SUPERCRITICAL EXTRACTION OF OMEGA-3 RICH OIL FROM TROUT WASTE

L. Fiori[a,*], M. Solana[a], P. Tosi[b], M. Manfrini[c], C. Strim[b], G. Guella[b]

[a] DICA, Università di Trento, via Mesiano 77, 38123 Trento (TN), Italy

[b] Dipartimento di Fisica, Università di Trento, via Sommarive 14, 38123 Povo (TN), Italy

[c] ASTRO, via Galilei 43, 38015 Lavis, Trento (TN), Italy

E-mail address: luca.fiori@ing.unitn.it (L. Fiori); fax: +39 0461 282672

Abstract

In this paper the extraction of oils from trout waste is investigated. Trout heads, viscera and spine, obtained from a fish-processing company, have been separately freeze-dried and then extracted. Two different solvent extraction techniques have been utilized and compared: the common soxhlet extraction, which utilizes *n*-hexane at atmospheric pressure as solvent, and the supercritical CO_2 extraction. To maximize the solubility of the oil into the solvent, the supercritical extraction tests have been conducted at 500 bar and 60 °C.
During the supercritical extraction, the collected oil has been sequentially separated into several fractions, in order to verify possible variations in composition for the same fractions.
The results are the following:
1) for each substrate the oil yield was significantly higher by using supercritical extraction than via soxhlet apparatus.
2) The fatty acid profile was similar for the various substrates (which were mainly constituted by TAGs) and practically independent on the extraction technique utilized, but crude oil obtained from viscera contains significant amount (13%) of DAGs and FFA.
3) The fatty acid profile testified an important presence of the most important omega-3 fatty acids (DHA and EPA) in the oil from fish waste (comparable with their amount in the oil from fresh fish filet).
4) Presence of lipids with omega-1 polyunsaturated fatty chains was observed in all the samples, although in low relative amount (about 3%)
5) The composition of the oil showed a slight dependence on the extraction time: for instance, the last oil fractions contained more DHA than the first fractions. Actually, the differences in compositions are small and therefore fractionating in this way did not result very effective.

Keywords: fish oil; omega 3 fatty acids; trout waste; supercritical extraction.

INTRODUCTION

In recent years there has been a growing interest in value-added specialty oils enriched with different omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6) for human nutrition and long-term health benefits. Fish is the most important source of omega-3 fatty acids in the diet, specially fish oil.
The basic idea of this study was the valorisation of the fish waste resulting from industrial processing, by recovering its oil content: on one hand, this will allow reducing the amount of

the waste whose elimination represents an important problem for fish industries; on the other, it will allow obtaining an EPA and DHA enriched product for food supplementation.

Extraction and purification of the lipids by conventional methods present some problems because they use flammable or toxic solvents and high temperature. These methods may have adverse health effects [1]. They also cause protein denaturalization and loss of functional properties [2]. An alternative to them, it is represented by the use of supercritical CO_2, which allows obtaining solute (oil in the specific case) free of solvent, operating in a not oxidizing atmosphere and using near ambient temperature. In the case of the fish oil, this is essential because its high grade of un-saturation makes it unstable. CO_2 has mild critical conditions (T_c=304.15 K, P_c=7.38 MPa) and it is compatible with food supplementation because of an easy elimination of the non-toxic CO_2.

In the literature it has been reported that different waste from different kinds of fish could be extracted with supercritical CO_2 to produce fish oil - salmon's viscera and heads [3], sardine heads [1], off cut from peeling hake [4], trout powder [5] - and that it is possible to concentrate omega-3 fatty acids in the extracted oil [6].

Fish waste oil extraction has been analyzed by a few authors. Recently, Létisse et al. [1] studied the supercritical fluid extraction of omega-3 rich oil from sardine heads. Fresh sardine samples were freeze-dried to reduce their water content to about 3%. For each extraction test, lasting 45 min, an amount of 2.5 g of substrate was extracted. Pressure, temperature and CO_2 flow rate were set at 300 bars, 75 °C and 2.5 ml/min, respectively. The amount of extracted oil resulted equal to 10.36% of the freeze-dried material and the concentration in EPA and DHA in the oil was equal to 10.95 and 13.01%, respectively.

Skara T. et al. [7] reported that by-products from aqua-cultured salmon could be used to produce fish oil of a quality suitable for human consumption. Filleting by-products (heads, frame bones, skin, and down-graded gutted fish) from farmed Atlantic salmon were separated into a solid/aqueous phase and a lipid phase (oil) using a scraped-surface heat exchanger (90 °C to 95 °C) and a decanter centrifuge (93 °C). Salmon oil is a stable product, and even more when it is stored at appropriate conditions (under nitrogen atmosphere and refrigerated).

Nuria Rubio-Rodriguez et al. [4] studied the supercritical fluid extraction of the omega-3 rich oil contained in the off cut from peeling hake. The raw material was grounded to different sizes and freeze-dried to different moisture contents. They concluded that moisture affects negatively the extraction yield. Particle reduction seemed not to have a large influence on the extraction yield, although the type of by-product and its pre-treatment is important. About 100 g of substrate were extracted in each test. The optimum extraction conditions were found: 25 MPa, 40 °C, 13.75 kg CO_2/h. Under these conditions, the extraction yield obtained after 3 h was 96.4 % of the total oil contained in the raw material and the extracted oil presented a high percentage of omega-3 and omega-6 (respectively about 6% and 14% of the total fatty acids contained in the hake by-products used in this work).

Ted H. Wu et al. [3] determined the FFA level in oil extracted from aging pink salmon heads and viscera maintained at two temperatures (6 °C and 15 °C). Results suggested that even after 4 days of storage at 15 °C, oil extracted from salmon heads and viscera is a good source of omega-3 fatty acids, especially EPA and DHA. Moreover, this oil had FFA values lower than 7%, and it retained approximately 25% of its antioxidant activity. This supports the concept of salmon by-products from remote locations being transported within 4 days to a centralised processing facility for oil extraction.

Hamzeh Zakizadeh Nei Nei et al. [5] developed in 2008 a mathematical model capable to predict the supercritical fluid extraction of oil from trout powder. They performed also experimental tests. Trout fish was cut into small pieces and freeze-dried at -50 °C for 24 h.

The effect of the pressure, temperature and bed void fraction on the oil extraction yield was examined. The best results were obtained with a pressure of 34 MPa, a temperature of 45°C and a bed void fraction of 0.45. A fluid flow rate of 0.80 ml/min was used and the extractor volume was equal to 10 ml.
The fractionation of fish oil with supercritical carbon dioxide in order to concentrate polyunsaturated omega-3 fatty acids was studied by Ana Paula Antunes Correa in 2008 [8]. The best operational conditions to fractionate the oil were 7.8 MPa and 28°C. They concluded that supercritical CO_2 can fractionate oils depending of the composition of triacylglycerols in the sample.
Besides salmon, sardine and hake by-products, also by-products from other fish species with high fat content like the trout could be used as raw material source for the oil recovery.
In this work we focused on aquaculture trout waste. About 30-40 wt % of the trout are not utilized for human consumption and remain as leftovers from industrial processing.
The fish waste have been at first freeze-dried. Then they have been extracted with supercritical CO_2 and, for comparative purposes, also at atmospheric pressure in a soxhlet apparatus using *n*-hexane as solvent. The extracted oils has been analyzed with different techniques.
The trout waste resulted to contain significant amounts of extractable lipids, and their oil a significant amount of EPA and DHA.
Our results suggest that also industrial waste from aquaculture trout are a good source of lipids and could be utilized to produce omega-3 rich oil by means of the supercritical technology.

MATERIALS AND METHODS

Solvents and fish waste

CO_2 (4.0 type, purity greater than 99.99 %) used as supercritical solvent was purchased by Rivoira. *n*-Hexane for the soxhlet extraction was purchased from Fluka (USA). All chemical used in the analytical methods were of "Reagent Grade".
Fish waste were furnished by Astro, a trout processing company located in Trento, Italy.

Extraction procedure

Prior to the extraction, trout by-products have been separately freeze-dried, because the pre-treatment of fish by-products affects the extraction yield [4,9]. The residual moisture resulting from freeze-drying has been measured by means of a thermo-balance: it resulted equal to 1.5%, 7.9% and 0.2% for the spine, head and viscera, respectively.
The freeze dried by-products have been kept refrigerated at a temperature of −20 °C until the experiments were performed.
Before extraction, the spine and head samples were milled by a grinder (Sunbeam Osterizer blender), and then sieved so that the particles utilized for the experiments had linear dimensions lower that 2 mm. Viscera samples were not milled because they appeared as a oily substrate.
The supercritical extraction equipment and procedure have been previously reported [10]. The temperature and the pressure were maintained constant for the various tests, at 60 °C and 500 bar, respectively. Solvent flow rate was fixed at about 12 g/min. For each test, about 50 g of freeze-dried trout waste were extracted.

In order to obtain the extraction kinetic curve, every 10 min the extracted oil was weighed. The vessel utilised for the oil collection was changed whenever 5-8 grams of oil were collected with the aim of separating sequentially during the extraction different oil fractions. In Table 1 the extraction time and the weigh of the different fractions are reported.

Table 1
Extraction time and weigh of the different fractions extracted

Fraction	Spine		Head		Viscera	
	time (min)	weigh (g)	time (min)	weigh (g)	time (min)	weigh (g)
1	50	6.3	70	7.36	30	6.92
2	90	6.37	160	7.13	60	6.97
3	180	5.13	230	1.63	100	6.31
4					160	7.25
5					220	6.97
6					370	7.8

The conventional extraction was carried out with a Randall apparatus. The equipment is a conventional soxhlet extractor modified in different aspects to increase the temperature of the solvent coming into contact with the sample to be extracted in order to reduce the extraction time. In the Randall extractor, a porous sample container is at first directly immersed into the boiling solvent. The direct immersion step allows a fast wetting of the sample and an extraction of soluble components, obviating the need for long periods of condensed vapour extraction which is slow and inefficient [11]. The immersion step is followed by a washing step where the extraction is finished according to the standard soxhlet technique. The solvent used was *n*-hexane. The temperature of extraction was 69 °C (boiling temperature of the solvent at atmospheric pressure). The total time of extraction was 2 h: 1 h for immersion and 1 h for washing.

Conversion of crude oil lipids into FAME

The transesterification was carried out on 200μL of crude oil both in basic and in acid media, in the former case at room temperature by adding 5mL of a 0.5M solution of KOH in methanol for 3 h, and in the latter by adding 5mL of a solution of a 3mM H_2SO_4 in methanol at 70°C for 24 hours. Both the reactions were carried out avoiding any contamination of water and were monitored using TLC (*n*-hexane/ethyl acetate 93:7). The process carried out in basic conditions was finally chosen as routinely transesterification method for the different batches of fish oil. After neutralization of the basic solution with sulphuric acid and i.v. evaporation of the organic solvent (yield 90%) the FAMEs were isolated by subjecting crude reaction material to a flash chromatography on Silica gel with *n*-hexane/ethyl acetate gradient elution. FAMEs were collected in the first 1-3 fractions (overall yield 85%) whereas free fatty acids and sterols in the last ones (7-9).

TLC analysis

Thin-Layer Chromatography (TLC) was performed on Merck Si60 PF_{254} plates (025 mm × 5 cm × 10 cm) in a TLC chamber by using 10 mL of a 9:1 (v/v) solution of *n*-hexane/ethyl acetate as mobile phase. After developing (about 10 min), the plate was dried and examined under a UV lamp (λ_{obs} = 254 nm); lipids lacking efficient UV absorption at the observed

wavelenght, can only be revealed as dark spot at R_f 0.62 by using the universal stain reagent $Ce(SO_4)_2/H_2SO_4$. A 100 mL solution of this reagent can be safely prepared by adding 30 mL of 98% sulfuric acid (v/v) to 70 mL of distilled water in a flask kept in an ice-bath. When the temperature of this solution is near room temperature, solid $Ce(SO_4)_2$ salt can be added with stirring in small aliquots until saturation. The TLC plate is first treated by an aerosol spray of this staining reagent, then heated at 110-120 ^{0} C for few seconds to afford products charring, leading eventually to the appearance of distinctive dark spots for each lipid components.

NMR analysis

NMR spectra (^{1}H NMR, ^{13}C NMR) were recorded on a Bruker-Avance 400 MHz NMR spectrometer by using a 5 mm BBI probe with 90^0 proton pulse length of 8 μs at a transmission power of 0 db. All spectra were taken at 298K in 50-100 mM solution of the different samples obtained from fish oil in $CDCl_3$ (700 μL); the chemical shift scales (δ) were calibrated on the residual signal of $CDCl_3$ at δ_H 7.26 ppm and δ_c 77.00 ppm.

NMR analysis was carried out both on the crude fish oils samples and on the corresponding purified FAMEs as obtained from FC chromatography.

Chromatographic analysis

HPLC-UV-ESIMS

HPLC separation was performed using a high-pressure liquid chromatograph Hewlett-Packard model HP1100 series, (Walderbron, Germany). A Rheodyne 7725 (Rohnert Park, CA, USA) injection valve equipped with a 20 μL internal loop was used for the injections. The liquid chromatograph was coupled to the mass spectrometer by means of an online ESI source on an Esquire™ LC Bruker-Daltonics ion trap mass spectrometer. (Bruker, Germany) The system was controlled by the softwares DataAnalysis (Version 3.0, Bruker Daltonik GmbH) and LC/MSD ChemStation (Agilent Technologies). This analysis was carried out only on purified FAMes as obtained from FC chromatography of transesterification reaction mixtures. FAMEs were analysed by HPLC-UV-MS techniques by using a RP18 Kinetex column and acetonitrile/water gradient elution.

GC-FID analysis

The fatty acid composition after transesterification was determined by gas chromatography.

A Carlo Erba mega series gas chromatograph, model 5300, equipped with a flame ionization detector was used. The carrier gas was Helium.

The chromatographic column used was a DB-WAX 30 m x 0.324 mm x 0.25 μm. The temperature of the column, injector and detector were maintained constant at 200 °C, 250 °C and 300 °C, respectively. Each analysis had a duration of 2 hours and was carried out in triplicate in order to quantify the precision of the results.

RESULTS AND DISCUSSION

Extraction

The extraction yield Y is expressed as the ratio between the weight of the extracted oil and the weight of freeze-dried and milled fish waste placed in the extractor vessel (in the following referred to as charge).

The supercritical extraction kinetic curves have been obtained by plotting the yield against the ratio between solvent consumption and charge (m_{CO2}/m_{charge}).

Fig. 1 shows typical extraction kinetics: in the first part of the extraction the increase of the yield (the slope of the curve) is maximal, then it lowers until the maximum yield is approached. The latter is about 0.78 for the viscera, 0.40 for the head and 0.41 for the spine. The fact that the slope of the curve relevant to the viscera is higher than the slope of the curves relevant to the spine and head could be due to the different characteristic of the charge. Because of the oily nature of the viscera, we can not exclude that some oil drops escaped from the extraction bed (we are working in the down-flow mode) and were transported in the liquid state to the oil collecting vessel.
Comparative soxhlet extractions have been conducted. The yields (0.57 for the viscera and 0.33 for the head and the spine) were in all the cases lower than the yields obtained by the supercritical extraction.

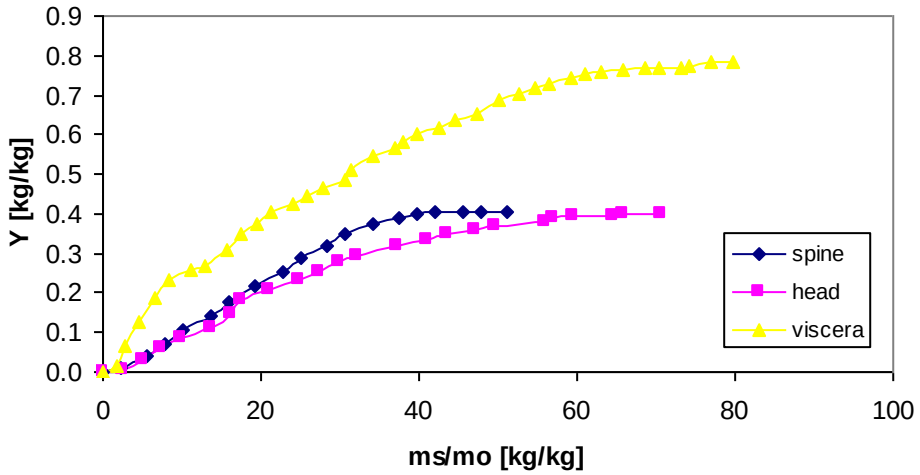

Fig 1. Extraction curves obtained by spine, head and viscera from trout at 60 °C and 500 bar.

Characterization of extracted oils

TLC analysis

After charring with staining reagent, the TLC analysis (Fig. 2) of dilute hexane solutions (~5 mg/ml) of the 3 crude oil extracts (viscera, head and spine) revealed wide dark spots at $R_F \approx 0.3$ (R_F the retardation factor, i.e. the ratio of the distance travelled by the center of the spot to the overall distance travelled by the mobile phase) attributable to TAGs and smaller spots at $R_F \approx 0.1$ (more polar metabolites) due to the presence of isomeric DAGs and free fatty acids (FFA).

By this simple and inexpensive technique it is possible to point out macroscopic difference in metabolites distribution in different samples. In particular, remarkable difference in DAG and FFA amounts can be appreciated between the 3 oil extracts with viscera fish extract containing higher amount of polar metabolites than head and spine extracts, an outcome confirmed by NMR analysis. Moreover, the FFA relative amount in viscera extracts (5%) were found by NaOH

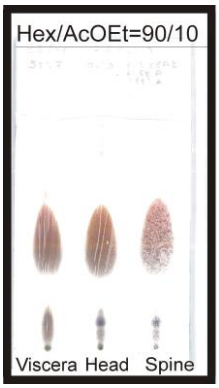

Figure 2: TLC analysis of crude oil extracts

titration of their ethanol solutions (by using phenolphthalein as indicator) to be significantly higher than in head and spine extracts (1%).

NMR analysis of the raw fish oils

The ^{1}H-NMR spectrum of the raw oil extract as obtained from fish heads (sohxlet) indicated that : i) the mean unsaturation index is 1.54 ± 0.05; ii) the relative content of unsaturated TAGs is 46% and thus the relative molar ratio of all the saturated chains is 54% ; iii) the presence of tri-unsaturated ω-3 lipids is confirmed by the presence in the ^{1}H-NMR spectrum of a sharp triplet at 0.97 ppm, a structural feature for homo-allylic Me group in unsaturated ω-3 fatty chains. In particular the ratio of this peak area to that of the Me group resonance as a broad triplet at 0.88 ppm of all the other chain (including ω-6 species) allowed us to establish the relative abundance of ω-3 lipids (15%).

Significant difference were noticed in relative content of TAG/DAG/FFA in the different samples. Whereas in extracts from head and spines the oil is essentially constituted by TAGs (overall 2% contribution of the other lipid species) in the viscera crude extracts the molar distribution ratio becomes TAG:DAG:FFA = 87%: 8%: 5%. In these samples both 1,3 DAG (5%) and 1,2 DAG (3%) were present.

NMR analysis of the corresponding FAMEs

From the ^{1}H-NMR spectrum of the purified fractions containing FAMEs we establish i) the mean unsaturation index (1.90 ± 0.02); ii) the relative content (65%) of all the unsaturated chains thus leaving at 35% the population of the saturated chains; iii) the presence of ω-3 species (20:5 and 22:6, mainly) in an estimated molar ratio 22% and iv) the presence of ω-1 species (16:4 or 18:5) in an estimated molar ratio of 3% .

Its worth of note that the unsaturation index is greater for the FAME mixture (no matter if obtained by acid or base hydrolysis) than for the original lipid mixture thus suggesting a preferential or faster hydrolysis process for unsaturated fatty lipids.

LC-MS analysis and isolation of FAMEs

FAMEs were analysed by HPLC-MS techniques by using a RP18 Kinetex column and acetonitrile/water gradient elution. The UV chromatogram as detected at 215 nm is shown in Fig. 3.

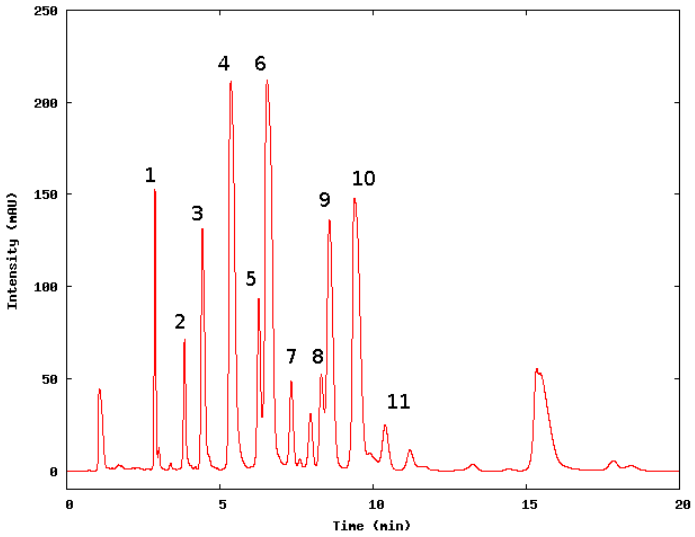

Figure 3 : RP-18 HPLC-UV chromatogram (λ 215 nm) of the transesterification products obtained from head oil extract

Every component of the FAMEs mixture (numbered from 1 to 11) was identified by on-line mass spectrometer working in ESI positive ion-mode through detection of the characteristic m/z values of any compound corresponding to their $[M+H]^+$ and $[M+Na]^+$ adducts.
Furthermore, HPLC methodology has been exploited not only to assign definite structure to every peak in the chromatographic run but also, in separate experiments, to collect every peak containing FAMEs (1-11) for using in the quantitative GC-FID measurements reported below.

GC-FID analysis of FAME mixture and purified peaks
A representation of a typical chromatogram of trout oil is presented in Fig.4.

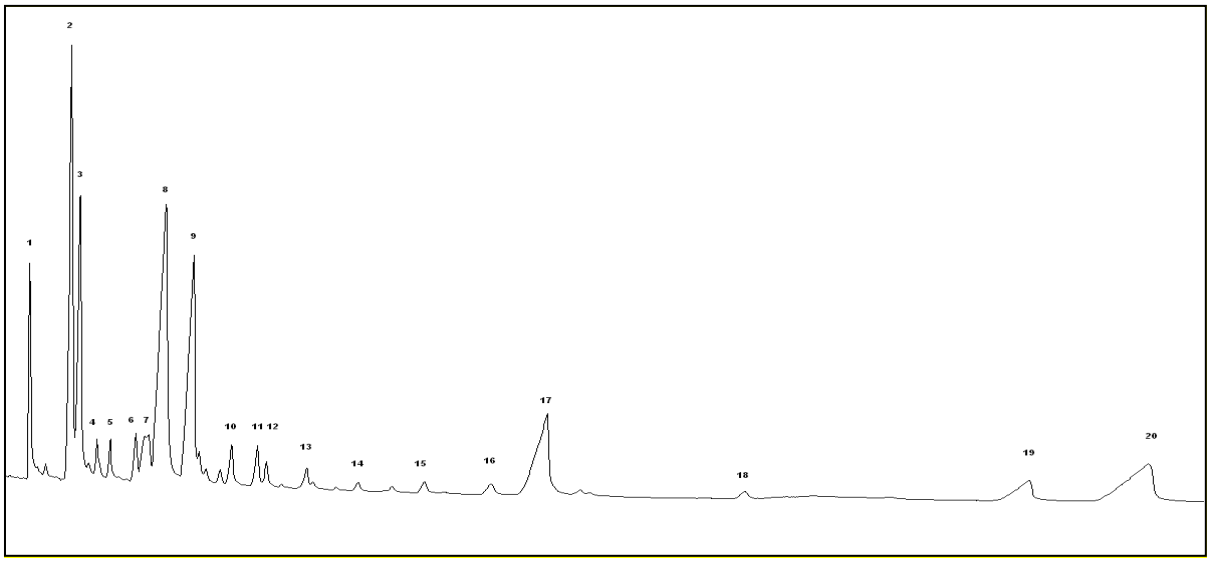

Fig.4. Chromatogram of the second fraction of the trout head oil extracted by supercritical CO_2. Peak numbers and the correspondent representative FA are: (1) 14:0, (2) 16:0, (3) 16:1, (4) 16:2, (5) 16:3, (6) 17:0, (7) 18:0, (8) 18:1, (9) 18:2, (10) 18:3n-3, (11) 18:4n-3, (12) 22:0, (13) 20:1, (14) 20:3, (15) 20:4n-6, (16) 20:4n-3, (17) 20:5, (18) 23:0, (19) 22:5n-3, (20) 22:6n-3.

The fatty acid profiles of trout spine, viscera and head oil extracted with the soxhlet are reported in Table 2. The oil contains mainly C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:1 (oleic acid), C18:2 (linoleic acid), C20:5 (EPA) and C22:6 (DHA). The fatty acid composition of the oils from trout spine, head and viscera resulted extremely similar. The fatty acid composition of the oils from trout waste has been compared with the composition of the oil from the fillet of the fresh trout (data furnished by ASTRO). As shown in Table 2, the FA composition of the oils from fish waste is very similar to that of the fresh fillet. The percentage of one of the most important omega-3 fatty acids extracted from trout waste, i.e eicosapentanoic acid (EPA, 20:5), resulted even greater than the value found out for the trout-fillet (about 9 % and 4.20 %, respectively).
The fatty acid composition of the trout waste oil extracted by supercritical extraction and by soxhlet were compared. The composition of the oil extracted by supercritical extraction was calculated multiplying the percentage of every fatty acid in every fraction by the weigh of the relevant fraction and then dividing what obtained by the total weigh of the oil extracted. As shown in Table 2, the percentage of EPA and DHA is slight higher in the oil extracted by supercritical extraction, but there are not high differences between the fatty acid composition of the oils extracted by the different methods. In the literature, it has been reported that the oil extracted with SC-CO_2 at 40 °C presents a higher value of unsaturated fatty acids than the oil extracted by hexane [4]. In this study, supercritical extraction was carried out at 60°C and the

soxhlet extraction at 69 °C, so the thermal degradation of the fatty acids can be hypothesized as being similar and consequently the fatty acid profile is practically the same.
As we have shown previously, CO_2 extraction permitted to obtain a yield higher than conventional extraction. So, the efficiency of SFE was higher of that of hexane extraction. 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 who consumes these products. For all these reasons, we think that the application of supercritical CO_2 without any organic modifier is interesting for such valuable molecules from waste of canned fish industry [1].

Table 2
Fatty acid composition of the spine, head and viscera trout oil extracted by different methods and comparison with the fatty acid composition of the fresh trout oil

Fatty acid	Composition (%)						
	soxhlet			SCE-CO_2			fresh trout
	spine	head	viscera	spine	head	viscera	
c14:0	5.24 ± 0.58	5.39 ± 0.59	5.07 ± 0.22	5.16 ± 0.42	5.38 ± 0.53	5.14 ± 0.43	4.70 ± 0.55
c15:0	0.41 ± 0.02	0.43 ± 0.19	0.37 ± 0.03	0.42 ± 0.03	0.43 ± 0.15	0.35 ± 0.24	
c16:0	14.36 ± 0.76	14.95 ± 1.27	14.57 ± 0.70	14.69 ± 0.66	14.57 ± 0.68	13.98 ± 0.71	18.67 ± 1.25
c16:1n7	8.33 ± 0.75	9.00 ± 0.44	8.92 ± 0.21	8.32 ± 0.41	9.28 ± 0.71	9.17 ± 0.53	6.23 ± 0.57
c16:2	1.37 ± 0.10	1.48 ± 0.18	1.39 ± 0.09	1.35 ± 0.03	1.53 ± 0.13	1.52 ± 0.25	
c16:3	1.21 ± 0.13	1.29 ± 0.14	1.21 ± 0.18	1.16 ± 0.07	1.36 ± 0.10	1.33 ± 0.09	
c17:0	1.42 ± 0.00	1.52 ± 0.16	1.44 ± 0.06	1.37 ± 0.05	1.52 ± 0.08	1.44 ± 0.07	0.54 ± 0.52
c18:0	3.28 ± 0.05	3.34 ± 0.02	3.31 ± 0.06	2.97 ± 0.42	3.23 ± 0.11	3.39 ± 0.22	4.41 ± 0.48
c18:1	20.99 ± 0.24	20.65 ± 0.11	21.67 ± 0.33	21.40 ± 0.40	20.95 ± 0.85	22.28 ± 0.63	21.84 ± 1.32
c18:2n6	15.50 ± 0.52	13.81 ± 0.13	15.60 ± 0.05	15.31 ± 0.40	14.47 ± 0.42	13.34 ± 0.41	15.44 ± 2.1
c18:3 n-6	0.40 ± 0.06	0.43 ± 0.10	0.25 ± 0.21	0.40 ± 0.05	0.53 ± 0.13	0.43 ± 0.22	0.36 ± 0.04
c18:3 n-3	**1.59 ± 0.08**	**1.39 ± 0.14**	**1.64 ± 0.28**	**1.55 ± 0.06**	**1.27 ± 0.41**	**1.43 ± 0.54**	**2.44 ± 0.52**
c18:4 n-3	**1.28 ± 0.02**	**1.36 ± 0.06**	**1.16 ± 0.11**	**1.27 ± 0.05**	**1.31 ± 0.08**	**1.36 ± 0.07**	**1.21 ± 0.12**
c20:0	0.82 ± 0.04	0.83 ± 0.02	0.78 ± 0.07	0.79 ± 0.03	0.98 ± 0.32	0.87 ± 0.21	0.39 ± 0.17
c20:1 n-9	1.18 ± 0.20	1.01 ± 0.19	0.82 ± 0.62	1.04 ± 0.15	1.10 ± 0.11	1.06 ± 0.13	3.28 ± 0.37
c20:3 n-6	0.43 ± 0.04	0.36 ± 0.05	0.25 ± 0.15	0.43 ± 0.09	0.27 ± 0.04	0.47 ± 0.03	0.25 ± 0.04
c20:4 n6	0.56 ± 0.05	0.36 ± 0.22	0.49 ± 0.08	0.34 ± 0.15	0.62 ± 0.26	0.41 ± 0.13	0.51 ± 0.07
c20:4 n3	**0.90 ± 0.21**	**0.61 ± 0.16**	**0.86 ± 0.23**	**0.73 ± 0.11**	**0.71 ± 0.11**	**0.73 ± 0.13**	**0.94 ± 0.06**
c20:5 n3	**8.94 ± 0.55**	**9.55 ± 0.80**	**8.60 ± 0.41**	**9.22 ± 0.59**	**9.72 ± 0.50**	**9.06 ± 0.53**	**4.20 ± 0.44**
c21:5 n3	**0.49 ± 0.06**	**0.48 ± 0.04**	**0.52 ± 0.05**	**0.56 ± 0.13**	**0.51 ± 0.15**	**0.77 ± 0.16**	**1.83 ± 2.39**
c22:5n3	**2.80 ± 0.17**	**2.95 ± 0.32**	**2.71 ± 0.29**	**2.81 ± 0.20**	**2.96 ± 0.31**	**2.89 ± 0.23**	**1.62 ± 0.17**
c22:6n3	**8.50 ± 0.83**	**8.82 ± 1.02**	**8.36 ± 0.69**	**8.69 ± 0.57**	**8.82 ± 0.75**	**8.61 ± 0.64**	**11.50 ± 1.21**

Fig. 5 and 6 show the composition of the different fractions of the head and spine trout oil extracted by supercritical CO_2. The first fraction is the richest in the fatty acids with short chain. The EPA and DHA are more abundant in the fractions 2 and 3. So, in order to obtain an oil relatively richer in these compounds, the oil extracted in the second part of the extraction could be used. Anyway, the differences are not very high. Thus, in order to increase the percentage of EPA e DHA, it would be necessary to proceed with the transesterification of the oil before utilizing supercritical CO_2 for the fractionation, in a fractionation column, of the esterified fatty acids.

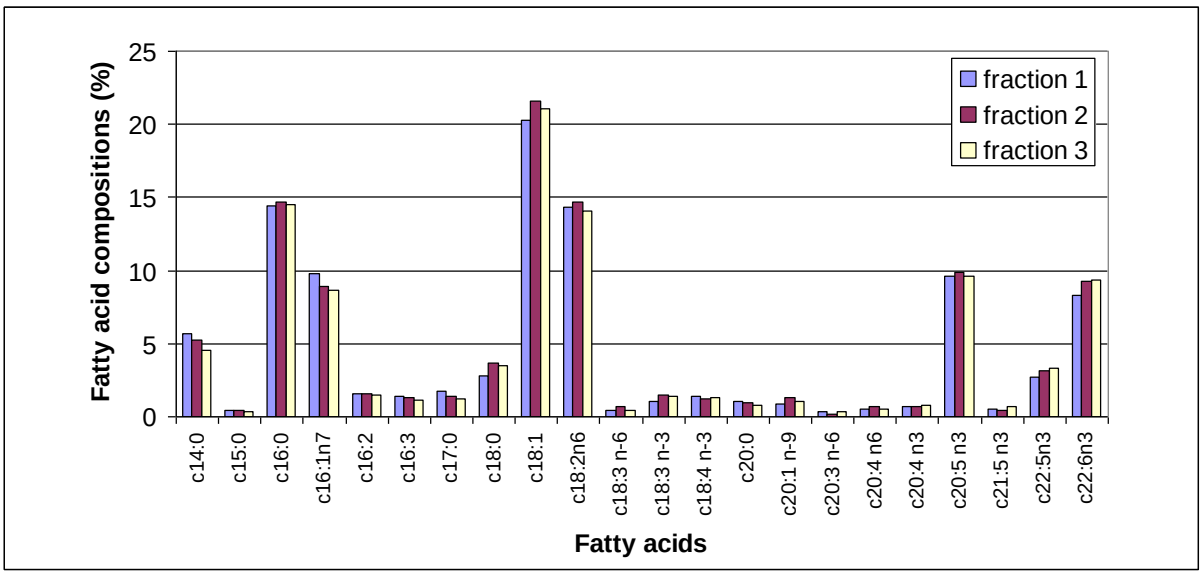

Fig. 5. Comparison of fatty acid composition of the different fractions from the supercritical extraction of the trout head oil.

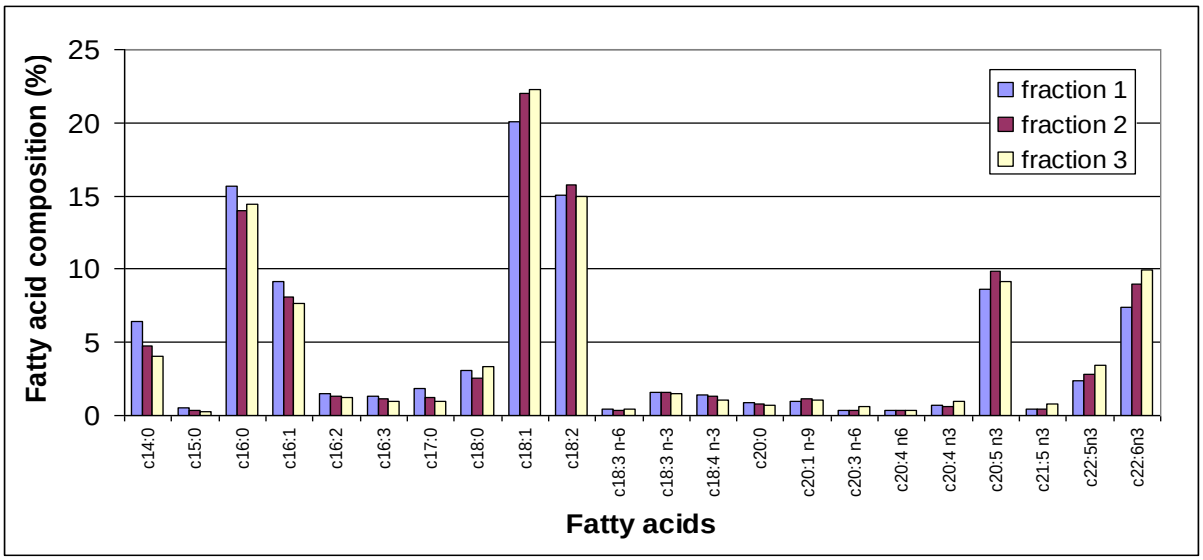

Fig. 6. Comparison of fatty acid composition of the different fractions from the supercritical extraction of the trout spine oil.

Table 3 resumes the lipid classes in the trout oil, calculated from the fatty acid profile. It shows that the unsaturated fatty acids contents in waste oils represent about 74 % of the total fatty acids, and 72% in fillet oil. Therefore, they are a rich source of unsaturated fatty acids. The omega-3/omega-6 ratio in spine, head and viscera oils extracted by soxhlet were 1.45, 1.68 and 1.44, respectively, and 1.51, 1.59 and 1.70 in the oil obtained by supercritical extraction. The omega-3/omega-6 ratio in the total lipids of freshwater fish is usually between 0.5 and 3.8 [12]. The omega-3/omega-6 ratio has been suggested as a useful indicator for comparing relative nutritional values of fish oils. An increase in the human dietary omega-3/omega-6 fatty acid ratio is essential in the diet, to help prevent coronary heart disease by

reducing plasma lipids, and to reduce cancer risk. An increase in the dietary omega-3/omega-6 ratio also seems to be effective in preventing toxic shock syndrome and cardiomyopathy [13].

Table 3
Lipids classes (% of total fatty acids) in trout spine, head, viscera oils obtained by two processes

	soxhlet			$SCE\text{-}CO_2$			fresh trout
	spine	head	viscera	spine	head	viscera	
saturated fatty acids	25.52	26.46	25.54	25.40	26.11	25.17	28.70
unsaturated fatty acids	74.48	73.54	74.46	74.60	75.43	74.83	71.64
saturated/unsaturated	0.34	0.36	0.34	0.34	0.35	0.34	0.40
unsaturation index	1.98	1.99	1.95	1.99	2.03	1.98	1.89
omega 3	24.52	25.16	23.86	24.84	25.31	24.84	23.74
omega3/(tot-omega3)	0.32	0.34	0.31	0.33	0.34	0.33	0.31
omega 6	16.89	14.95	16.59	16.49	15.90	14.65	16.56
omega-3/omega-6	1.45	1.68	1.44	1.51	1.59	1.70	1.43

Finally, the percentage of the most important omega 3 fatty acids, EPA and DHA, was compared with that of some other fish oils. The trout waste oil has a lower proportion of EPA and DHA than mackerel and sardine oil, but a higher content of those fatty acids than the oil from the salmon and the carp waste, as shown in Table 4.

Table 4
Comparison of EPA and DHA concentration (%) in fish oil from different waste

Fatty acid	Composition respect to the total fatty acid content (%)								
	salmon viscera [14]	mackerel viscera [15]	carp viscera [16]	trout viscera	mackerel head [15]	sardine head [1]	trout head	mackerel skin [15]	trout spine
c20:5n3 (EPA)	7.91	9.98	3.82	8.60	10.82	10.95	9.72	14.28	8.94
c22:6n3 (DHA)	6.99	9.31	1.2	8.36	10.34	13.01	8.82	12.51	8.50

CONCLUSIONS

The supercritical extraction of trout waste was compared with the conventional extraction. The yield obtained by supercritical extraction was in all the cases higher than the yield obtained by conventional extraction. The differences in the percentage of fatty acids extracted using the different extraction methods were not significant.
The differences in the percentage of fatty acids extracted in the different fractions of supercritical extraction were not very significant.
These results suggest that also industrial waste from aquaculture trout are a good source of lipids and could be utilized to produce omega-3 rich oil by means of the supercritical technology. Use of by-products as a source of fish oil can also benefit the environment by reducing waste dumping.
The presence in all the investigated trout extracts of omega-1 lipids, although in relative low amount (3%), has been also established by NMR analysis. Since no commercial reference sample are available for these lipids, further investigations are needed to establish their real structure but TAGs with 18:4 fatty chains are the most plausible candidates.

Significant difference were noticed in relative content of TAG/DAG/FFA in the different extracts with viscera being much more rich in FFA and DAGs than heads and spines.
We are currently working on the fractionation of the PUFAs by using supercritical CO_2 in order to obtain a better purity in EPA and DHA in the product for food supplementation.

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