EXTRACTION OF PUFAs RICH OILS FROM ALGAE WITH SUPERCRITICAL CARBON DIOXIDE.

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Abstract
Recently, attention has focused on n-3 PUFAs, especially eicopentaenoic acid (EPA) and docosahexaenoic acid (DHA), due to their association with the prevention and treatment of several diseases (atherosclerosis, thrombosis, arthritis, cancers, etc.). The conventional source of EPA and DHA is marine fish oil, but higher amount of EPA and some DHA can be produced by the use of algae. The extraction of oil from algae (*Isochrysis galbana* Parke) was studied using supercritical carbon dioxide at different extraction temperature and pressure, using ethanol as cosolvent. Extraction yield at different conditions was compared with traditional organic solvents Soxhlet extraction (hexane, petroleum ether, chloroform/ethanol), and the composition in fatty acids was compared with data in literature. Lyophilized samples collected by flocculation and by centrifugation were tested. 4-10% lipid extraction yield was obtained by using neat carbon dioxide at 690 bar and 40°C. 5-11% was obtained by using carbon dioxide/ethanol. The higher lipid extraction yield (15-28%) was obtained by using chloroform/ethanol 1/1 (v/v), while 5-16% and 5-15% was obtained by using hexane and petroleum ether respectively. Some differences on the composition of extracts have been found. Results allow us to conclude that supercritical fluid extraction can be considered a process to obtain active compounds form algae with favorable extraction time, number of handling steps and quantity and quality of solvents used although lower extraction yields. The composition of extracts leads to consider them as nutraceuticals that is one of the trends in EU policies as reported in the recent VI Framework Program.

Introduction
Since the sustainable development is a stringent problem at the beginning of the third millennium, every research conducted on the direction of reducing the use of toxic chemicals, saving energy and managing wastes and by-products, is welcomed at international level (www.johannesburgsummit.org; www.europe.en.int). Moreover, the market for nutraceuticals is growing quickly worldwide, and it is this global scope that particularly attracts marketers. Both the food and pharmaceutical industries are interested in and aggressively developing this product category. This sector has the potential to grow and capitalize on tremendous global marketing opportunities. It is thus important to characterize the industry and identify what it needs to help it take advantage of the growing market for nutraceuticals, both as final products and as food ingredients, around the world (www.agr.gc.ca). Examples of these compounds include Poly Unsaturated Fatty Acids (PUFAs). Recently, attention has focused on n-3 PUFAs, especially eicopentaenoic acid (EPA) and docosahexaenoic acid (DHA), due to their association with the prevention and treatment of several diseases (atherosclerosis, thrombosis, arthritis, cancers, etc.) (Shahar *et al*., 1994; von Schacky *et al*., 1999). The conventional source of EPA and DHA is marine fish oil, but higher amount of EPA and some DHA can be produced by the use of algae (Choi *et al*., 1987; Lopez Alonso *et al*., 1992; Balaban *et al*., 1996; Liu and Lin, 2001; www.irishscientist.ie; www.fao.org). Indications for cholesterol prevention indicate supplying 1-1.5 g die of EPA and DHA, with EPA>DHA and 60% concentration or more; no particular need is required for fatty acid source. The use for

dietary supplements in pregnancy or retina protection requires DHA>EPA and the same total concentration.
The purpose of this research was to study extraction of lipids from a marine phytoflagellate algae (*Isochrysis galbana* Parke) using Supercritical Carbon Dioxide (SC-CO_2) at different extraction temperature and pressure, using ethanol as cosolvent to propose novel products (oils) with higher value in comparison with current available products on the market. Experiments on lipids extraction were conducted on laboratory scale through apparatuses able to collect the extracts to be subsequently evaluated. In some traditional PUFAs rich oils extraction, organic solvents (generally n-hexane) are used for different materials. The procedure usually requires a relatively long time, and the conventional priced solvents used are toxic and polluting. SC-CO_2 introduces for PUFAs rich oils extraction a clean technology, with negligible environmental impact (Rizvi *et al*., 1988; Temelli *et al*., 1995; Taylor, 1996). Method application concerned the realization of SC-CO_2 extraction of lipids from algae. Analyses of extracts were conducted. Extraction yield at different conditions was compared with traditional organic solvents

Materials and methods

A quantity of the strain LB2307 of *Isochrysis galbana* Parke algae were supplied by the Agri-food Science Park in Pantalla di Todi – PG (Italy). The matrix was grown in photo-bio-reactor, harvested after 3 days with centrifugation (cream separator) or flocculation (ferric chloride 180mg/L), and lyophilized for storing.

(http://www.nhm.ac.uk/hosted_sites/ina/CODENET/galleries/DICimages/source/iso.htm)

The following chemicals were used: n-hexane (HPLC), ethanol 99.8%, of ACS degree, and petroleum ether (40°-60°C) (analytical grade) were purchased from Carlo Erba (Milan, Italy). Water (HPLC) and methanol (HPLC) were purchased from J.T. Baker (Deventer, NL). Liquid CO_2 (99.998%) was purchased in cylinders from Linde-Caracciolossigeno (Perugia, Italy).
The following **instruments** were used. A Soxhlet unit was used, composed by common reflux distillation glassware and thermostated water bain. "HR 200" analytical balance was from AeD Instrument Ltd (Oxon, UK). For the separation of fatty acids a Jasco PU-1580 pump (Jasco Co. Tokyo, Japan), and an ELSD 500, MKIII, evaporative light scattering detector (Alltech Associates Inc., Deerfield, IL, USA) with two reversed-phase C18 columns (Inertsil ODS-3), in series, were used. The injector was a Rheodyne Model 7725 (Rheodyne Inc., Cotati, CA, USA) with a 50 µL loop.
Supercritical fluid laboratory extractor SFX3560^{TM} (ISCO Inc. Lincoln, Ne, USA). The SFX3560 extractor is a laboratory plant with a sample reel holding up to 24 sample cartridges (extractors) and 24 collection vials (separators). The instrument has software, which enables a dynamic method-programming scheme, and is able to programming up to 24 different extractions. Two model 100DX ISCO pumps, capable of 690 bar were used to deliver CO_2 and the modifier, into the SFX 3560. An automatic restrictor is capable to control static and dynamic extractions with flow from 1.0 to 2.0 mL/min., heating up to 200°C. Collections are in 3 to 10-mL extraction separators. A screening of the following pressures, temperatures and flows was used. Algae extractions were conducted on lyophilized samples using the SFX3560 apparatus with the 10 mL vessel, and equipments for lipids extraction with organic solvents (Soxhlet). The neat SC-CO_2 extractions were conducted with the following parameters: pressure of 300 and 690 bar; temperature of 40° and 50°C, static extraction time of 10 and 60

min., dynamic extraction time of 240 min.. The extractions were conducted with CO_2 and modifier (ethanol) to verify its ability to increase significatively the extraction yield and modifying the extract composition. The following parameters were used: 0.038 ethanol molar fraction (5% v/v), pressure of 690 bar, temperature of 40° and 50°C (at 40°C and 690 bar the solvent mixture is in subcritical conditions; at 50°C and 690 bar the phase is in supercritical conditions), static extraction time of 10 min., dynamic extraction time of 240 min.. Solvent flow rate was 2mL/min. (liquid CO_2 with or without modifier, compressed at extraction pressure conditions, and at 5°C). Collection was at 5°C and atmospheric pressure, in ethanol refilled automatically by the SFX3560 software in the glass vials. n-Hexane, petroleum ether, chlorophorm/ethanol 1/1 (v/v) were used with reflux distillation in traditional Sohxlet apparatus following the AOCS Ai 3-75 method (1996). The distillation temperature was that of ebullition for the different solvents.

Analyses: fatty acids methyl esters were prepared by transesterification of the collected extracts, dissolved in n-hexane, with methanolic KOH. n-Hexane was evaporated under nitrogen, and the methyl esters were finally redissolved in methanol, filtered with a 0.2 um nylon filter (Whatman, Maidstone, UK) and injected. The used eluent was methanol:water 97:3; flow rate, 1 mL/min. The separation of fatty acids methyl esters is achieved using two reversed-phase C_{18} columns (250 mm x 4.6 mm, 5 µm, Inertsil ODS-3) in series, and ELSD was used. The ELSD drift tube temperature was set at 75°C. Nitrogen gas flow of the nebulizer was set at 2.75 L/min., and nitrogen gas pressure was 1.38 bar (Lin *et al*., 1995).

Data from duplicate analysis were analyzed with Excel worksheets (Microsoft, Redmond, WA, USA); means are reported. They were evaluated for standard deviation and reported as percentage Relative Standard Deviation (RSD%) for intra-laboratory repeatability assessment. The Unpaired t-test (SigmaStat, SPSS Science, Chicago, IL USA) was used at $P<0.05$ to determine the statistically significant difference between analytical methods.

Results and discussion

The results obtained for the application of SFE method are here reported. Initial trials were performed with a SFE screening system (50mL extractor vessel) to verify the extractability of the analytes from matrix. These trials were necessary to study the new approach with SFE (data not reported). The utilized conditions were the same reported in literature for similar products. The use of lyophilized samples enables extractions without the interference of water in extracting and collecting only lipids. Moreover, the matrix is available in a powder form leading to efficient diffusion and activity of SC-CO_2 and no particular matrix preparation is needed. For the centrifuged samples, the higher extraction yield was obtained by neat SC-CO_2 (10.40 g/100g) at 690 bar and 40°C with 10 min. of static extraction before the dynamic one (**table 1**). In these conditions a 72% (10.40/14.48) recovery was obtained in comparison to the petroleum ether, of 66% (10.40/15.84) in comparison to n-hexane, of 38% (10.40/27.45) in comparison to the mixture chlorophorm/ethanol. The extraction yield improves (11.15 g/100g) giving respectively a 77% (11.15/14.8), 70% (11.15/15.84) and 41% (11.15/27.45) recovery, adding 5% of ethanol as a modifier, and bringing the extraction temperature at 50 °C to reach supercritical conditions of the mixture. The efficiency of the modifier however is not statistically significant. The study of static extraction time leads to ineffective results for the extraction yield. The mixture chlorophorm/ethanol is surely influenced by its polarity allowing extracting numerous polar compounds increasing the total weight of the collected extract. Instead, the use of a modifier improves the extracting ability of CO_2 because few polar lipids are solubilized over what the neat CO_2 can do, without interesting with not lipidic fractions as the chlorophorm/ethanol mixture can do. A confirmation of such hypothesis is

found in literature (Choi *et al*., 1987; Lopez Alonso *et al*., 1992), and will be confirmed when the characterization analyses will be completed. It is evident the low yield obtained in the subcritical conditions (only 9.65 g/100g).

solvent and conditions (bar/°C)	**Collected extraction yield** (g/100g)	**SD**	**RSD%**
n-hexane	15.84[a]	0.21	1%
petroleum ether	14.48[ab]	0.09	1%
chlorophorm/ethanol 1/1	27.45[a]	1.65	6%
300/40, 10' static, neat CO_2	9.86[ac]	2.16	22%
690/40, 10' static, neat CO_2	10.40[bc]	0.56	5%
690/40, 60' static, neat CO_2	9.67[ac]	1.63	17%
690/50, 10' static, neat CO_2	9.95[a]	3.77	38%
690/40, 10' static, 5%EtOH*	9.65[a]	0.74	8%
690/50, 10' static, 5%EtOH**	11.15[a]	0.98	9%

n=2; RSD%: relative standard deviation.; [abc]Superscriptive letters in each sample row indicate significant difference at $P<0.05$; * liquid solvent; ** supercritical solvent.

Table 1. Algae oil extraction yield at different extraction and solvent conditions (centrifuged samples).

For the flocculated samples, the higher extraction yield (4.33 g/100g) was again obtained at 690 bar, but increasing the temperature at 50°C (**table 2**). The high error does not allow finding statistically significant differences between 40° and 50°C for the centrifuged and the flocculed samples. Since the fractions of *Isochrysis* raised in same conditions, it is evident from the analysis of **table 1** and **2** that the kind of algae harvesting, influenced the initial lipids content. An overall decrease of lipids content is evident for flocculated algae. The effect of $FeCl_3$ to coagulate the microalgae cells lead to clusters with reduced surface of the matrix to solvent activity during extraction.

solvent and conditions (bar/°C)	**Collected extraction yield** (g/100g)	**SD**	**RSD%**
n-hexane	4.93[a]	0.56	11%
petroleum ether	4.70[a]	0.24	5%
chlorophorm/ethanol 1/1	15.05[a]	1.91	13%
300/40, 10' static, neat CO_2	4.39***	-	-
690/40, 10' static, neat CO_2	3.74[ac]	1.57	42%
690/40, 60' static, neat CO_2	3.21[ac]	0.85	26%
690/50, 10' static, neat CO_2	4.33[a]	1.19	27%
690/40, 10' static, 5%EtOH*	1.92[abc]	0.02	1%
690/50, 10' static, 5%EtOH**	5.24[ac]	1.75	33%

n=2; *** n=1;RSD%: relative standard deviation; [abc]Superscriptive letters in each sample row indicate significant difference at $P<0.05$; * liquid solvent; ** supercritical solvent.

Table 2. Algae oil extraction yield at different extraction and solvent conditions (flocculated samples).

In such conditions and neat SC-CO_2 a 92% (4.33/4.70) recovery was obtained in comparison to the petroleum ether, 88% (4.33/4.93) in comparison to n-hexane, of 29% (4.33/15.05) in comparison to chlorophorm/ethanol. Adding 5% ethanol as a modifier, the extraction yield improves, and the recovery was respectively 112% (5.24/4.70), 106% (5.24/4.93) and 35% (5.24/15.05). The low lipids concentration for this flocculated fraction, and the influence of flocculation on the matrix structure increased the error. Considering the general behavior of traditional solvents, neat SC-CO_2 and SC-CO_2 mixed with ethanol, it is confirmed the general behavior of changing the extracting ability, mainly in terms of polar compounds extraction.

The concentration of fatty acids in collected extracts at different extraction conditions is reported in **table 3** for centrifuged samples, and **table 4** for flocculated samples. No particular differences are found in consideration of different static extraction time.

solvent and conditions (bar/°C)	**Fatty acids** (mg/mL of collected extracts)					
	myristic	**palmitic**	**palmitoleic**	**stearic**	**oleic**	**linoleic**
n-hexane	9.024	2.599	n.d.	n.d.	1.310	0.965
300/40, neat CO_2	8.979	n.d.	1.841	n.d.	1.191	0.939
690/40, neat CO_2	9.162	n.d.	1.839	n.d.	1.216	1.021
690/50, neat CO_2	9.071	2.481	1.872	n.d.	1.237	1.148
690/40, 5% EtOH	8.943	2.552	1.857	1.414	1.263	0.891

n.d.: not detectable

Table 3. Fatty acid composition of centrifuged samples.

Myristic acid content in collected extracts is not influenced by the solvent or conditions used. Palmitic acid is extracted only by hexane, and liquid CO_2/ethanol mixture or supercritical CO_2 at 10.000 psi and 50°C; only these last conditions allow the extraction of stearic acid. For oleic and linoleic acids, no particular differences arises from the use of different solvents and extraction conditions for centrifuged samples.

solvent and conditions (bar/°C)	**Fatty acids** (mg/mL of collected extracts)					
	myristic	**palmitic**	**palmitoleic**	**stearic**	**oleic**	**linoleic**
chloroform/ethanol 1/1	9.188	2.583	2.008	1.326	2.259	0.975
300/40, neat CO_2	8.962	2.572	1.847	1.413	1.249	1.004
690/40, neat CO_2	9.115	2.646	n.d.	1.429	1.971	1.204
690/50, neat CO_2	8.971	2.555	1.832	n.d.	1.390	0.900
690/40, 5% EtOH	8.971	2.560	1.830	1.412	2.342	1.306
690/50, 5% EtOH	9.026	2.555	1.828	n.d.	1.188	0.895

n.d.: not detactable.

Table 4. Fatty acid composition of flocculated samples.

The use of neat SC-CO_2 at 690 bar and 50°C increases the content of unsatured fatty acids in comparison with the use of cosolvent. When the temperature of the solvent mix (CO_2 and ethanol) decreases to 40°C (liquid solvent) the concentration of unsatured fatty acids is higher in comparison with supercritical mixture. Concentration of octadecatertraenoic acid (C18:4), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) will be available when standard calibration curves will be completed. Chromatogram areas are now available and here discussed (data not reported). For centrifuged samples, the use of traditional solvent (hexane) leads to do not extract EPA, while it is possible with the use of liquid CO_2/ethanol mixture, supercritical carbon dioxide at 300 bar, or better when 690 bar are used. The use of 40°C instead of 50°C allows high content of C18:4 and EPA in collected extracts. C18:4 can be extracted in minor quantity when hexane or liquid mixture is used. For flocculated samples, DHA is extracted and collected only when chlorophorm/etanol mixture is used. The use of neat SC-CO_2 at 690 bar and 40°C allows the higher extraction of EPA in flocculated samples, while, the use of 50°C, or the use of cosolvent, leads to minor concentration in this unsatured fatty acid and in C18:4. Liquid sc-CO_2/ethanol mixture allows higher unsatured fatty acid content for flocculated samples in comparison with centrifuged ones. Overall range of collected fatty acids is higher for centrifuged samples in comparison with flocculated ones, confirming the general behavior found for total collected extract.

Conclusions

The study of SFE on *Isochrysis* allows us to draw some considerations.

The use of supercritical fluids allow the extraction of lipids from microalgae with interesting composition in terms of extraction yield and fatty acid concentration in comparison with traditional solvents. More analysis on collected extracts will determine if the different extraction conditions can influence the enrichment in PUFAs for different novel products targeted to consumers.

The scaling up of processing will indicate how the proposed technology can be economically effective.

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