolakowska et al. (2006) employed viscera, backbones and fins from rainbow trout (Oncorhynchus mykiss), so that an oil including C 16:0, C 18:1n-9 and C 20:1n-9 as major FA was obtained, with a PUFA content of 25.9 %. Linder et al. (2005) employed salmon heads and obtained an oil including C 18:1n-9, C 16:0, C 22:6n-3 and C 20:4n-3 as the major FA; in this case, a 41.6 % of PUFA was attained. Pando et al. (2014), employed Coho salmon belly muscle, which led to an oil with C18:1n-9, C 18:2n-6 and C 16:0 as the major fatty acids, with a EPA+DHA content of 10.3-11.0 %, while total PUFA content was 34-36 %.

In agreement with its low rancidity development degree, as well as its high n-3 PUFA content, it is concluded that the oil corresponding to the actual belly by-product represents a valuable alternative for the PUFA concentrates production.
Effect of process variables on lipid oxidation indices: Pareto charts and multiple regression analysis

Results obtained in each experimental run for each of the response variables (lipid oxidation indices) are expressed in Table 1. Based on such results and in order to describe the effect of the process variables on the response variables, Pareto charts and multiple regression analyses were employed.

Figure 1 describes the process variables that influence the oxidation parameters by means of the Pareto charts. Both PV and TI (Panel a and Panel b, respectively) proved to be dependent (p<0.05) on the crystallisation temperature (quadratic effect, BB), the crystallisation time (lineal effect, C), the urea/FA contents ratio (quadratic effect, AA), the crystallisation temperature (lineal effect, B), and the interaction between the urea/FA contents ratio and the crystallisation time (AC). Concerning the PI (Panel d), this parameter showed to be affected by the urea/FA contents ratio (lineal effect, A) and by the interaction between the urea/FA contents ratio and the crystallisation time (AC). Contrary to the results obtained for the PV, TI and PI, the Pareto chart for the AV (Panel c) showed that this variable was not modified by the different process variables considered (p > 0.05). Additionally, no effect of the stirring speed of the urea/FA mixture (D) on the different oxidation parameters tested could be implied under the present experimental conditions.

Lipid oxidation is a complex process involving the formation of different classes of compounds, most of them unstable and thus susceptible to breakdown, forming lower weight compounds or reacting with other molecules (Aubourg, 1993; Howell, 1994; Rodríguez et al., 2010). According to the present results, it is concluded that during the PUFA concentrates production by urea complexation, formation of primary lipid oxidation compounds is markedly more important than formation of secondary compounds. In
agreement with this, the TI would mainly depend on the PV. Thus, the ANOVA for simple regression between TI and PV scores provided a $p$-value lower than 0.05, this showing a statistically-significant relationship between these parameters. Thus, the fitted multiplicative model is expressed in Figure 2. This fitted model explained 91.997 % of the variability of the TI ($R^2 = 91.99$), while the correlation coefficient was 0.96, this indicating a strong relationship between the two variables. Figure 2 shows the plot for the TI assessment from the PV, where all the experimental points are placed inside the region delimited by the dotted lines at both sides of the diagonal. Consequently, it could be possible to predict the TI from the PV without carrying out the analysis of the AV. Since the objective of the actual research is to prevent the rancidity development during the LC-PUFA concentrates production, it is concluded that most efforts ought to be focussed on the inhibition of the primary oxidation development.

An opposite result was obtained by García-Moreno et al. (2013); in it, the optimisation of the bleaching process condition for sardine oil was analysed. As a result of the high temperatures needed (90-130 ºC), a high secondary lipid oxidation development was observed, this leading to a higher dependence of the TI on the AV than on the PV. In agreement with the present results, Schelenk & Holman (1950) indicated that urea complexation protects n-3 PUFA from autoxidation; this positive effect may explain the fact that secondary lipid oxidation had a scarce significance in the actual research.

Previous research accounts for a wide range of studies concerning the PUFA concentrates preparation by urea complexation by optimising the different process variables. Among such studies, sea blubber (*Phoca groenlandica*) oil (Wanasundara & Shahidi, 1999), sardine (*Sardinops sagax*) oil (Gámez-Meza *et al.*, 2003) and tuna (*Thunnus albacares*) oil (Liu *et al.*, 2006) can be mentioned. However, scarce attention has
been devoted to the rancidity development during such process. The present research provides, to our knowledge, the first optimisation study concerning this damage pathway during the urea complexation process applied to a seafood by-product.

In the case of the PI, this response variable was affected mainly by the urea/FA contents ratio, and secondly by the interaction between the urea/FA contents ratio and the crystallisation time. This indicates that this parameter would mainly depend on the content of urea and does not depend on factors such as the stirring speed and the crystallisation temperature. This independency from the crystallisation temperature and low dependence on the crystallisation time is considered highly beneficial in maintaining the EPA and DHA contents in the PUFA concentrates. The strong effect of the urea complexing process on the PV can be explained on the basis of the special lability of highly unsaturated fatty acids (i.e., EPA and DHA) to peroxide formation; as expected, separation of urea complexes from the non-urea complexing fraction effectively removes saturated and long-chain MUFA and enriches the liquid fraction with highly unsaturated FA (Zuta et al., 2003).

In order to express the three response variables as a function of the process variables, the experimental data were firstly fitted to a complete quadratic model. Statistical testing of the model was performed by the Fisher’s statistical test for analysis of variance (ANOVA). Thus, the polynomial coefficients for the response-surface model were calculated by multiple regressions. Finally, the following equations were obtained:

\[
PV = 0.3529 - 1.4242A - 0.0815B + 0.3985C + 0.5413AA - 0.0863AC + 0.0067BB
\]

(adjusted \(R^2 = 0.35\))
TI = 1.8469 – 4.1372A – 0.2157B + 0.9505C + 1.4048AA – 0.2138AC + 0.0181BB
(adjusted $R^2 = 0.40$)

PI = – 79.0542 + 72.5534A – 4.0170B + 3.4630C – 0.2117D + 0.0002DD – 1.7798AC + 0.1302BC
(adjusted $R^2 = 0.50$)

In agreement with such equations, the three response variables showed to be dependent on the same process variables as previously expressed in the Pareto analysis.

**Effect of process variables on lipid oxidation indices: Analysis by RSM charts**

Canonical analysis allows examining the shape of the response surface and contouring curves on the predicted quadratic polynomial models. This analysis is a mathematical approach used to locate the stationary point of the response surface and to determine whether it represents a maximum, minimum or saddle point (Wanasundara & Shahudi, 1999). Figure 3 shows the effect of different process variables on the PV by means of charts including the estimated response surfaces with its contours (Panels a, c and e); in such curves, equal response values at different levels and marks represent optimal values for estimated minimum PV scores (Panels b, d and f). A similar canonical analysis was also carried out on the TI values. In agreement with the high correlation between TI and PV parameters, similar plots were obtained so that their presentation in the actual study was not considered necessary.

The shape of response surface is known to be characteristic when the lineal, quadratic or interaction effects participate in the models. Thus, Figure 3 (Panels a and b) shows the PV as a function of the urea/FA contents ratio and the crystallisation
temperature. From the shape of Panel a, the quadratic effects of the urea/FA contents ratio and the crystallisation temperature effects on PV can be observed, as well as the lineal effect of the crystallisation time. Additionally, contour surface (Figure 3, Panel b) shows an ellipsoidal shape in the estimated PV minimum. Meantime, Panels c and d describe PV as a function of the urea/FA contents ratio and the crystallisation time; in this case, the shape of the response surface is given by the negative effect of the urea/FA contents ratio and the positive effect of the crystallisation time on the PV. In practical terms, the response obtained for the PV reaches a minimum value in the case that scores for the urea/FA contents ratio and the crystallisation time are also minimum. Finally, Panels e and f represent the PV as a function of the crystallisation temperature and the crystallisation time. As in the previous cases, the characteristic shape of the crystallisation temperature effect is observed. Thus, the stationary point for the PV predicts a minimum value of 0.005 at scores of 1.56 for the urea/FA contents ratio, 6.1 ºC for the crystallisation temperature and 3.05 h for the crystallisation time.

**Optimisation of the process variables by means of the RSM**

To minimise the scores obtained for PV and TI and maximise those of the PI, an individual and multiple optimisation process (Derringer’s desirability function) of the four process variables was developed. Results obtained from this optimisation process on the PV are shown in Table 2. Optimal scores for the three process variables showing a significant effect on the PV are depicted, as well as the predicted optimal value. In the same way, optimised values of process variables for TI and PI are also expressed in this Table. The results concerning the multiple optimisation or desirability function were 2.27, 5.09 and 196.86 for PV, TI and PI, respectively.
Figure 4 (Panels a and b) shows the PI as a function of the urea/FA contents ratio and the crystallisation time. As it can be observed, both variables significantly affect the PI score, although the crystallisation time only does it when the interaction with the urea/FA contents ratio occurs, in agreement with the Pareto analysis. The response surface shows a maximum score for the PI at a urea/FA contents ratio of 6 and at a crystallisation time of 3.05 h; consequently, the PI reaches its highest score at maximum urea/FA contents ratio and at minimum crystallisation time.

The same situation occurs when a minimum PV score is to be predicted from the two same process variables. In this case, the urea/FA contents ratio affects positively the PI due to the fact that a higher ratio allows a greater PUFA retention in the complexation urea process, as a result of an increase of the DHA and EPA values in the PI formula (C 20:5\textit{n}-3 + C 22:6\textit{n}-3)/C 16:0). Main effects of the process variables on PV are illustrated in Figure 5. In agreement with this Figure, lower urea/FA contents ratio (variable A) and crystallisation temperature (variable B) provide a higher PV response; meanwhile, crystallisation time (variable C) shows a linear relationship with PV.

**CONCLUSIONS**

PUFA concentrates preparation by urea complexation of oil obtained from rainbow trout belly was studied. The RSM analysis of the effect of the different process variables indicated that PV and TI showed to be dependent on the crystallisation time and temperature and on the urea/FA contents ratio, while no influence of the stirring speed of the urea/FA mixture was detected on either index; additionally, PI was affected by the urea/FA contents ratio and its interaction with the crystallisation time. On the contrary, AV did not show to be influenced by any of the processing variables tested. In order to
minimise the PV and TI and maximise the PI, the desirability function of the four process variables was optimised to a score near to 1.0, provided that values of 2.8 °C, 3.05 h and 3.57 were applied for crystallisation temperature, crystallisation time and urea/FA contents ratio, respectively.

With the aim of obtaining high-quality concentrates from seafood by-products, present results outline the need of setting a previous experimental design based on the optimisation of the response variables (i.e., lipid oxidation indices) encountered in the process involved. Previous studies have focused on the optimisation of different process variables relating to the PUFA concentrates preparation by urea complexation. However, to the best of our knowledge, no previous research has addressed the optimisation of rancidity inhibition during such a process. Present research has shown the possibility of obtaining a PUFA concentrate with a low-rancidity level from a rainbow trout by-product (i.e., belly muscle).

**ACKNOWLEDGEMENTS**

The authors thank Salmones Antártica, S. A. (Aysen, Chile) for kindly providing the rainbow trout belly. The work was supported by the FONDECYT program (Government of Chile) throughout the Project Nº 1120627.
REFERENCES


**LEGEND TO FIGURES**

**Figure 1**: Standardised Pareto charts showing lineal, quadratic and interaction effects of the different process variables (A, B, C and D)* on the response variables: peroxide value (meq. active oxygen kg\(^{-1}\) oil; Panel a), TOTOX index (Panel b), anisidine value (Panel c) and polyene index (Panel d).

* Process variables: A (urea/fatty acids contents ratio, w/w), B (crystallisation temperature, °C), C (crystallisation time, h) and D (stirring speed of the urea/FA mixture, rpm).

**Figure 2**: Plot of fitted model for the TOTOX index (TI) assessment as a function of the peroxide value (PV; meq. active oxygen kg\(^{-1}\) oil).

**Figure 3**: Effect of different process variables (A, B and C)* on the peroxide value (PV, meq. active oxygen kg\(^{-1}\) oil): process variables A and B (Panels a and b, response and contour surfaces, respectively), process variables A and C (Panels c and d, response and contour surfaces, respectively) and process variables B and C (Panels e and f, response and contour surfaces, respectively).

* Process variables: A (urea/fatty acids contents ratio, w/w), B (crystallisation temperature, °C), C (crystallisation time, h) and D (stirring speed of the urea/FA mixture, rpm).

**Figure 4**: Effect of urea/fatty acids contents ratio (w/w) (variable A) and crystallisation time (variable C, h) on the polyene index (PI)*: response surface (Panel a) and contour surface (Panel b).

* Other process variables: variable B (crystallisation temperature, °C) and variable D (stirring speed of the urea/FA mixture, rpm).
Figure 5: Plot description of the main effects of urea/fatty acids contents ratio (w/w) (variable A), crystallisation temperature (variable B, °C) and crystallisation time (variable C, h) on the peroxide value (PV, meq. active oxygen kg$^{-1}$ oil).
TABLE 1

Central composite rotatable design $2^4 +$ star and values obtained for the different response variables*

<table>
<thead>
<tr>
<th>Run</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>PV</th>
<th>AV</th>
<th>TI</th>
<th>PI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>−15</td>
<td>14.3</td>
<td>250</td>
<td>6.12</td>
<td>0.94</td>
<td>13.17</td>
<td>8.76</td>
</tr>
<tr>
<td>2</td>
<td>4.5</td>
<td>−15</td>
<td>14.3</td>
<td>250</td>
<td>9.20</td>
<td>11.53</td>
<td>29.94</td>
<td>242.44</td>
</tr>
<tr>
<td>3</td>
<td>1.5</td>
<td>15</td>
<td>14.3</td>
<td>250</td>
<td>2.29</td>
<td>2.56</td>
<td>7.14</td>
<td>6.61</td>
</tr>
<tr>
<td>4</td>
<td>4.5</td>
<td>15</td>
<td>14.3</td>
<td>250</td>
<td>2.97</td>
<td>0</td>
<td>5.95</td>
<td>105.45</td>
</tr>
<tr>
<td>5</td>
<td>1.5</td>
<td>−15</td>
<td>36.8</td>
<td>250</td>
<td>11.73</td>
<td>0</td>
<td>23.46</td>
<td>9.68</td>
</tr>
<tr>
<td>6</td>
<td>4.5</td>
<td>−15</td>
<td>36.8</td>
<td>250</td>
<td>12.21</td>
<td>0</td>
<td>24.41</td>
<td>63.42</td>
</tr>
<tr>
<td>7</td>
<td>1.5</td>
<td>15</td>
<td>36.8</td>
<td>750</td>
<td>4.54</td>
<td>1.48</td>
<td>10.57</td>
<td>28.53</td>
</tr>
<tr>
<td>8</td>
<td>4.5</td>
<td>15</td>
<td>36.8</td>
<td>750</td>
<td>5.79</td>
<td>8.30</td>
<td>19.53</td>
<td>21.90</td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>−15</td>
<td>14.3</td>
<td>750</td>
<td>2.97</td>
<td>0</td>
<td>3.57</td>
<td>9.37</td>
</tr>
<tr>
<td>10</td>
<td>4.5</td>
<td>−15</td>
<td>14.3</td>
<td>750</td>
<td>9.55</td>
<td>1.21</td>
<td>20.31</td>
<td>276.44</td>
</tr>
<tr>
<td>11</td>
<td>1.5</td>
<td>15</td>
<td>14.3</td>
<td>750</td>
<td>2.90</td>
<td>3.57</td>
<td>9.37</td>
<td>9.05</td>
</tr>
<tr>
<td>12</td>
<td>4.5</td>
<td>15</td>
<td>14.3</td>
<td>750</td>
<td>8.91</td>
<td>0</td>
<td>17.82</td>
<td>145.62</td>
</tr>
<tr>
<td>13</td>
<td>1.5</td>
<td>−15</td>
<td>36.8</td>
<td>750</td>
<td>15.89</td>
<td>9.53</td>
<td>41.31</td>
<td>6.02</td>
</tr>
<tr>
<td>14</td>
<td>4.5</td>
<td>−15</td>
<td>36.8</td>
<td>750</td>
<td>13.64</td>
<td>2.97</td>
<td>30.24</td>
<td>32.74</td>
</tr>
<tr>
<td>15</td>
<td>1.5</td>
<td>15</td>
<td>36.8</td>
<td>750</td>
<td>7.20</td>
<td>2.83</td>
<td>17.24</td>
<td>7.48</td>
</tr>
<tr>
<td>16</td>
<td>4.5</td>
<td>15</td>
<td>36.8</td>
<td>750</td>
<td>6.72</td>
<td>0</td>
<td>13.44</td>
<td>143.13</td>
</tr>
<tr>
<td>17</td>
<td>0</td>
<td>0</td>
<td>25.55</td>
<td>500</td>
<td>12.44</td>
<td>5.97</td>
<td>30.85</td>
<td>1.49</td>
</tr>
<tr>
<td>18</td>
<td>6.0</td>
<td>0</td>
<td>25.55</td>
<td>500</td>
<td>2.43</td>
<td>0</td>
<td>4.87</td>
<td>12.19</td>
</tr>
<tr>
<td>19</td>
<td>3.0</td>
<td>−30</td>
<td>25.55</td>
<td>500</td>
<td>7.29</td>
<td>8.00</td>
<td>22.58</td>
<td>27.43</td>
</tr>
<tr>
<td>20</td>
<td>3.0</td>
<td>30</td>
<td>25.55</td>
<td>500</td>
<td>9.84</td>
<td>0.76</td>
<td>20.43</td>
<td>16.96</td>
</tr>
<tr>
<td>21</td>
<td>3.0</td>
<td>0</td>
<td>3.05</td>
<td>500</td>
<td>1.87</td>
<td>0</td>
<td>3.73</td>
<td>6.97</td>
</tr>
<tr>
<td>22</td>
<td>3.0</td>
<td>0</td>
<td>48.05</td>
<td>500</td>
<td>1.50</td>
<td>0</td>
<td>3.01</td>
<td>22.14</td>
</tr>
<tr>
<td>23</td>
<td>3.0</td>
<td>0</td>
<td>25.55</td>
<td>0</td>
<td>2.60</td>
<td>0</td>
<td>5.21</td>
<td>87.85</td>
</tr>
<tr>
<td>24</td>
<td>3.0</td>
<td>0</td>
<td>25.55</td>
<td>1,000</td>
<td>3.19</td>
<td>4.12</td>
<td>10.51</td>
<td>100.77</td>
</tr>
<tr>
<td>25</td>
<td>3.0</td>
<td>0</td>
<td>25.55</td>
<td>500</td>
<td>3.51</td>
<td>0</td>
<td>7.02</td>
<td>79.88</td>
</tr>
<tr>
<td>26</td>
<td>3.0</td>
<td>0</td>
<td>25.55</td>
<td>500</td>
<td>6.07</td>
<td>0</td>
<td>12.14</td>
<td>50.22</td>
</tr>
</tbody>
</table>

* Process variables: A (urea/fatty acids contents ratio, w/w), B (crystallisation temperature, °C), C (crystallisation time, h) and D (stirring speed of the urea/FA mixture, rpm). Response variables: PV (peroxide value, meq. active oxygen kg$^{-1}$ oil), AV (anisidine value), TI (TOTOX index) and PI (polyene index).
TABLE 2

Optimised values of the independent variables that minimise the peroxide value (PV) and the TOTOX index (TI), and maximise the polyene index (PI): Individual and multiple optimisations by employing the desirability function*

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Optimization of the process variable</th>
<th>Multiple optimisation of the response variable</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Process variable</td>
<td>Stationary point</td>
</tr>
<tr>
<td>PV</td>
<td>1.56</td>
<td>6.10</td>
</tr>
<tr>
<td>TI</td>
<td>1.70</td>
<td>5.96</td>
</tr>
<tr>
<td>PI</td>
<td>6.00</td>
<td>–30.00</td>
</tr>
<tr>
<td>Maximum desirability</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Process variables (A, B and C) as expressed in Table 1.
FIGURE 2

TI = \exp (0.828082 + 1.0089 \times \ln PV)
FIGURE 3

Panel a
Variable C = 3.1; Variable D = 697.8

Panel b
Variable C = 3.1; Variable D = 697.8

Panel c
Variable B = 6.1; Variable D = 697.8

Panel d
Variable B = 6.1; Variable D = 697.8

Panel e
Variable A = 1.6; Variable D = 697.8

Panel f
Variable A = 1.6; Variable D = 697.8