SUPERCRITICAL FLUID EXTRACTION OF MICROALGAE *SPIRULINA PLATENSIS*. CHEMO-FUNCTIONAL CHARACTERIZATION

Jose A. Mendiola[1], F. Javier Señoráns[1], Elena Ibañez*[2], Laura Jaime[1] and Guillermo Reglero[1]

1: Área de Tecnología de Alimentos, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain

2: Instituto de Fermentaciones Industriales, CSIC, España. C/ Juan de la Cierva, 3 28006 Madrid

E-mail: elena@ifi.csic.es Fax: +34-91 564 4853

The goal of the present work was to develop clean technologies for the isolation of new functional ingredients from alternative sources such microalgae. Clean technologies were based on the use of supercritical fluids such as carbon dioxide and ethanol as modifier. Both are GRAS (Generally Recognized As Safe) solvents, therefore, both of them can be used in the food industry.

In this work, a complete study of the parameters that control supercritical fluid extraction (pressure, temperature, density and use of modifier) was performed. Pressures tested ranged from 78 to 361 bar, temperatures from 26 to 83 ºC and densities from 0.195 to 0.923 g/ml. All experiments were done under an experimental design strategy. The design was done twice, differing in the use of 10% ethanol as co-solvent.

The chemical characterization of the extracts was performed using liquid chromatography with Diode Array Detector while the fractions were also functionally characterized by *in vitro* antioxidant activity assays (using the β-carotene bleaching method).

The possibility of the combined used of functional and chemical characterization of the extracts provides a complete information about both, the activities expected and the compounds responsible for such activity.

INTRODUCTION

Spirulina is considered a traditional food in the Mexican and African cultures. It is a planktonic blue-green algae (from Cyanobacterium gender) found in alkaline water of volcanic lakes. *Spirulina* has a 62% amino acid content being the world's richest natural source of vitamin B_{12}, it also contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments. *Spirulina* has a soft cell wall made of complex sugars and proteins. Actually it is gaining more attention because of its nutritional and various medicinal properties [1].

The use of Spirulina's pigments as colorants has been explored by the pharmaceutical and food industries mainly because the new world trends towards the substitution of synthetic colorants by natural ones. The first experimental plant for *S. platensis* production was developed during the 60's in the Institut Francaise du Peetrole. Nowadays, Japan and United States of America have industrial scale production and more than 40 kind of products derived from this algae are available on the market [2].

Supercritical fluid extraction (SFE) is a well-recognized alternative to conventional solvent-based extraction techniques. SFE has the main advantages of being environmentally

benign and available as fully automated systems. CO_2 has been the most used supercritical solvent, because the compounds can be obtained without contamination by toxic organic solvents and without thermal degradation, and it requires mild conditions to become a supercritical fluid. Nevertheless, due to its poor solvating power sometimes is necessary the use of polar modifiers. In the present work, ethanol was selected as modifier due to its GRAS (Generally Recognized As Safe) character that makes it suitable to be used for food ingredients production.

I - MATERIALS AND METHODS

Sample & Reagents: Microalgae *Spirulina platensis* used in present work was obtained freeze dried from Algamar (Pontevedra, Spain). Ethanol (HPLC) was obtained from Panreac (Barcelona, Spain). CO_2 (N-48 quality) was supplied by AL Air Liquide España S.A. (Madrid, Spain).

Instrumentation-Extraction method: A schematic diagram of the SFE pilot plant employed in this study is shown in Figure 1. The SFE pilot plant has the following features: an extraction vessel (270 ml), two separator cells (270 ml capacity each) for sequential decompression, and a cryogenic trap at atmospheric pressure. The plant has computerized PLC-based instrumentation and control system, with alarms and rupture disks. The CO_2 and modifier pumps are from Dosapro Milton Roy.

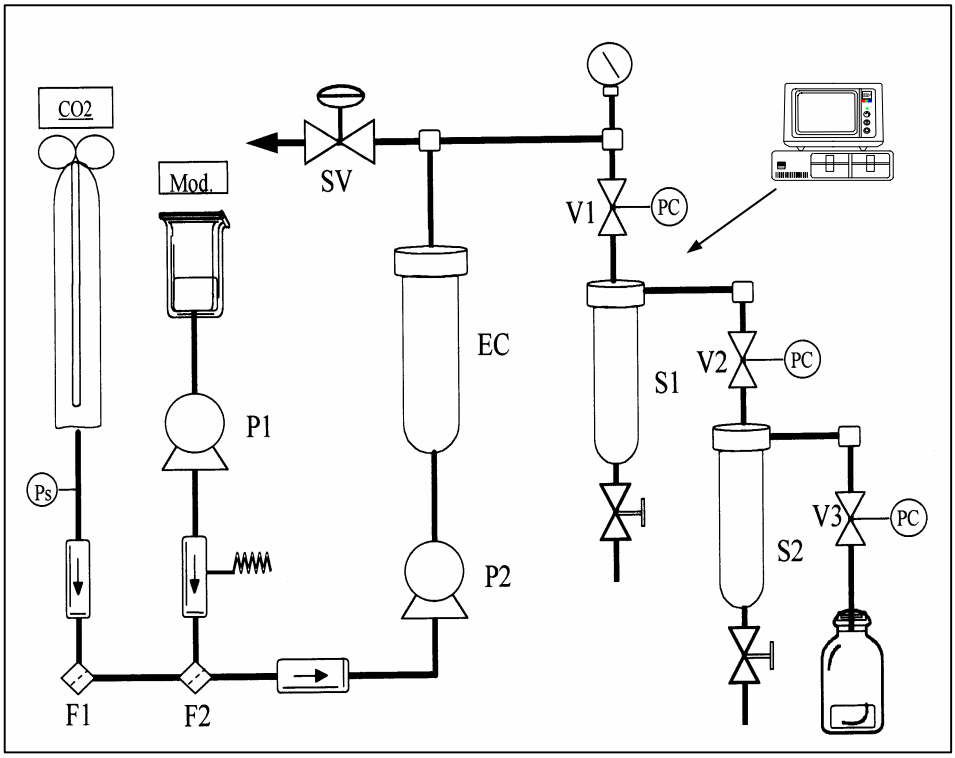

Figure 1: Schematic diagram of the countercurrent SFE pilot plant used in the present study. P1= modifier pump; P2= CO_2 pump; SV= safety valve; EC= Extraction vessel ; S1= separator 1; S2= separator 2; V1, V2, V3= micrometering valves; PC= pressure controller, Mod.= modifier deposit

Experimental design: Table 1 shows the matrix of the experimental design (centered, second order) used in this study. Two variables, extraction pressure and temperature, were selected with a total of 10 experiments considering those of a complete factorial 2^2 (+1, -1) plus 4 star points (levels +1.44, -1.44) and 2 replicates in center (level 0). Thus, several pressures and temperature conditions were tested with values of extraction pressure ranging from 78 to 320 bar and values of extraction temperature from 26.7 to 83.3 °C. Conditions were selected to cover a wide range of extraction densities based on data found in the literature [3] and experimental limitations of our pilot scale plant. The experimental design were done twice, one using pure CO_2 as extracting agent and another one using CO_2 with 10% ethanol as modifier. In all the experiments, the pressure of separator 1 was selected as 50% of the extraction pressure while pressure in separator 2 was set as 50% of the pressure of separator 1. The temperatures in both separators were set at the corresponding extraction temperature. In all cases 75 g of freeze dried microalgae mixed with 100 g of sea sand (Panreac, Barcelona, Spain) were used. The solvent flow rate was set at 3000 ml CO_2/h.

Table 1.- Matrix of the experimental design (centered, second order) used to study the supercritical fluid extraction conditions of *Spirulina platensis*, showing the physical values tested

Exp.	P Level	T Level	Real Pressure	Real Temp.	CO_2 Density
1	0	0	220	55	0.781
2	0	0	220	55	0.781
3	1	1	320	75	0.785
4	-1	1	120	75	0.318
5	1.44	0	361	55	0.888
6	0	-1.44	220	26.7	0.920
7	-1.44	0	78	55	0.195
8	0	1.44	220	83.3	0.618
9	-1	-1	120	35	0.768
10	1	-1	320	35	0.940

Pressures given in bar, temperatures given in ºC and densities given in g/ml

Analysis by Liquid Chromatography (LC-DAD): in order to determine the chemical composition of the different fractions obtained by SFE, all the extracts were injected in an HPLC (HP1090 Liquid Chromatograph, Agilent) equipped with Diode Array Detector (DAD) and automatic injector (20 µL of a solution containing 40 mg/L of extract diluted of ethanol). The system was coupled to an Agilent software (Chemstation LC 3D). A Column Novapack C_{18} 3.9x150 mm with 4 µm of particle diameter (Waters) was used. Two different chromatographic conditions were employed depending on the fraction analyzed. For extracts collected in separator 2 (when using ethanol as modifier) the mobile phase was a mixture of solvent A (water) and solvent B (methanol) according to a step gradient changing from 50 % B to 60 % B at 10 minutes and to 100 % B at 30 minutes and keeping that conditions for 10 minutes, at a flow rate of 0.8 mL/min. The rest of the fractions were analyzed using also a mixture of solvent A (water) and B (methanol) with the following gradient: 80% B changing to 100% B in 25 minutes and then keeping that conditions constant 15 minutes. In all cases the spectral data was collected using a Diode Array Detector working from 200 to 600 nm. Data obtained was used to determine the family of the detected compounds. Quantitative

determination was done by previously selecting different wavelengths (230 nm; 280 nm and 440 nm).

Antioxidant Assays: The antioxidant activity of supercritical fluid extracts was determined according to the β-carotene bleaching method described by [4] with some modifications. 0.2 ml of *Spirulina platensis* supercritical extracts or 0.2 ml of pure ethanol (as control) were added to a reagent mixture, containing 0.2 ml of β-carotene (Sigma) solution (1 mg/mL in chloroform), 20 mg of linoleic acid (Sigma), and 200 mg of Tween 20 (Sigma) and the final mixture was evaporated to dryness under a nitrogen stream. Fifty millilitres of distilled water were added and the mixture was vigorously shaken to form a liposome solution. The samples were kept at 50ºC for 3 hours for thermal autooxidation. The absorbance of these solutions was measured at 470 nm using a spectrophotometer. All samples were assayed in triplicate. Butyl-hydroxy-toluene (BHT) (Sigma) (0.5 mg/mL) was used as standard. The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using equation (1):

$$AA = [(R_{control} - R_{sample})/R_{control}] \times 100 \quad (1)$$

Where: $R_{control} = (\log_n Abs(t_0)/Abs(t_{120})/180$

II- RESULTS AND DISCUSSION

Supercritical fluid extraction: Due to the different conditions used to perform the study, the collected fractions were very different both in aspect and in extraction yield. In general, extractions using pure CO_2 provided lower yields and red-orange look while extractions done with CO_2 plus 10% of ethanol give higher extractions yields and dark green-brown color. Overall, yields were very low due to the high protein content of *Spirulina* (more than 60% weight). As for the extraction yield, the best extraction conditions were those of experiment 7 (78 bar, 55 ºC) of the modifier series, that was the experiment with the lowest CO_2 density (0.1947 g/ml), providing a yield of 8.08% w/w. It is important to consider that, at these subcritical extraction conditions with 10% ethanol as modifier, the extraction was performed with two phases (CO_2 + ethanol) instead of using a mixture of one phase [5]. On the other hand, using pure CO_2 the highest extraction yield was obtained at the conditions of experiment 3 (320 bar, 75 ºC, 0.785 g/ml) with 0.85% w/w.

Functional analysis: The fractions with higher antioxidant activity were those obtained in separator 1 in experiments 1, 2 and 3 with modifier and 3 without modifier, as can be seen on Table 2. In order to compare these activities with a well-known antioxidant, the test was repeated using BHT, which gave a percentage of inhibition of 95.26 ± 1.25 using 0.5 mg/ml, and 80.85% ± 1.63, for 0.25 mg/ml.

Chemical analysis: The aim of the present work was to identify the compounds, or families of compounds, associated to the functional activities of the supercritical extracts. As a first approach, a tentative identification was done based on spectral data. At present, studies are being conducted in our laboratory to further identify the compounds found in the extracts using liquid chromatography coupled to mass spectrometry (LC-MS).
As can be seen in Figure 2, there are important differences between LC profiles of extracts obtained in separator 1 (chromatograms A and B obtained at two different wavelengths, 230 and 440 nm) and 2 (chromatograms C and D). By observing the major compounds found in each fraction it can easily be seen that in separator 1 the main compounds extracted and

recovered belong to the chlorophylls family while in separator 2 carotenoids are found in highest concentration.

Table 2. - Antioxidant activity of *Spirulina platensis* sub- and supercritical carbon dioxide extracts measured by β-carotene bleaching test calculated in terms of percent inhibition relative to the control

Conc.*	CO_2 12.5 mg/ml	CO_2 + Ethanol 12.5 mg/ml
1.S1	68.37 ± 1.16	94.74 ±0.78
1.S2	63.15 ±0.57	78.44 ±3.04
2.S1	71.96 ±1.13	94.17 ±0.21
2.S2	64.53 ±0.91	83.72 ±0.54
3.S1	94.56 ±0.12	94.16 ±0.09
3.S2	88.72 ±0.39	–
4.S1	52.65 ±0.69	76.57 ±1.33
4.S2	48.17 ±3.4	37.57 ±2.3
5.S1	82.98 ±0.54	87.45 ±1.68
5.S2	90.22 ±0.19	47.62 ±1.6
6.S1	84.18 ±0.18	84.01 ±0.50
6.S2	56.96 ±3.41	61.00 ±0.82
7.S1	55.27 ±4.91	83.92 ±0.28
7.S2	19.76 ±2.66	15.68 ±1.42
8.S1	85.16 ±0.03	89.43 ±0.69
8.S2	38.07 ±1.56	61.80 ±2.25
9.S1	65.20 ±5.66	67.82 ±1.29
9.S2	47.29 ±16.84	35.73 ±4.79
10.S1	26.19 ±1.68	93.27 ±0.04
10.S2	75.70 ±0.41	74.13 ±0.24

* Concentrations given in mg of dry extract per ml ethanol

III- CONCLUSIONS

In the present study the possibilities of extracting antioxidant compounds from Spirulina platensis by using an environmentally friendly process (such SFE) are reported. Several extracting conditions were evaluated via an experimental design and the extracts obtained were chemically and functionally characterized using LC-DAD and ß-carotene bleaching test, respectively. Results obtained demonstrated the interest of the SFE as a clean technique to selectively isolate different type of compounds, with different activities, from natural sources such microalgae.

IV- ACKNOWLEDGEMENT

This work has been financed under a R+D program of the Spanish Ministry of Science and Technology, with a project called “New food ingredients obtained by sub- and supercritical fluid extraction from microalgae” (AGL-2002-04621-C02-02). J. A. Mendiola acknowledges the Ministry of Science and Technology for a grant.

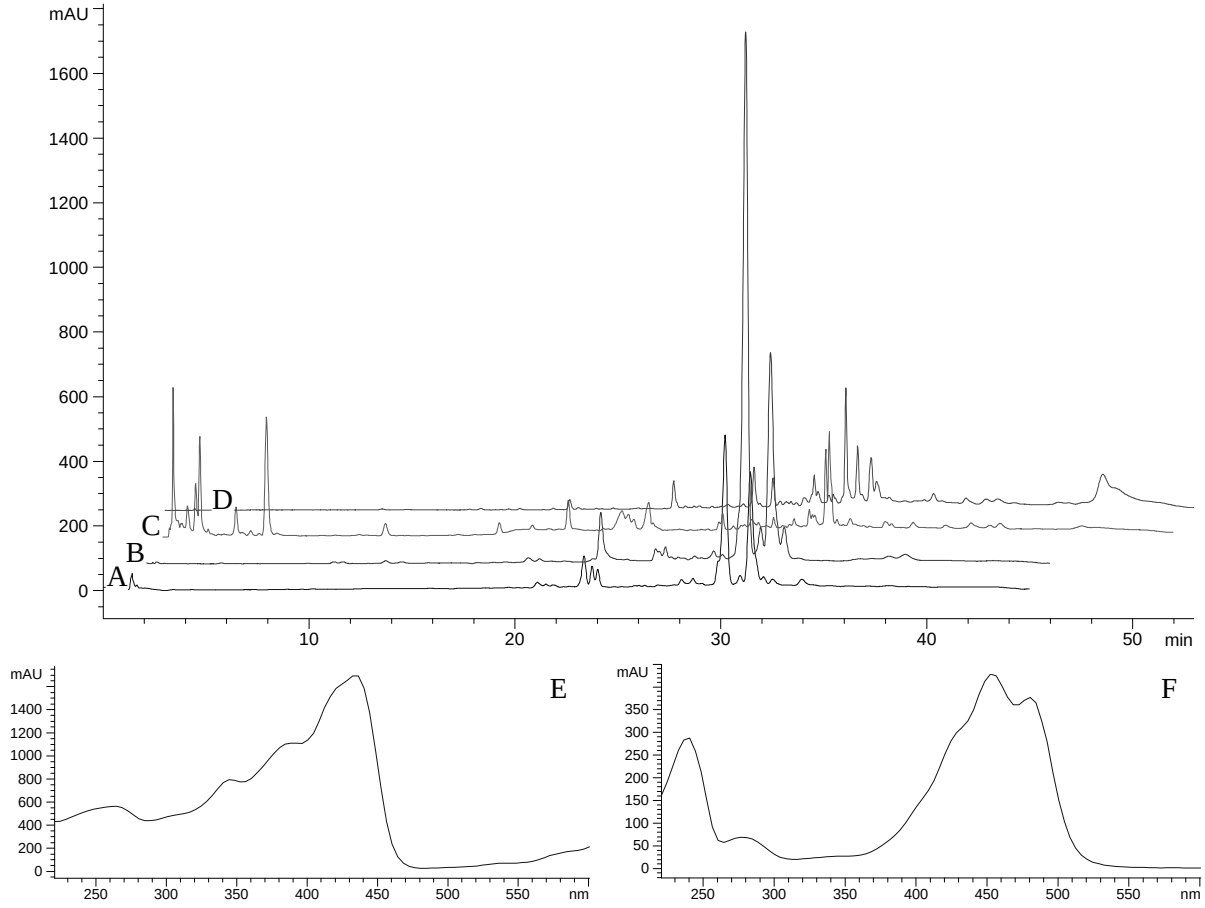

Figure 2.- Chromatograms from experiment 2 with modifier. A and B are fractions in separator 1 at 230 and 440 nm, respectively. C and D are fractions in separator 2 at 230 and 440 nm respectively. All at the same absorbance scale. E is the spectra of the major peak in separator 1, which shows the typical profile of chlorophylls. F is the spectra of the main peak in separator 2 which shows a typical profile of carotenoids.

V- REFERENCES

[1] Piñero Estrada, J.E.; Bermejo Bescós P., et al., Il Farmaco, 56, **2001**, p. 497

[2] Pelizer, L.H.; Danesi E.D.G. et al, Journal of Food Engineering, 56, **2003**, p. 371

[3] Careri M.; Furlattini L., et al., Journal of Chromatography A, 912, **2001**, p. 61

[4] Velioglu, Y.S; Mazza, G. et al., Journal of Agricultural and Food Chemistry, 46, **1998**, p. 4113

[5] Joung, S.N.; Yoo, C.W. et al., Fluid Phase Equilibria, 185, **2001,** p. 219