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# **Enrichment of EPA and DHA from sardine by supercritical fluid extraction without organic modifier : I. Optimization of extraction conditions.**

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## **Abstract :**

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) play an important role in the prevention of a lot of human diseases, and the supplementation in the alimentation of such fatty acids is thus recommended in certain diets. EPA and DHA were obtained by extraction and fractionation of fish industries waste, such as fish heads, produced for fish canning. Supercritical fluid extraction (SFE) of sardine oil was performed to optimize various parameters such as pressure, temperature, CO<sub>2</sub> rate and time in order to obtain the highest yield of extracted oil with the highest amount of EPA and DHA in the extraction product.

An experimental design was planned and the experiments were performed with a supercritical pilot (Hewlett Packard), while the composition of each extract was determined by GLC.

In a first approach, the oil yield was determined. Then, a quadratic model with three variables was studied to maximise the EPA and the DHA concentrations. A multicriteria optimization, using the desirability function, was performed to determine the best level for each parameter. Pressure, temperature and CO<sub>2</sub> rate were respectively set at 300 bars, 75°C and 2.5 ml/min during 45 min extraction. A yield of 10.36% of extracted oil (compared with the dry material) was achieved with an amount of 10.95% of EPA and 13.01% of DHA (compared with all fatty acids of the extract). This result was compared with a traditional solvent extraction and the solvent extraction yield seemed to be better. In fact, the advantages of SFE were a shorter extraction time, a prevention of heating, and a better organoleptic aspect by excluding the use of legally not allowed organic solvents.

**Key words** : supercritical CO<sub>2</sub> extraction, PUFAs, fish oil, GLC, experimental design.

## 1. Introduction

*n*-3 polyunsaturated fatty acids (*n*-3 PUFAs), especially eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) have been shown to be important for the prevention of a range of human diseases and disorders [1] [2]. The main dietary sources of EPA and DHA are fish, such as sardines, mackerels, anchovies. The fish canning industry produces an high amount of waste constituted notably by fish heads and tails. Nevertheless, this cheap raw material could be valorised by extracting and fractioning the valuable PUFAs. The aim of this study is thus double: in one hand, it reduces the processing waste whose elimination represents an important problem for fish industries. In second hand, it supplies an EPA and DHA enriched product for food supplementation. Previous researches in our laboratory showed an enrichment of EPA and DHA from sardine oil with enzymatic systems [3] [4] [5].

Extraction and purification of the lipids by conventional methods, such as hexane extraction, vacuum distillation, urea crystallization or conventional crystallization, present some problems because they use flammable or toxic solvents. These methods may have adverse health effects. They may also generate the deterioration of the PUFAs because of the high-temperature processing [6] [7].

Supercritical carbon dioxide is a promising process for the extraction and fractionation of edible oils containing labile PUFAs. The extraction can be carried out at low temperature. Besides, supercritical fluid extraction (SFE) offers new opportunities thanks to the fact that CO<sub>2</sub> is a non-toxic, non-flammable, inexpensive and clean solvent.

Supercritical fluid extraction and fractionation of fish oil fatty acids have been studied by many authors during the last ten years [8-11]. An enrichment of EPA was achieved after a saponification and esterification of the fatty acids [12]. In order to improve the sardine oil extraction yield, with the greatest amount of EPA and DHA in the lipid extract, we optimized the extraction conditions without organic co-solvent.

To optimize the conditions of supercritical extraction of sardine oil, we had first to determine the parameters that may significantly influence the extraction yield. Several studies showed the influence of parameters which included pressure, temperature, CO<sub>2</sub> rate and time. Pressure, temperature and time have been studied by Özden [13], pressure and temperature by Esquivel et al. [8] and Fleck et al. [14], while temperature, pressure and CO<sub>2</sub> rate have been studied by Dunford et al. [15]. In general, pressure, temperature, CO<sub>2</sub> rate and time seemed to be important parameters for the optimization of extraction. For the pressure, a space was determined between 100 and 350 bars according to the capacities of the pilot, and for the

same reasons, the CO<sub>2</sub> rate was limited to between 1 and 3.5 ml/min. For the temperature, one more constraint was considered, namely, the deterioration of the polyunsaturated fatty acids because of heating. Thus, temperature was limited to between 40 to 80°C. Finally, the limits of time was determined according to the consumption of CO<sub>2</sub>. The time was limited to between 5 and 60 min. The goal of this study was not to evaluate the influence of each parameter, but to determine the influence of the combination of the 4 parameters cited above. For the best parameters selection, as previously reported, a design of experiments was established [16].

In a preliminary study in laboratory conditions, we observed that lyophilisation permitted to dry efficiently sardine heads without altering PUFAs, and that this efficiency was significantly increased by grinding sardine heads before drying. By this method, we managed to reduce the quantity of water at 3%, that was necessary to increase the extraction yield as Dunford showed in 1998 [15]. The results obtained with the supercritical fluid extraction were compared with hexane extraction or Bligh and Dyer extraction, knowing that the extracted lipid classes were not the same because of the polarity of the solvents used in each case.

## **2. Experimental procedures**

### *2.1. Reagents*

Acetic acid, glacial acetic acid and methanol were provided by Carlo Erba. Chloroform, diethylether, ethanol and hexane were of analytical grade and were provided by Sigma. Sulphuric acid was provided by Prolabo. The CO<sub>2</sub> used for the supercritical fluid extraction was of technical grade (purity > 99%, provided by Linde Gas). The azote and hydrogen used for GLC were of technical grade and provided by Linde Gas. Karl Fischer reagent was provided by Merck, like the Silica gel 60 F<sub>254</sub> plates used for TLC. A PUFAs standard (marine source n°1) and the free saturated fatty acids (C10 – C24) were provided by Supelco, the cholesterol, the L- $\alpha$ -phosphatidylethanolaminedipalmitoyle and the phosphatidylcholine from egg yolk were provided by Sigma, the diglyceride and monoglyceride mixture by Arlès and the tripalmitine by Fluka.

## 2.2. Sample preparation

Sardine heads were provided by Société Nouvelle Dominique Ferrigno (Port Saint Louis du Rhône, France). Fresh sardine samples were lyophilized with a Virtis 12SL lyophilizator to obtain the water content of about 3%. The percentage of water was measured with the Karl Fischer method, with a DL18 Mettler.

## 2.3. Supercritical CO<sub>2</sub> extraction

### a- Principle of SFE processing

The SFE extractor used was an automatic Hewlett Packard 7680T (figure 1).

The thimble containing the sample was placed in a thimble carousel which carried it into the extraction chamber equipped with a heating jacket. The system was pressurized to the target pressure. During this step, the pump head temperature was decreased to 4°C. Then, the system was equilibrated until pressure, CO<sub>2</sub> rate and temperature became constant to begin extraction. Temperature regulation was assured by automatic electrical heating while pressure stabilization was assured by opening or closing the valve V6. CO<sub>2</sub> rate was maintained constant at operating value by the supercritical fluid pump. The components were then extracted by the pre-heated supercritical CO<sub>2</sub> and entered the trap through a nozzle where CO<sub>2</sub> was depressurized. The components were collected on the trap while CO<sub>2</sub> exited the trap to the vent. Then, the rinse solvent was applied to the sample to remove components from the sample matrix and deposit them on the trap. The extracted fractions were collected into vials in a fraction output tray.

A software program (HP Chemstation B.02.04.) permitted to set all the parameters and to control the extraction status.

### b- Parameters of SFE experimentation

2.5 g of sample were used to fill the extraction thimble. The nozzle temperature was 45°C, while the trap temperature was 40°C. The trap packing was stainless steel, with a compensation volume of 1.0 ml. The rinse solvents used were hexane as solvent A and ethanol as solvent B. The rinse rate was 2.0 ml/min and 4 fraction outputs of 1.8 ml for each solvent were filled up. The equilibration time was 2 min, and the pump head temperature was 4°C. Concerning pressure, temperature, time extraction and CO<sub>2</sub> rate, they were set according to the experimental design (see table 1, columns 3 to 6).

After each extraction, the rinse solvents were eliminated of the output fractions under vacuum evaporation before the extraction yield was determined (Eq. 1). An aliquot of the lipid extract was then used for TLC and GLC analysis.

$$\text{oil yield} = \frac{\text{mass of extracted oil}}{\text{mass of dried material}} \times 100 \quad (1)$$

#### 2.4. Experimental design

To obtain the best compromise between the quantitative and the qualitative aspect, we had to know the evolution of the results according to the variation of the studied parameters.

For this purpose, we could perform a serie of experiments covering the whole experimental domain, or we could use a tool, giving the same information but with less experiments. This tool is an empirical mathematical model (Eq. 2). In this case, the model is written as follows :

$$\eta = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{14} X_1 X_4 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4. \quad (2)$$

where  $\eta$  represents the response,  $\beta_i$  the coefficients of the model and  $X_i$  the coded variables. To estimate precisely the coefficients of the model and to obtain a good prediction in the experimental design, an ordinary set of experiments could not be realized. In our case, among the optimal designs of experiments for a model of a second degree, we chose a Doehlert uniform shell design [17], with 5 test points evenly distributed over the experimental domain. Some experiments were replicated in order to estimate the variance of the experimental error. Then, to minimize the effect of systemic errors, experiments were carried out in a random fashion. The isoresponse curves and the desirability function [18] were used to determine the best compromise in the experimental domain.

For the exploitation of the analytical results, a specific software program was used [19].

#### 2.5. Solvent extraction

Solvent extraction was carried out using 30 ml of hexane to extract the non-polar compounds of 2 g of dried and ground sardine head sample. The sample and the solvent were placed in an erlenmeyer under nitrogen, at room temperature (20°C) and a magnetic agitation was maintained during 24 hours. Then the sample was filtered on celite under vacuum, dried with anhydrous sodium sulphate, and the solvent was evaporated under vacuum with a

rotative evaporator. The resulting fish oil was recuperated and placed at + 4°C under nitrogen for further analysis.

In parallel, another solvent extraction was performed on fresh sardine, using the Bligh and Dyer method [20], in order to determine the total lipid composition of the sardine head oil. In this experiment, 2 g of fresh material was placed in an erlenmeyer with 20 ml of chloroform/methanol (2:1, v/v) under nitrogen at room temperature (20°C) and a magnetic agitation was maintained during 24 hours. Then the sample was filtered on celite under vacuum, dried with anhydrous sodium sulfate and the solvent was evaporated under vacuum with a rotative evaporator. The fish oil was recuperated and placed at + 4°C under nitrogen for further analysis.

For both methods, the oil yield was calculated (Eq. 1).

### *2.6. Thin layer chromatography of the lipid extracts*

Before GLC analysis, the lipids from the SC-CO<sub>2</sub> extractions and those from solvent extractions were separated and identified by TLC. The elution of each product was achieved by using a mixture of hexane/diethyl ether/acetic acid (70/30/1), and the visualisation by pulverizing sulphuric acid 30% before leaving the plate into an oven at 100°C during 15 min. Standards permitted a better identification by calculating the frontal reports of each spot. As shown in figure 2, the total lipid content was constituted by triglycerides, diglycerides, monoglycerides, free fatty acids, sterols, phospholipids (PL) and other carbures. We improved the PL migration by optimizing elution solvent : chloroform/methanol/glacial acetic acid (65/25/8). In addition, we used the modified method of Vaskovsky and Kostetsky with a mercury-based preparation [21].

### *2.7. Quantitative determination of fatty acids in the sample by GLC*

The fatty acid composition of sardine oil triacylglycerol was directly analyzed by GLC after methyl esterification. A gas chromatograph (Shimadzu CG-14A) was used with an oven temperature set at 180°C, while the injector temperature was set at 250°C. Nitrogen was used as the carrier gas in an omegawax-320 fused silica capillary column provided by Supelco (30 m x 0.32 mm x 0.25 µm). The detector used was an FID set at 260°C and the integration was achieved by a Shimadzu C-R4A. Each peak was identified by its Equivalent Chain Length value (ECL) determined with a marine source standard. Then, by an internal calibration with heptadecanoic acid methyl ester, each fatty acid was quantified. The mass composition of a

fatty acid or mixture Y was given by the general equation (Eq. 3). A representation of a typical chromatogram is presented in figure 3.

$$Y \text{ concentration} = \frac{\text{mass of } Y}{\text{mass of the total fatty acids}} \times 100 \quad (3)$$

### 3. Results and discussion

#### 3.1. Optimization of conditions for the supercritical extraction of sardine oil

To optimize the conditions for the supercritical extraction of sardine oil, we used a Doehlert uniform shell design, as explained in chapter 2.4. and the experiments corresponding to this design were performed. This Doehlert design is shown with the experimental results in table 1.

The analysis of the results showed a discontinuity of the phenomenon. Indeed, some experiments presented an extracted oil yield near 0%, that did not allowed further analyses. These experiments were distinguished by a low pressure, that led us to do the study again, considering only the experiments that present the 3 experimental results. For this purpose, we used one of the interesting properties of the Doehlert design, which was the sequential approach in the space of the variables. In fact, the subset of experiments 1 to 12 and 26 to 29 constituted an optimal experimental design for a quadratic model, for three variables (temperature, CO<sub>2</sub> rate, time), while pressure remained constant.

From these results, the estimation of the coefficients of the model were calculated using multilinear regression from NEMROD-W [19]. The models describing yield Y<sub>1</sub> (Eq. 4), EPA Y<sub>2</sub> (Eq. 5) and DHA Y<sub>3</sub> (Eq. 6) were :

$$Y_1 = 9,625 - 0,764 X_1 + 2,913 X_2 + 3,928 X_3 + 0,025 X_1^2 - 5,416 X_2^2 - 2,358 X_3^2 + 0,764 X_1X_2 + 0,084 X_1X_3 - 3,529 X_2X_3. \quad (4)$$

$$Y_2 = 11,475 + 0,063 X_1 - 1,348 X_2 + 0,284 X_3 + 0,525 X_1^2 - 2,136 X_2^2 - 0,601 X_3^2 + 3,229 X_1X_2 + 1,116 X_1X_3 + 1,065 X_2X_3. \quad (5)$$

$$Y_3 = 9,550 - 0,950 X_1 + 0,952 X_2 + 1,644 X_3 + 0,550 X_1^2 - 0,781 X_2^2 - 1,079 X_3^2 + 1,845 X_1X_2 + 1,420 X_1X_3 + 2,551 X_2X_3. \quad (6)$$

with X<sub>1</sub> = temperature, X<sub>2</sub> = CO<sub>2</sub> rate, X<sub>3</sub> = time.

After validation by using variance analysis, these equations were used to represent the responses in the whole domain. We usually represent the response surface graphically, such as shown in figure 4. EPA or DHA was plotted versus the levels of X<sub>2</sub>, X<sub>3</sub> (respectively CO<sub>2</sub>

rate, time) with temperature fixed. In the contour plot, lines of constant response were drawn in the  $X_2, X_3$  plane, with  $X_1$  fixed ( at 60°C).

In this contour plots, we saw that for increasing DHA, the CO<sub>2</sub> rate and time had to be increased. For increasing EPA, CO<sub>2</sub> rate should not be too high and time was not very influent.

The maximisation of each response was not obtained at the same conditions. One way of overcoming this difficulty was to define an acceptable range for each response. The satisfactory zone was that part of the domain for which the value of each response was acceptable. To determine this acceptable zone, we used a multicriteria optimization procedure based on desirability function. For each response, we associated a partial desirability function. This varied from 0 to 1, according to the closeness of the response to its target value. For example, on oil yield, the target value was 10% and the desirability  $d_1$  at this point was equal to 1. An oil yield between 9 and 10% was acceptable, but the desirability was equal to 0 if the oil yield was lower than 9%. The partial desirabilities  $d_1, d_2$  or  $d_3$  were combined together, as the geometric mean, to obtain the overall desirability function  $D$  (Eq. 7) for the system whose maximum value could be looked for within the domain [19] :

$$D = (d_1 \cdot d_2 \cdot d_3)^{1/3} \quad (7)$$

This function was physically mapped over the domain (figure 5) giving a reliable general picture of the acceptable region. From this desirability function, it was possible to predict the expected values of each response.

Özden [13] and Esquivel [8] set the optimized temperature at 40°C, while Dunford [15] set it at 35°C. The values were quite similar but we determined a great influence of temperature on the extraction yield and the experimental design permitted us to set the optimized temperature at 75°C. Although this temperature seemed to be high, we didn't observe a deterioration of PUFAs by following oxidized products.

Respect to CO<sub>2</sub> rate, the influence of this parameter was not very important as Dunford [15] also showed. He tested three CO<sub>2</sub> rate degrees : 0.5 l/min, 1.0 l/min and 1.4 l/min. The amount of oil extracted in each case were quite similar as we showed with the experimental design. We set thus CO<sub>2</sub> rate in our system at 2.5 ml/min.

We also tested the extraction time, which was influent on the response. We set our extraction time at 45 min. We tested until 55 min, and 45 min was sufficient for obtaining an optimized extraction yield.

Concerning pressure, the disparity of the results was more important. Esquivel [8] determined an optimized pressure of 180 bars, while Dunford [15] set it at 345 bars.

Özden[13] obtained his best yield with a pressure of 280 bars, that was nevertheless the highest tested pressure studied by Özden among three pressure levels. Whereas we could not study the influence of pressure by the Doehlert design, we could observe the influence of pressure on the oil yield (only one experiment could not be carried out). We saw that pressure was an influent factor for the extraction optimization and we showed that the higher is pressure, the better is the yield. The pressure was set at 300 bars, according to the results of this study and to the apparatus capacity.

With pressure set at 300 bars, if temperature was set at 75°C, CO<sub>2</sub> rate at 2.5 ml/min and time at 45 min, an oil yield of 10.78% could be achieved. At the same conditions, 12.65% of EPA and 11.59% of DHA in the extract could in theory be obtained. These are the values presented in the tables of figure 6. Each figure showed the optimal zone to increase the responses and by combination of the 3 figures, the optimal value of each parameter was determined.

The corresponding experiments were performed and the results are given in table 2. The first experiment used the best levels for temperature, CO<sub>2</sub> rate and time for a pressure set at 225 bars (the pressure used for the Doehlert design), and at 300 bars for the second one. These two experiments permitted to validate the mathematical model. As shown in this table, the results obtained in the second case were better than in the first one : the oil yield (Eq. 1), EPA concentration (Eq. 3) and DHA concentration (Eq. 3) were higher under these conditions.

Moreover, we did a statistical test on the optimal conditions to compare the expected values to the experimental results, taking into account the variance of the prediction and for each response, this test was no significant. Concerning oil extraction, the experimental yield was 10.36% instead of 10.78% and a composition of 12.65% of EPA and 11.59% of DHA was expected while we obtained 10.95% of EPA and 13.01% of DHA. The experimental results fitted the expected values.

### *3.2. Comparison of SFE yield with traditional solvent extraction yield*

The most common lipid extraction is the Bligh and Dyer method. We applied this method on the sardine heads and the yield of extracted oil achieved was 63% of the dried material (1). The Bligh and Dyer method may permit the extraction of all lipids, including polar lipids, phospholipids, and probably lipids bound with other components from cellular membranes. The yield is high but its application for food supplementation is not conceivable because of the toxicity of the solvents used.

Hexane extraction was also carried out on dried sardine heads and a yield (Eq. 1) of 21% was achieved. The hexane extraction favoured the extraction of non-polar compounds, that may explain why the yield was lower than those obtained with the Bligh and Dyer method. However, hexane extraction is commonly used in food industry because hexane is the only organic solvent allowed for oil extraction or transformation. Furthermore, because of its non-polar property, hexane extraction was more similar with CO<sub>2</sub> extraction.

As we showed previously, CO<sub>2</sub> extraction permitted to obtain a yield of average 10.5% of the dried material in the optimal experiments. So, the efficiency of SFE was almost 50% of that of hexane extraction.

### *3.3. Fatty acid composition of extracted oils*

Quantitative analysis by GLC of supercritical fluid optimal extraction and traditional solvent extractions is given in table 3. The fatty acids composition of extracted oils by hexane, Bligh and Dyer and by SFE was quite similar for each method. By a global analysis of lipid classes (saturated FA, mono-unsaturated FA, PUFAs), we could see equivalent concentrations for the three methods, except in the case of hexane extraction, where the concentration of saturated FA was slightly lower than that for SFE or Bligh and Dyer method, and the concentration of PUFAs was slightly higher: average 35.4% in the hexane extraction instead of 31.2% in the SFE and 32.7% in the Bligh and Dyer extraction.

Considering the alimentary application of extracted oil, the Bligh and Dyer method remained an experimental procedure that could not be applied in our research application. The data showed that SFE preserved the fatty acids composition, especially EPA and DHA concentrations at respectively 11% and 13% of the total fatty acids.

Although hexane is still used in alimentary oil production and transformation, its elimination requires drastic process to have in the product less than 0.001% of hexane. Moreover, hexane can be charged in heavy metals and other impurities that could have bad effects on the health of whom consume these products. For all these reasons, we think that the application of supercritical CO<sub>2</sub> without any organic modifier is interesting for such valuable molecules from waste of canned fish industry.

#### **4. Conclusions**

In this study, an experimental design was used to dispose of a statistical tool for determining the best levels of pressure, temperature, CO<sub>2</sub> rate and extraction time, because of the disparity of results presented in the years.

The overall results showed that the extraction of fish oil was successfully performed by supercritical CO<sub>2</sub> without any modifier. The yield of extracted oil, like the mass compositions of EPA and DHA, depended on the operating conditions. An optimized experiment was carried out : pressure was set at 300 bars, temperature at 75°C, CO<sub>2</sub> rate at 2.5 ml/min and time at 45 min. The extracted oil yield achieved 10.36% of the dried material and the concentration in EPA and DHA was respectively 10.95% and 13.01%.

To estimate the performance of the mathematical model, we compared the expected values with the experimental results, by a statistical test taking into account the variance of the prediction. We observed that the experimental results fitted the expected values. The design of experiments used for this study was an efficient statistical tool for optimizing the conditions of a one-step supercritical fluid extraction.

This study showed a possible application of SFE technology for valorising waste products of canned fish industry by extracting PUFAs, and especially EPA and DHA. Although the operating costs for such SFE plant remain higher than for a continuous counter current column, we think that SFE presents several interesting advantages for such application : it is compatible with food supplementation because of an easy elimination of the non-toxic CO<sub>2</sub>. That is the reason why we chose to carry the extraction without using any organic modifier. Moreover, EPA and DHA are high quality dietary labile molecules that could be altered by a too high temperature necessary in chemical procedures.

We are currently performing the fractionation of the PUFAs by using supercritical CO<sub>2</sub> in order to obtain a better purity in EPA and DHA in the product for food supplementation.

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## **Legends**

Figure 1 : Schematic representation of SFE process

Figure 2 : TLC of the lipid extracts from sardine heads

Figure 3 : GLC chromatogram of fatty acids contained in sardine oil extracted by supercritical CO<sub>2</sub>.

Table 1 : Doehlert design and experimental results

Figure 4 : Response surface projections

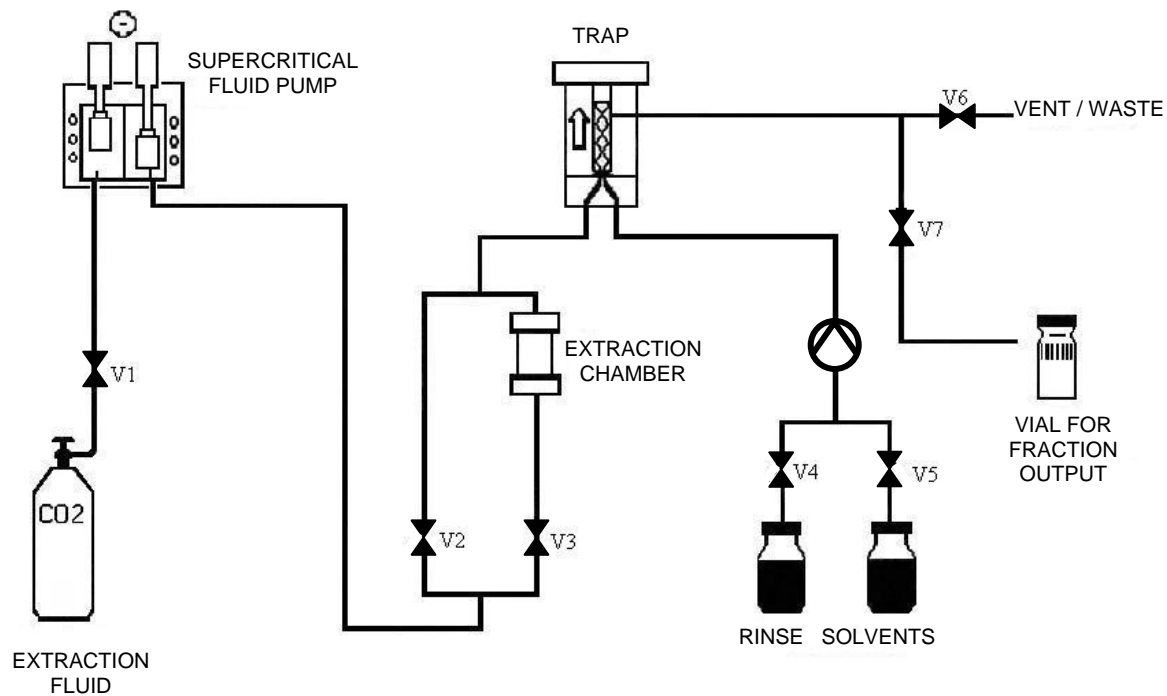
Figure 5 : Graph of the desirability function : Unilateral left Derringer

Figure 6 : Study of the desirability

Table 2 : Experimental results of optimized experiments for two levels of pressure compared with calculated values. Determination of oil yield and EPA and DHA concentrations

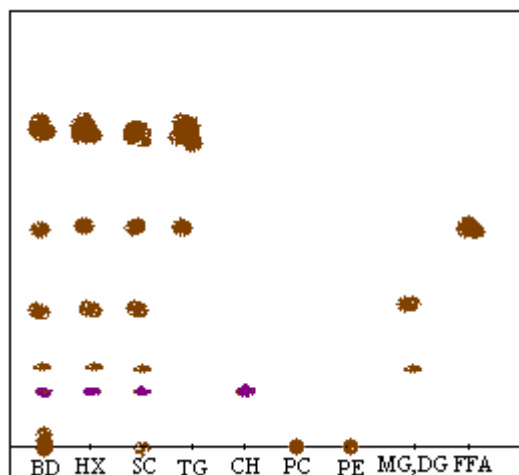
Table 3 : Fatty acid composition of oil extracted by supercritical CO<sub>2</sub> compared to that of oil extracted by Bligh and Dyer or by hexane.

**Figure 1 : Schematic representation of SFE process (V = valve)**



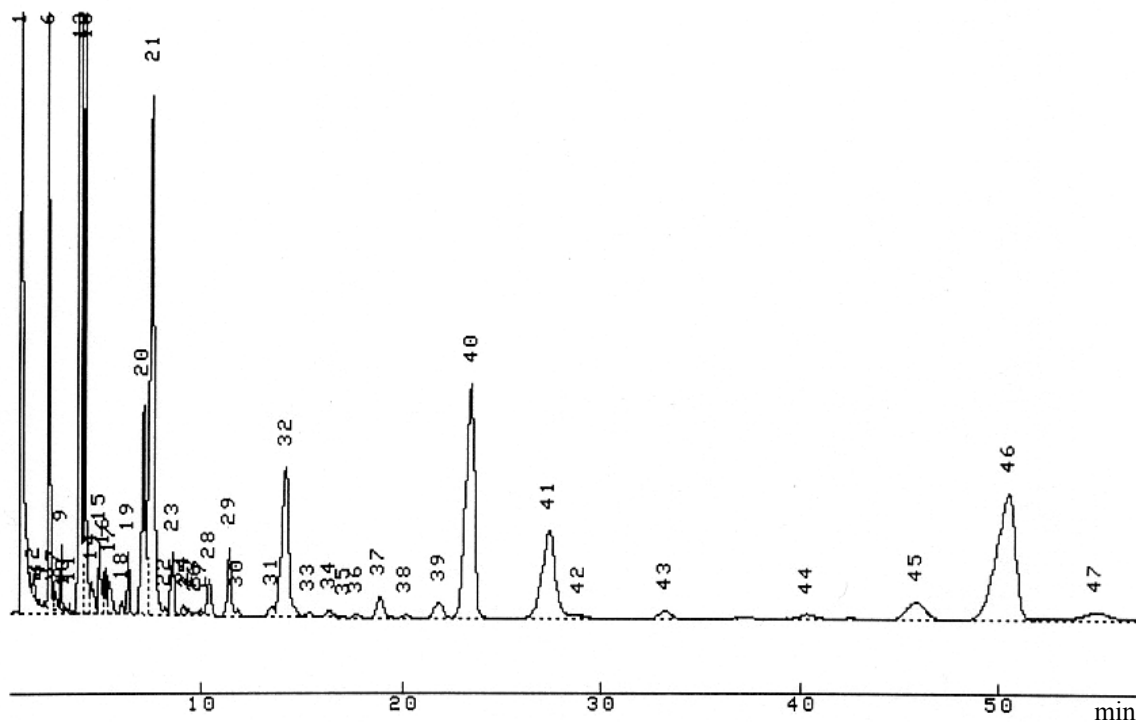
## Figure 2 : TLC of the lipid extracts from sardine heads

BD = oil extracted by Bligh and Dyer method. HX = oil extracted by hexane. SC = oil extracted by supercritical CO<sub>2</sub>. TG = standard of tripalmitine. CH = standard of cholesterol. PC = standard of phosphatidylcholine. PE = standard of phosphatidyl-ethanolamine. MG,DG = standard of mono- and diglycerides. FFA = standard of free fatty acid. Solvent elution : hexane/diethylether/acetic acid (70/30/1).



**Figure 3 : GLC chromatogram of fatty acids contained in sardine oil extracted by supercritical CO<sub>2</sub>**

Conditions of supercritical CO<sub>2</sub> extraction : pressure = 300 bars. temperature = 75°C. CO<sub>2</sub> rate = 2.5 ml/min. time = 45 min. Conditions of GLC as described in paragraph 2.7.  
Pick number 40 = EPA. Pick number 46 = DHA.



**Table 1 : Doehlert design and experimental results**

Doehlert design experiments are in bold.

Note : N.D. = Not Determined.

<sup>1</sup>mean and standard deviation were calculated with the experiments 26 to 29 performed in the same conditions.

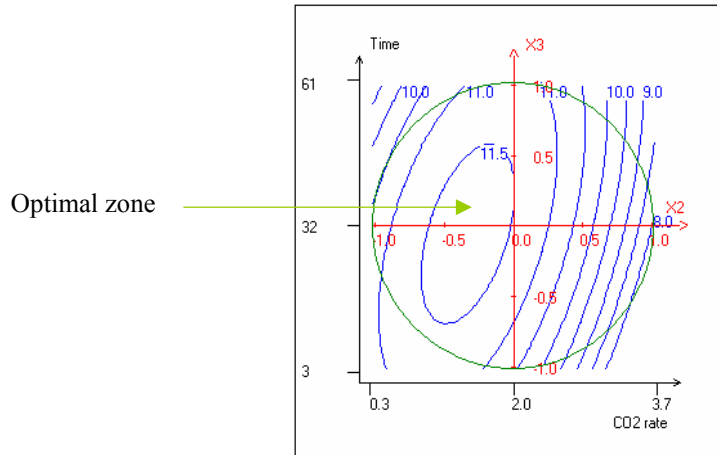
Experiments 14 to 17 : pressure was so small that a low quantity of oil was collected and it was not possible to do the qualitative analysis by GLC. Experiment 6 : the oil yield was too small to do further analyses. Experiment 18 : pressure coupled with CO<sub>2</sub> rate conditions were too high for the supercritical extractor. This experiment could not be carried out.

Rand = randomization.

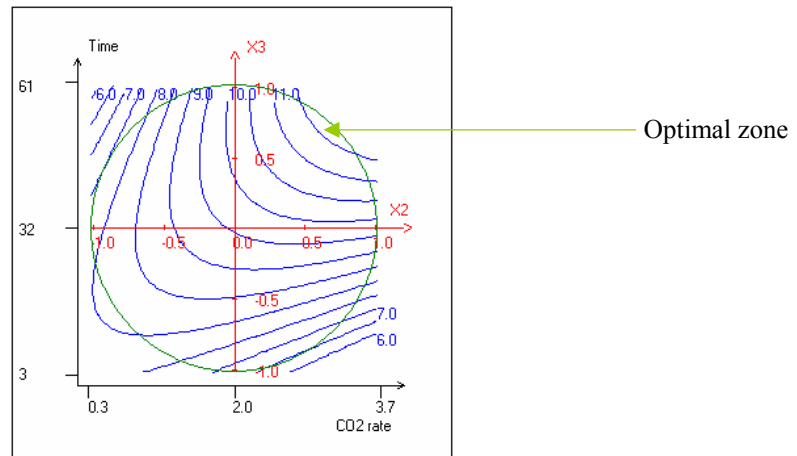
N°Exp	Rand	Temperature (°C)	CO <sub>2</sub> rate (ml/min)	Time (min)	Pressure (bars)	Oil yield (Eq. 1) (%)	EPA concentration (Eq. 3) (%)	DHA concentration (Eq. 3) (%)
<b>1</b>	<b>26</b>	<b>80</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>8.56</b>	<b>11.3</b>	<b>8.1</b>
<b>2</b>	<b>6</b>	<b>40</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>10.74</b>	<b>12.7</b>	<b>12.1</b>
<b>3</b>	<b>8</b>	<b>70</b>	<b>3.50</b>	<b>32</b>	<b>225</b>	<b>8.37</b>	<b>10.9</b>	<b>11.2</b>
<b>4</b>	<b>2</b>	<b>50</b>	<b>0.50</b>	<b>32</b>	<b>225</b>	<b>3.41</b>	<b>11.9</b>	<b>8.6</b>
<b>5</b>	<b>15</b>	<b>70</b>	<b>0.50</b>	<b>32</b>	<b>225</b>	<b>2.32</b>	<b>9.8</b>	<b>7.0</b>
<b>6</b>	<b>9</b>	<b>50</b>	<b>3.50</b>	<b>32</b>	<b>225</b>	<b>0.07</b>	<b>N.D.</b>	<b>N.D.</b>
<b>7</b>	<b>23</b>	<b>70</b>	<b>2.50</b>	<b>55</b>	<b>225</b>	<b>10.57</b>	<b>12.2</b>	<b>11.6</b>
<b>8</b>	<b>1</b>	<b>50</b>	<b>1.50</b>	<b>9</b>	<b>225</b>	<b>3.22</b>	<b>12.2</b>	<b>9.1</b>
<b>9</b>	<b>3</b>	<b>70</b>	<b>1.50</b>	<b>9</b>	<b>225</b>	<b>2.48</b>	<b>11.3</b>	<b>7.6</b>
<b>10</b>	<b>21</b>	<b>60</b>	<b>3.00</b>	<b>9</b>	<b>225</b>	<b>6.02</b>	<b>8.2</b>	<b>5.6</b>
<b>11</b>	<b>20</b>	<b>50</b>	<b>2.50</b>	<b>55</b>	<b>225</b>	<b>10.73</b>	<b>9.4</b>	<b>9.7</b>
<b>12</b>	<b>22</b>	<b>60</b>	<b>1.00</b>	<b>55</b>	<b>225</b>	<b>9.78</b>	<b>11.5</b>	<b>9.1</b>
13	28	70	2.50	38	350	7.68	12.4	15.6
14	16	50	1.50	26	100	0.07	N.D.	N.D.
15	12	70	1.50	26	100	0.00	N.D.	N.D.
16	10	60	3.00	26	100	0.54	N.D.	N.D.
17	18	60	2.00	49	100	0.03	N.D.	N.D.
18	11	50	2.50	38	350	N.D.	N.D.	N.D.
19	17	60	1.00	38	350	10.02	9.4	9.2
20	5	60	2.00	15	350	10.91	12.3	11.9
21	7	52	1.61	27	205	9.76	11.6	9.3
22	24	68	1.61	27	205	4.69	10.5	6.9
23	19	60	2.79	27	205	9.67	9.3	6.7
24	4	60	2.00	46	205	10.29	12.4	9.3
25	25	60	2.00	32	304	10.33	11.8	13.7
<b>26</b>	<b>13</b>	<b>60</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>9.98</b>	<b>11.3</b>	<b>9.2</b>
<b>27</b>	<b>27</b>	<b>60</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>9.75</b>	<b>11.6</b>	<b>9.7</b>
<b>28</b>	<b>29</b>	<b>60</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>9.13</b>	<b>11.7</b>	<b>9.8</b>
<b>29</b>	<b>14</b>	<b>60</b>	<b>2.00</b>	<b>32</b>	<b>225</b>	<b>9.64</b>	<b>11.3</b>	<b>9.5</b>
mean <sup>1</sup>						9.63	11.5	9.6
standard deviation <sup>1</sup>						0.359	0.206	0.265

#### Figure 4 : Response surface projections

Variation of the response - EPA concentration in the plane: CO<sub>2</sub> rate, Time  
FIXED FACTORS : - Temperature = 60 °C

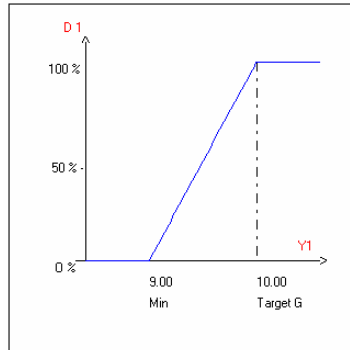


Variation of the response - DHA concentration in the plane: CO<sub>2</sub> rate, Time  
FIXED FACTORS : - Temperature = 60 °C

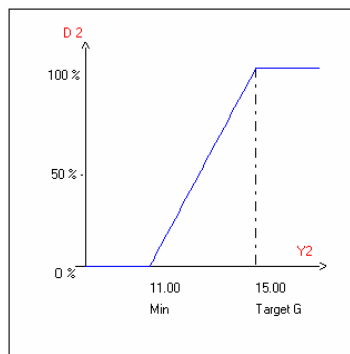


**Figure 5 : Graph of the desirability function : Unilateral left Derringer**

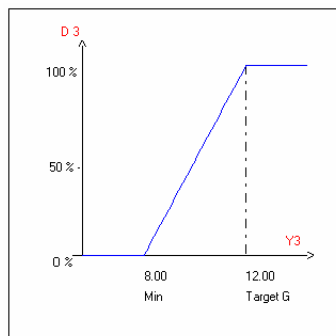
*Elementary desirability function of the response (Y1 ) : oil yield*



*Elementary desirability function of the response (Y2 ) : EPA concentration*



*Elementary desirability function of the response (Y3 ) : DHA concentration*



**Figure 6 : Study of the desirability**

*Coordinates of the maximum*

Pressure was set at 300 bars

Variable	Value	Factor	Value
X1	0.794539	Temperature	75
X2	0.394486	CO <sub>2</sub> rate	2.5
X3	0.497881	Time	45

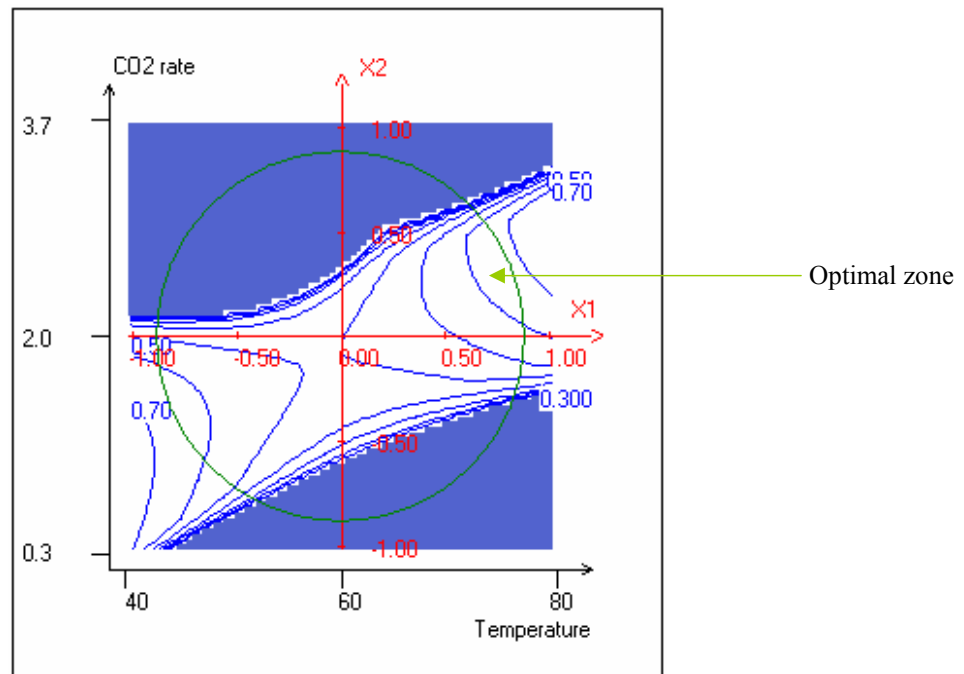
*Optimal conditions*

Response	Name of the response	Value	di %	weight	di min %	di max %
Y1	Oil yield	10.78	100.00	1	100.00	100.00
Y2	EPA concentration	12.65	41.18	1	36.02	46.33
Y3	DHA concentration	11.59	89.71	1	83.09	96.32
Y4	(EPA+DHA) concentration	25.26	100.00	1	100.00	100.00
	DESIRABILITY		77.96		73.97	81.73

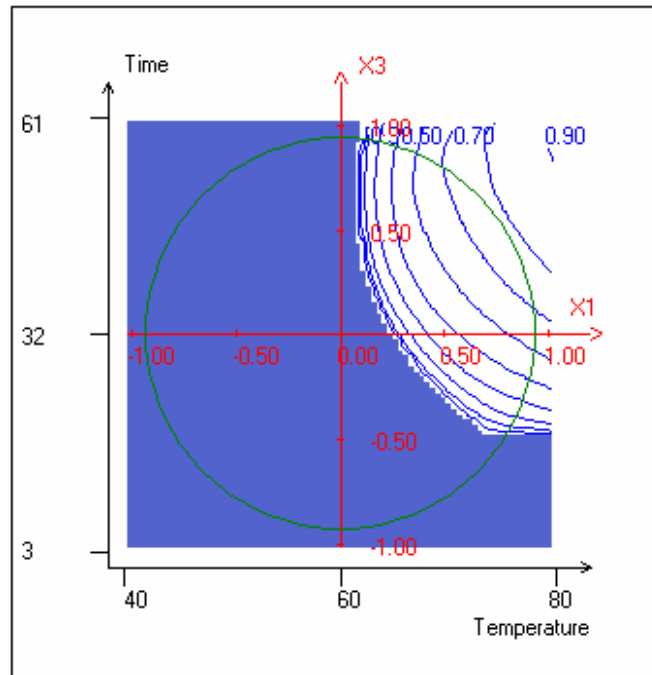
di : partial desirability of response Yi. di min : minimal partial desirability of response Yi. di max : maximal partial desirability of response Yi.

*Variation of the desirability function in the plane: Temperature, CO<sub>2</sub> rate*

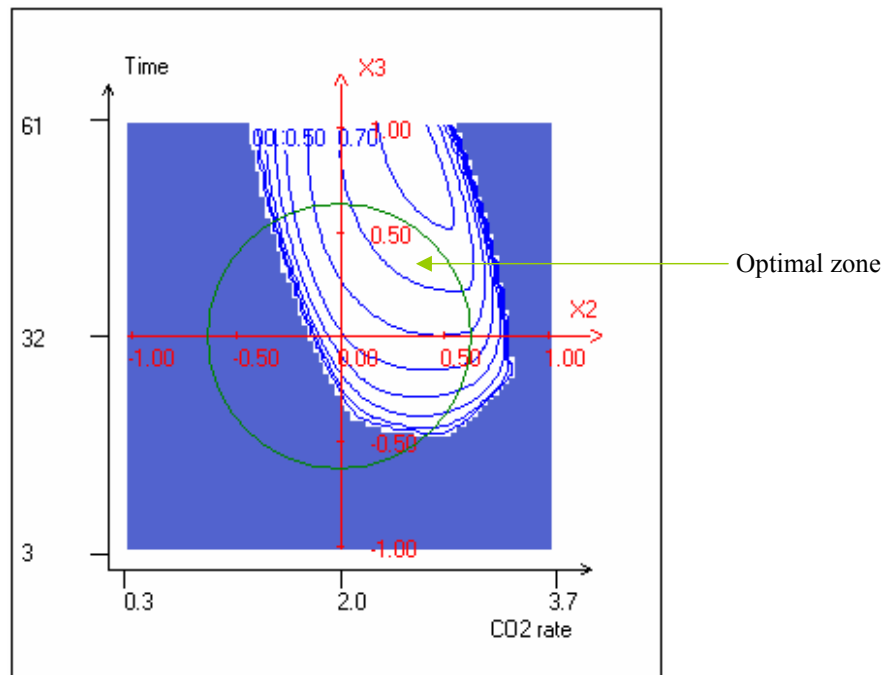
*FIXED FACTORS : - Time = 45 min*



Variation of the desirability function in the plane: Temperature, Time  
 FIXED FACTORS : - CO<sub>2</sub> rate = 2.5 ml/min



Variation of the desirability function in the plane: CO<sub>2</sub> rate, Time  
 FIXED FACTORS : - Temperature = 75 °C



**Table 2 : Experimental results of optimized experiments for two levels of pressure compared with calculated values. Determination of oil yield and EPA and DHA concentrations.**

Conditions : temperature = 75°C ; CO<sub>2</sub> rate = 2.5 ml/min ; time = 45 min ; P<sub>1</sub> = 225 bars ; P<sub>2</sub> = 300 bars.

<sup>1</sup>exp : experimental results

<sup>2</sup>num : numerical values : expected result from the desirability function.

Experiment	Oil yield (Eq. 1) (%)		EPA concentration (Eq. 3) (%)		DHA concentration (Eq. 3) (%)	
	exp <sup>1</sup>	num <sup>2</sup>	exp <sup>1</sup>	num <sup>2</sup>	exp <sup>1</sup>	num <sup>2</sup>
1	9.74	-	10.60	-	8.41	-
2	10.36	10.78	10.95	12.65	13.01	11.59

**Table 3 : Fatty acid composition of oil extracted by supercritical CO<sub>2</sub> compared to that of oil extracted by Bligh and Dyer or by hexane.**

<sup>1</sup> SFE parameters : Temperature = 75°C ; Pressure = 300 bars ; time = 45 min ; CO<sub>2</sub> rate = 2.5 ml/min.

Fatty acids	Hexane	SFE <sup>1</sup>	Bligh and Dyer
C14:0	5.48	6.56	7.22
C15:0	1.25	0.57	0.6
C16:0	18.26	18.86	19.05
C16:1 (n-7)	7.01	7.72	7.77
C16:2	3.20	0.91	0.98
C18:0	2.40	3.93	3.99
C18:1 (n-9)	5.96	N.I.	N.I.
C18:1 (n-7)	13.81	12.31	11.35
C18:2 (n-6)	1.43	1.05	0.98
C18:3 (n-3)	0.49	0.76	0.71
C18:4 (n-3)	1.19	1.53	1.59
C20:1 (n-9)	3.78	5.56	4.77
C20:4	1.38	0.96	0.85
<b>C20:5 (n-3)</b>	<b>10.01</b>	<b>10.95</b>	<b>11.73</b>
C22:1 (n-11)	2.84	5.85	5.23
C22:4	1.16	0.47	0.36
C22:5	1.34	1.71	1.60
<b>C22:6 (n-3)</b>	<b>15.16</b>	<b>13.01</b>	<b>13.97</b>
C24:1	N.I.	0.92	0.86
<b>saturated FA</b>	<b>27.39</b>	<b>29.93</b>	<b>30.86</b>
<b>mono-unsaturated FA</b>	<b>33.40</b>	<b>32.36</b>	<b>29.98</b>
<b>PUFAs</b>	<b>35.36</b>	<b>31.21</b>	<b>32.65</b>