Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 102 (2007) 1357–1367
Food Chemistry
www.elsevier.com/locate/foodchem

# Screening of functional compounds in supercritical fluid extracts from *Spirulina platensis*

J.A. Mendiola [a], L. Jaime [a,*], S. Santoyo [a], G. Reglero [a], A. Cifuentes [b], E. Ibañez [b], F.J. Señoráns [a]

[a] *Sección Departamental de Ciencias de la Alimentación, Facultad de Ciencias, Módulo C-XVI, 505, Universidad Autónoma de Madrid, Ciudad Universitaria de Cantoblanco, 28049 Madrid, Spain*
[b] *Instituto de Fermentaciones Industriales, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

Received 13 June 2005; received in revised form 7 June 2006; accepted 7 June 2006

**Abstract**

Supercritical fluid extraction and fractionation of *Spirulina platensis* were carried out in order to obtain functional extracts with antioxidant and/or antimicrobial activities. The β-carotene bleaching method and DPPH· free radical-scavenging assay were used to determine the optimal extraction conditions for antioxidant compounds. The best antioxidant extract was obtained in the first fraction when using intermediate pressures and temperatures (220–320 bar, 55 °C), with $CO_2$ plus 10% ethanol as cosolvent, whereas higher pressures and temperatures (320 bar, 75 °C) were needed when pure $CO_2$ was used. Besides, antimicrobial activities of microalgae extracts were tested against four different microbial species, including a gram positive bacterium (*Staphylococcus aureus*), a gram negative bacterium (*Escherichia coli*), a yeast (*Candida albicans*) and a fungus (*Aspergillus niger*). The most active fraction against all the microorganisms tested, was the one collected in the second fraction in the experiment performed at 220 bar and 26.7 °C with 10% of ethanol.


*Keywords:* *Spirulina platensis*; Supercritical fluid extraction; Antioxidant activity; Antimicrobial activity; DPPH; β-Carotene bleaching

## 1. Introduction

*Spirulina platensis*, a blue green microalga, has been used since ancient times as a source of food because of its high protein and nutritional value (Dillon, Phuc, & Dubacq, 1995). Recently, this alga is being widely studied, not only for nutritional reasons but also for its reported medicinal properties; thus, several studies have shown that *Spirulina* or its extracts could prevent or inhibit cancer in humans and animals and recent works have indicated that this specie has immuno-promoting effects (Hirahashi et al., 2002; Subhashini et al., 2004). *S. platensis* was also reported to present antimicrobial activity (Demule, Decaire, & Decano, 1996; Ozdemir, Karabay, Dalay, & Pazarbasi, 2004) as well as to inhibit replication of several viruses, such as Herpes simplex and HIV-1 (Ayehunie, Belay, Baba, & Ruprecht, 1998; Hernández-Corona, Nieves, Meckes, Chamorro, & Barron, 2002). Moreover, *Spirulina* contains a whole spectrum of natural mixed carotene and xanthophyll phytopigments which, together with phycocyanin, seem to be related to its antioxidant activity (Bhat & Madyastha, 2000; Miranda, Cintra, Barros, & Manchini, 1998; Piñeiro Estrada, Bermejo Bescós, & Villar del Fresno, 2001).

In recent years, supercritical fluid extraction has received increasing attention as an important alternative to the traditional solvent extraction methods, since this technique provides a high speed and efficiency of extraction, eliminates concentration steps and avoids the use of organic solvents which are potentially harmful in terms of environmental impact. Supercritical fluid extraction

* Corresponding author. Tel.: +34 914973777; fax: +34 914973778.
*E-mail address:* laura.jaime@uam.es (L. Jaime).

0308-8146/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2006.06.068

(SFE) is an extraction/fractionation method that exploits the unique properties of gases above their critical points to extract soluble components from a raw material. Carbon dioxide is an ideal solvent for the extraction of some classes of natural substances for food uses because is non-toxic, non-explosive, readily available and easy to remove from extracted products. Consequently, the quality of supercritical fluid extracts is higher than those obtained by liquid-liquid extraction with organic solvents or by steam distillation, since these methods can either induce thermal degradation or leave toxic residual solvent in the products.

Supercritical $CO_2$ extraction has been used by some authors to separate and purify active components from *Spirulina*, such as carotenoids and γ-linolenic acid, and these extracts have been compared with those obtained by solvent extraction (Careri et al., 2001; Mendes, Nobre, Cardoso, Pereira, & Palavra, 2003). Data provided by these authors demonstrate the advantages of SFE for extraction of compounds with pharmaceutical importance from these algae, since the developed process was easier and faster than was solvent extraction and thus more effective and convenient.

This research describes the results of primary screening for antioxidant and antimicrobial activities of SFE extracts of *Spirulina platensis* obtained using $CO_2$ and $CO_2$ plus ethanol as cosolvent under different conditions. The study was directed toward the optimization of the extraction and fractionation conditions to obtain extracts with antioxidant and/or antimicrobial activities.

## 2. Materials and methods

### 2.1. Samples and chemicals

Microalgae samples (*S. platensis*) consisted of air-dried microalgae with 6% moisture mass, from Algamar (Pontevedra, Spain), stored under dry and dark conditions.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH·, 95% purity) was obtained from Sigma–Aldrich (Madrid, Spain). Linoleic acid and β-carotene were purchased from Fluka (Madrid, Spain). $CO_2$ (N-38 quality) was obtained from Air Liquide España S.A. (Madrid, Spain).

### 2.2. Extraction method

All extractions were carried out in a pilot-scale plant for supercritical fluid extraction (Iberfluid, Spain) with a 285 ml extraction cell, previously described (Señorans, Ibáñez, Cavero, Tabera, & Reglero, 2000). The extraction cell was made of 316 steel and was equipped with a 0.5 μm frit at the inlet and a 2 μm frit at the outlet. The extraction pressure was controlled by micrometering valves, and the carbon dioxide pump was from Dosapro (France). Fractionation was achieved in two different separators, with independent control of temperature and pressure, by a decrease in pressure.

For each experiment, the extraction cell was filled with 75 g of microalgae and 120 g of washed sea sand (Panreac, Spain). Dynamic extractions were performed under the experimental conditions shown in Table 1. The extraction

Table 1
Conditions used for the experiments performed on a pilot plant scale

| Exp. | EtOH[a] (%) | $P_{ext}$[b] (bar) | $T_{ext}$[c] (°C) | $\rho_{ext}$[d] (g/ml) | $P_{s1}$[e] (bar) | $\rho_{s1}$[f] (g/ml) | $P_{s2}$[g] (bar) |
|---|---|---|---|---|---|---|---|
| 1 | 10 | 78 | 55 | 0.195 | 39 | 0.074 | 19.5 |
| 2 | 10 | 120 | 35 | 0.768 | 60 | 0.159 | 30 |
| 3 | 10 | 120 | 75 | 0.318 | 60 | 0.113 | 30 |
| 4 | 10 | 220 | 27 | 0.920 | 110 | 0.820 | 55 |
| 5, 6 | 10 | 220 | 55 | 0.781 | 110 | 0.416 | 55 |
| 7 | 10 | 220 | 83 | 0.618 | 110 | 0.248 | 55 |
| 8 | 10 | 320 | 35 | 0.940 | 160 | 0.828 | 55 |
| 9 | 10 | 320 | 75 | 0.785 | 160 | 0.507 | 55 |
| 10 | 10 | 361 | 55 | 0.888 | 180.5 | 0.724 | 55 |
| 11 | 0 | 78 | 55 | 0.195 | 39 | 0.074 | 19.5 |
| 12 | 0 | 120 | 35 | 0.768 | 60 | 0.159 | 30 |
| 13 | 0 | 120 | 75 | 0.318 | 60 | 0.113 | 30 |
| 14 | 0 | 220 | 27 | 0.920 | 110 | 0.820 | 55 |
| 15, 16 | 0 | 220 | 55 | 0.781 | 110 | 0.416 | 55 |
| 17 | 0 | 220 | 83 | 0.618 | 110 | 0.248 | 55 |
| 18 | 0 | 320 | 35 | 0.940 | 160 | 0.828 | 55 |
| 19 | 0 | 320 | 75 | 0.785 | 160 | 0.507 | 55 |
| 20 | 0 | 361 | 55 | 0.888 | 180.5 | 0.724 | 55 |

[a] % Ethanol added as modifier.
[b] Extraction pressure.
[c] Extraction temperature.
[d] Extractor density.
[e] Pressure in separator 1.
[f] Density in separator 1.
[g] Pressure in separator 2.

pressure ranged from 78 to 361 bar, and fractionation pressures were set in a first stage at 50% of extraction pressure and, in the second stage, were set, at 50% of the first stage, respecting a maximum of 55 bar. Extraction temperatures ranged from 27 to 83 °C and were kept constant in both separators at the corresponding extraction temperature. Extraction time was 75 min in all experiments.

As for the extractions using ethanol as modifier, the addition started after having reached the selected pressure during 75% of the extraction time. Ethanol was added in an amount corresponding to 10% of $CO_2$ (v/v). All extracts were kept under $N_2$, at −20 °C in the dark, and ethanol, when present as modifier, was eliminated at 35 °C in a vacuum rotary evaporator.

### 2.3. β-Carotene bleaching method

The procedure is based on a previously reported method with slight modifications (Velioglu, Mazza, Gao, & Oomah, 1998). 0.2 ml of *Spirulina* extracts (25 μg/ml and 50 μg/ml of ethanolic extracts in the liposome solution) or 0.2 ml of pure ethanol (as control) was added to a reagent mixture, containing 0.2 ml of β-carotene solution (1 mg/ml in chloroform), 20 mg of linoleic acid, and 200 mg of Tween 20 and the final mixture was evaporated to dryness under a nitrogen stream. Distilled water (50 ml) was added and the mixture was vigorously shaken to form a liposome solution. The samples were then subjected to thermal autoxidation at 50 °C for 3 h. The absorbance of these solutions was measured at 470 nm using a Shimazdu UV-120-01 spectrophotometer (Shimazdu, Kyoto, Japan). Due to the coloration of the extracts, blank extracts were prepared, where 0.2 m of chloroform was added instead of 0.2 of β-carotene solution. All samples were assayed in duplicate. Butylated-hydroxy-toluene (BHT) and ascorbic acid (Sigma) (1 μg/ml) were used as standards. The antioxidant activity (AA) was calculated in terms of percent inhibition relative to the control using the following equation:

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

where $R = \ln[\mathrm{Abs}(t_0)/\mathrm{Abs}(t_{180})]/180$.

### 2.4. DPPH· free radical-scavenging assay

The method, based on a procedure described by Brand-Willians, Cuvelier, and Berset (1995), consisted of the neutralization of DPPH· (1,1-diphenyl-2-picrylhydrazyl) free radicals by the antioxidant extracts. For each antioxidant extract, six different concentrations were tested (from 20 μg/ml to 350 μg/ml in the reaction mixtures). 3.9 m of DPPH· solution ($6 \times 10^{-5}$ mol/l in methanol) were placed in test tubes and 0.1 m of the different concentrations of *Spirulina* extracts were added. Absorbance was measured at 516 nm in a Shimazdu UV-120-01 spectrophotometer (Shimazdu, Kyoto, Japan) until the reaction reached a steady state. Methanol was used to adjust zero, DPPH·-methanol solution as a reference sample and ascorbic acid and BHT as standards. The radical scavenging activities of SF extracts in the reaction medium were calculated from a calibration curve at 516 nm. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or $EC_{50}$. Each determination was repeated twice.

### 2.5. TLC

Analytical-TLC was carried out in TLC plates (10 cm × 20 cm) cut from the commercially available sheets according to the Jaime et al. (2005) procedure. Twelve microliters of 30 mg/ml extract solution was applied to 1 cm of the base of the silica gel layer and allowed to dry for a few minutes. Afterwards, the plate was eluted in a closed chamber with mobile phase (petroleum ether:acetone (75:25)). Afterwards, the developed silica layers were stained with a 0.5 mM DPPH· radical, as described in the Jaime et al. (2005) procedure.

### 2.6. Microbial strains

The SFE extracts were individually tested against a panel of microorganisms, including *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 11775, *Candida albicans* ATCC 60193 and *Aspergillus niger* ATCC 16404.

Bacterial strain stock cultures were kept on nutrient agar at 4 °C. *C. albicans* was kept on Sabouraud dextrose agar at 4 °C. *A. niger* spores were obtained in vitro from monoconidial cultures after incubation (7 days, 24 °C) on potato dextrose agar, harvested in sterile distilled water containing 0.1% Tween 80 and stored at 4 °C until used as inocula.

### 2.7. Determination of minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC)

A broth microdilution method was used, as recommended by NCCLS, for determination of the minimum inhibitory concentration (NCCLS, 1999). All tests were performed in Mueller–Hinton broth supplemented with 0.5% Tween 20, with the exception of yeasts and fungi (Sabouraud dextrose broth + 0.5% Tween 20). The inocula of bacterial strains were prepared from overnight Mueller–Hinton broth cultures at 37 °C. Yeasts and fungi were cultured overnight at 25 °C in sabouraud dextrose broth. Test strains were suspended in Mueller-Hinton (bacteria) or Sabouraud dextrose (yeast and fungi) broth to give a final density $10^7$ cfu/ml. The *S. platensis* extract dilutions in DMSO ranged from 250 to 10 mg/ml.

The 96-microwell plates were prepared by dispensing, into each well, 165 μl of culture broth, 5 μl of the inocula and 30 μl of the different extract dilutions. The final volume of each well was 200 μl. Plates were incubated at 37 °C for 24 h for bacteria and at 24 °C for 48 h for yeast and fungus.

Negative controls were prepared, using 30 μl of DMSO, the solvent used to dissolve the microalgae extracts. Chloranphenicol and amphotericin B (Sigma, Madrid) were used as positive reference standards to determine the sensitivity of the microbial species used. After incubation, the MIC of each extract was determined by visual inspection of the well bottoms, since bacterial growth was indicated by the presence of a white "pellet" on the well bottom. The lowest concentration of the extract that inhibited growth of the microorganism, as detected as lack of the white "pellet", was designated the minimum inhibitory concentration. The minimum bactericidal and fungicidal concentration was determined by making subcultures from the clear wells which did not show any growth. Each test was performed in triplicate and repeated twice.

### 2.8. Lipid composition analysis

Gas chromatography, coupled to a flame ionization detector (GC-FID), was used to identify the free and esterified fatty acids of *S. platensis* extracts.

To prepare ethyl esters of free and esterified fatty acids, samples were mixed with chloroform/ethanol 2/1 (v/v) and ethylated by addition of 1 ml of a solution of sulfuric acid in ethanol (0.9 M). This mixture was allowed to stand overnight at 50 °C. After addition of 200 μl milliQ water, the resulting mixture was extracted with two 1 ml portions of *n*-hexane and the final extract was then dried with sodium sulfate.

One microlitre of derivatized sample was injected into a Perkin–Elmer autosystem XL (Wellesley, MA, USA) gas chromatograph with a 30 m BTR-Carbowax column (0.25 mm i.d.). Injector and detector temperatures were set at 220 and 230 °C, respectively. The temperature programme was as follows: starting at 100 °C and then heating to 180 °C at 20 °C/min followed by heating from 180 to 220 °C at 15 °C/min. The final temperature (220 °C) was held for 30 min. Identification of the ethyl esters of the various fatty acids was based on a menhaden oil fish standard (#4-7085) obtained from Supelco (Bellefonte, PA).

### 2.9. Statistical analysis

Statistical analyses were performed by using the statistical package StatGraphics version 5 (Manugistics Software).

## 3. Results and discussion

### 3.1. General

Since various parameters potentially affect the SFE process, the optimisation of the experimental conditions represents a critical step in the development of a SFE method. In fact, solubility of the different compounds can be controlled by the composition and density of the extraction fluid and thus depend on the extraction pressure and temperature used. In this study, a screening of different SFE conditions considering two factors (extraction pressure and temperature) was performed in order to optimize the extraction of antioxidants and/or antimicrobials from *S. platensis*. Experiments were chosen to cover a wide range of conditions, as shown in Table 1, always considering the experimental limitations of the pilot scale plant used in this study and previous data appearing in the literature for the extraction of valuable compounds from microalgae (Careri et al., 2001; Cocero, González, Perez, & Alonso, 2000; Mendes et al., 2003; Robles Medina, Molina Grima, Giménez Giménez, & Ibañez Gonzalez, 1998; Subra, Castellani, Jestin, & Aoufi, 1998). As mentioned, extraction pressures ranged from 78 to 361 and extraction temperatures between 27 and 83 °C, which implies using densities from 0.195 to 0.94 g/ml. The complete set of experiments was done in duplicate, using pure $CO_2$ and $CO_2$ plus 10% ethanol as modifier. Using modifiers allows increasing of the polarity of the extracting agent while changing the viscosity of the fluid. Sometimes a two-phase system is obtained (as in the present work when using 78 bar and 55 °C) which allows liquid and supercritical extraction simultaneously. Furthermore, a fractionation of microalgae extracts in two separation vessels by means of cascade depressurisation was carried out, allowing the recovery of two different extracts with different compositions and chemical characteristics.

Extraction time was fixed previously through sequential extractions of the same sample under selected conditions (220 bar and 55 °C without modifier). The extraction time was set at the beginning of the asymptotic curve yield (%)–time (min). Fig. 1 shows the evolution of the yield vs extraction time under the extraction conditions tested.

Dark green and dark maroon extracts were collected in fraction 1 with and without ethanol as modifier, respectively; meanwhile maroon-reddish and orange colours were found in fraction 2 extracts.

### 3.2. Antioxidant activity

Two different methods were used to find the optimal extraction conditions of antioxidant compounds. The β-carotene bleaching method was used as a reference of lipid peroxidation of unsaturated fatty acids that has been known to cause many pathological effects. On the other hand, DPPH$^{\cdot}$ is a purple stable radical that turns yellowish when it reacts with antioxidant analytes, and the degree of discoloration indicates the scavenging potential of the antioxidant extract.

The antioxidant activities of the extracts are shown in Tables 2 and 3 for the β-carotene bleaching and DPPH$^{\cdot}$ methods, respectively. Results indicated that supercritical fluid extracts obtained under different extraction conditions exhibited various degrees of antioxidant activity in both the DPPH$^{\cdot}$ the free radical-scavenging and β-carotene bleaching methods.

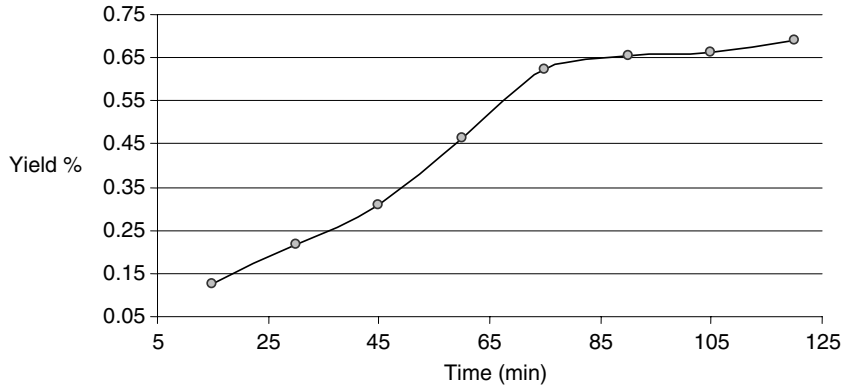


Fig. 1. Evolution of yield (%) vs extraction time. Extraction conditions: 220 bar, 55 °C, neat $CO_2$ as extracting solvent.

Table 2
Antioxidant activity of *S. platensis* SF extracts at different concentrations and BHT (1 μg/ml in the reaction mixture) in the bleaching β-carotene method (%)

| Exp. | Separator 1 | | Separator 2 | |
|---|---|---|---|---|
| | 25 μg/ml | 50 μg/ml | 25 μg/ml | 50 μg/ml |
| 1 | 54 ± 1 | 84 ± 1 | 30 ± 2 | 61 ± 1 |
| 2 | 55 ± 3 | 68 ± 1 | 8 ± 1 | 36 ± 4 |
| 3 | 52 ± 2 | 77 ± 1 | 21 ± 2 | 38 ± 2 |
| 4 | 70 ± 0 | 84 ± 0 | 1 ± 0 | 16 ± 1 |
| 5 | 90 ± 0 | 95 ± 1 | 47 ± 1 | 78 ± 3 |
| 6 | 93 ± 0 | 94 ± 0 | 60 ± 1 | 84 ± 1 |
| 7 | 80 ± 0 | 89 ± 1 | 29 ± 2 | 62 ± 2 |
| 8 | 91 ± 0 | 93 ± 0 | 43 ± 4 | 74 ± 0 |
| 9 | 87 ± 2 | 94 ± 0 | 32 ± 0 | 52 ± 2 |
| 10 | 77 ± 2 | 87 ± 2 | 13 ± 3 | 48 ± 2 |
| 11 | 60 ± 1 | 84 ± 0 | 21 ± 2 | 57 ± 3 |
| 12 | 17 ± 1 | 65 ± 6 | 28 ± 4 | 47 ± 7 |
| 13 | 27 ± 4 | 53 ± 1 | 24 ± 2 | 48 ± 3 |
| 14 | 31 ± 4 | 55 ± 5 | 4 ± 0 | 20 ± 3 |
| 15 | 41 ± 4 | 68 ± 1 | 21 ± 2 | 63 ± 1 |
| 16 | 38 ± 4 | 72 ± 1 | 23 ± 3 | 65 ± 1 |
| 17 | 57 ± 0 | 85 ± 0 | 19 ± 4 | 38 ± 2 |
| 18 | 35 ± 1 | 76 ± 0 | 16 ± 1 | 26 ± 2 |
| 19 | 91 ± 2 | 95 ± 0 | 62 ± 7 | 89 ± 0 |
| 20 | 50 ± 2 | 83 ± 1 | 72 ± 1 | 90 ± 0 |
| Ascorbic acid | 54 ± 1 | | | |
| BHT | 81 ± 2 | | | |

In relation to fractionation of *S. platensis* extracts from different supercritical extraction conditions (Table 2), in general, antioxidant activity was higher in the fraction 1 than in the fraction corresponding to separator 2. Moreover, the most active extracts in separator 1 also showed the highest antioxidant activities in fraction 2. The main difference between fractions 1 and 2 was the pressure in the vessel. This different pressure brought about a gradual precipitation of the extracts compounds on the basis of their solubility in the extracting solvent under the experimental conditions.

An analytical-TLC of the best antioxidant SF extracts was carried out to investigate inquire into the types of compounds responsible for that antioxidant activity (Figs. 2 and 3). Orange, yellow, orange-yellowish and green bands were found in the TLC plates eluted with petroleum ether:acetone (75:25), in agreement with the colours of the main pigments described in *S. platensis* (Becker & Venkataraman, 1984; Gireesh et al., 2001): chlorophyll a and different carotenoids, such as β-carotene, β-cryptoxanthin, zeaxanthin, echinenone, oscillaxanthin and myxoxanthophyll. Moreover, β-carotene and lutein standards showed $R_f = 0.99$ and 0.18, respectively (Fig. 2). These results are in agreement with several authors who have reported $R_f$ values of different carotenoid compounds, using similar eluents (Van den Hoek, Mann, & Jahns, 1995; Gireesh et al., 2001). In these studies, β- and α-carotene bands were always found at the top of the TLC, and lutein/zeaxanthin (that elute at the same $R_f$) showed $R_f$ values close to 0.2, whereas cryptoxanthin, asthaxanthin and

Table 3
DPPH· radical scavenging activity of *S. platensis* SF and ascorbic acid expressed as EC50 (μg/ml).

| Exp. | Separator 1 | Separator 2 |
|---|---|---|
| | EC50 (μg/ml) | EC50 (μg/ml) |
| 1 | 111.8 ± 5.6 | 204.5 ± 3.6 |
| 2 | 129.4 ± 3.8 | 166.8 ± 9.2 |
| 3 | 93.9 ± 3.6 | 107.0 ± 5.6 |
| 4 | 176.0 ± 15.8 | 112.4 ± 3.1 |
| 5 | 71.6 ± 2.5 | 73.9 ± 5.7 |
| 6 | 66.7 ± 8.4 | 63.1 ± 0.2 |
| 7 | 96.5 ± 2.1 | 122.5 ± 8.5 |
| 8 | 255.5 ± 9.6 | 64.5 ± 1.1 |
| 9 | 83.1 ± 1.4 | 287.5 ± 5.5 |
| 10 | 167.5 ± 8.3 | 131.9 ± 4.7 |
| 11 | 337.2 ± 89.0 | 297.8 ± 2.7 |
| 12 | 48.8 ± 7.7 | 239.9 ± 17.2 |
| 13 | 205.10 ± 24.85 | 171.1 ± 21.3 |
| 14 | 85.3 ± 2.5 | 213.9 ± 55.8 |
| 15 | 133.5 ± 6.0 | 186.3 ± 1.5 |
| 16 | 135.0 ± 9.3 | 187.4 ± 18.1 |
| 17 | 105.6 ± 7.5 | 139.7 ± 13.8 |
| 18 | 102.0 ± 2.8 | 207.7 ± 38.5 |
| 19 | 20.0 ± 1.7 | 129.4 ± 3.9 |
| 20 | 94.7 ± 7.5 | 92.5 ± 3.8 |
| Ascorbic acid | 4.4 ± 0.2 | |
| BHT | 18.1 ± 0.6 | |

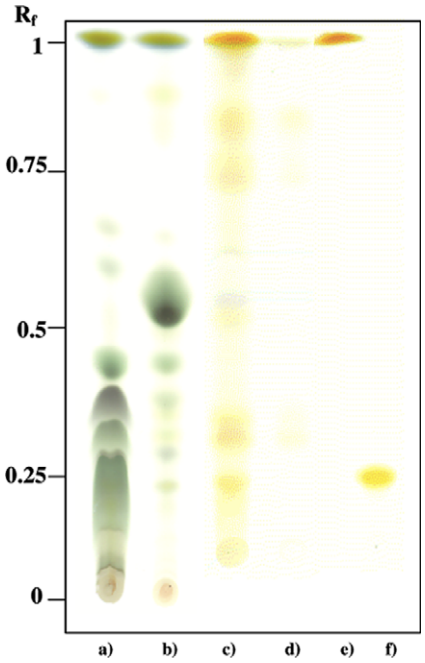


Fig. 2. Analytical-TLC of *S. platensis* SF extracts: (a) Exp. 5; S1, (b) Exp. 5; S2, (c) Exp. 19; S1, (d) Exp. 19 sep. 2, (e) β-carotene standard and (f) lutein standard.

other xanthophylls presented variable intermediate $R_f$ values, depending on their esterification. Thus, as zeaxanthin, instead of lutein, has been described in *S. platensis*, β-carotene and zeaxanthin are probably the main pigments

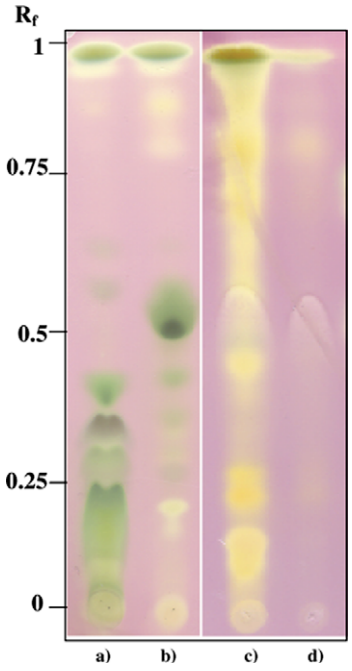


Fig. 3. Analytical-TLC of *S. platensis* SF extracts stained with 0.5 M DPPH· solution: (a) Exp. 5; S1, (b) Exp. 5; S2, (c) Exp. 19; S1 and (d) Exp. 19; S2.

found in SF extracts, together with other minor carotenoids. Furthermore, green wide bands were found in SF extracts with $CO_2$ as cosolvent, mainly in separator 1. Moreover, some of these green and grey-greenish bands might correspond to pheophytin-like compounds that come from the partial degradation of chlorophyll a (Quach, Steeper, & Griffin, 2004).

In a previous work done in our laboratory, an exhaustive characterization of supercritical fluid extracts of *S. platensis* by LC–MS was performed (Mendiola et al., 2005). β-Carotene, zeaxanthin, myxoxanthophyll and chlorophyll a were identified as the main constituents of the extracts. Other pigments, such as, pheophytin-like compounds, and siphonein and astaxanthin, together with other minor carotenoids were also found.

Developed TLC plate was stained with 0.5 M DPPH· solution to determine the compounds responsible for the antioxidant activity of the extracts. Using this procedure, compounds with antioxidant activity turned out to be brilliant yellow in the purple background of the plate. The intensity of the yellow colour depends on the amount and nature of radical scavengers present in the extract. Orange and orange-yellowish bands showed a high antioxidant capacity (Fig. 3). Therefore, carotenoids seemed to be the main antioxidant components of SF extracts.

The extract with $CO_2$ plus 10% ethanol as modifier not only gave a higher yield of the extract (1.9% on average compared to 0.23% when neat $CO_2$ was used) but also turned out to possess higher antioxidant activity than did the extracts with pure supercritical $CO_2$. This behaviour was confirmed by the two antioxidant methods and is in

agreement with data reported in the literature for supercritical fluid extraction of carotenoids from *S. platensis* (Careri et al., 2001). As reported by Marsili and Callahan (1993), an essential drawback in the use of supercritical $CO_2$ is its low polarity and the insufficient interaction between supercritical $CO_2$ and the matrix. Nevertheless, this limitation may be overcome by adding to the supercritical $CO_2$ small amounts of polar modifiers, such as ethanol in order to increase its solvating power, thus improving the extraction efficiency. In this way, ethanol has been described as the most effective modifier in the extraction of carotenoids from vegetables.

Since analytes with different polarity show a better recovery in those extractions using ethanol as cosolvent, the effect of the modifier results may be related, not only to the change in polarity of the extraction fluid, but also to its interaction with the matrix (Hawthorne, Krieger, & Miller, 1988). Thus, extracts obtained with $CO_2$ and 10% ethanol as modifier presented lower amounts of β-carotene but higher quantities of more polar xanthophylls. It is important to underline that several authors have reported that not only α- and β-carotene but other carotenoids such lycopene, zeaxanthin, lutein, echinenone, astaxanthin and canthaxanthin, well described in blue-green algae, show antioxidant activity against radicals, together with synergies between them, that probably contribute to the antioxidant activity of each fraction (Bohm, Puspitasari-Nienaber, Ferruzzi, & Schwartz, 2002; Van den Hoek et al., 1995).

In order to understand the behaviour of the SF extracts, antioxidant activity as affected by of extraction conditions (pressure, temperature and modifier), the response surfaces of antioxidant values with a concentration of 50 μg/ml of fraction 1 in the reaction mixture were plotted. Since both antioxidant methods showed similar estimated response surfaces, only those corresponding to the antioxidant activities obtained using the β-carotene bleaching method are shown (Fig. 4). As can be inferred from the three-dimensional plots, pressure of the supercritical fluid plays an important role in the SFE of antioxidant compounds from *S. platensis*.

Different estimated response surfaces were achieved with and without ethanol as modifier (Fig. 4a and b, respectively). With regard to antioxidant activities obtained using pure $CO_2$ (Fig. 4a), antioxidant activity of the extracts enhanced as pressure increased, especially at high temperatures. Opposite to this, rise in temperature at low pressure caused a loss of antioxidant capacity, whereas at high pressure antioxidant value increased with increasing temperature. Mendes et al. (1995) obtained similar results in carotenoid extraction with supercritical $CO_2$ from *Chlorella vulgaris*. The optimum extraction conditions would be 360 bar and 74 °C with pure $CO_2$.

On the other hand, a different response surface was achieved when using ethanol as modifier. Fig. 4b shows that antioxidant capacity was enhanced as extraction pressure increased up to 275 bar; moreover, better results were

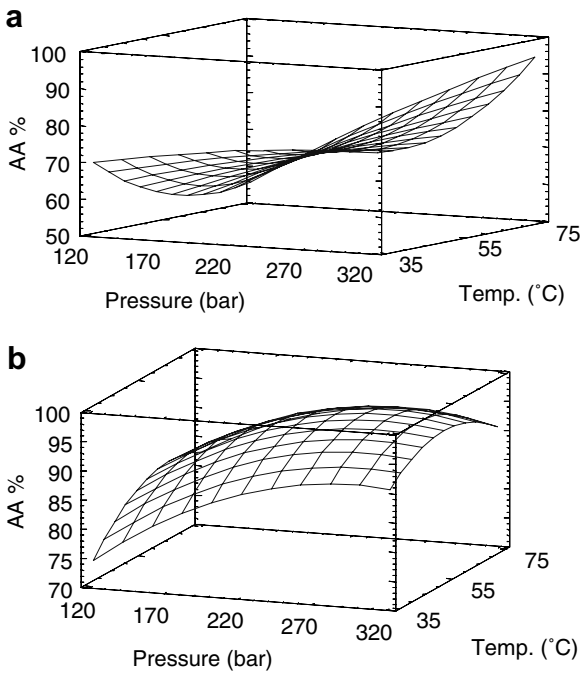


Fig. 4. Response surfaces for antioxidant activity (AA) of *Spirulina* SFE extracts (a) with pure $CO_2$ and (b) with $CO_2$ + 10% ethanol as modifier.

achieved at intermediate temperature whether the extraction pressure increased or not. The best extraction conditions would be obtained at 275 bar and 57 °C.

As Fig. 4a and b show, antioxidant activity of extracts was enhanced as the pressure increased. The increase in the extraction pressure caused an increase of the fluid density and thus it could have a double effect: an increase of the solvating power of the supercritical fluid, responsible for quantitative recoveries, and a higher interaction between the fluid and the matrix (Careri et al., 2001).

### 3.3. Antimicrobial activity of the SFE extracts

Four different microbial species, including a gram negative bacterium (*E. coli*), a gram positive bacterium (*S. aureus*), a yeast (*C. albicans*) and a fungus (*A. niger*), were used to screen the possible antimicrobial activity of *S. platensis* supercritical fluid extracts. In the extractions obtained with ethanol as modifier, the antimicrobial experiments were carried out with the fractions recovered in the first (S1) and in the second (S2) separators. On the other hand, in the extractions performed with pure $CO_2$, the antimicrobial activity was tested only in the fractions from the second separator, since the yield of the fraction recovered in the first one was not enough for the antimicrobial assays. The antimicrobial activity was quantitatively assessed by the determination of the minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC).

Results obtained (Table 4) showed that *C. albicans* was the most sensitive microorganism to all *Spirulina* SFE frac-

Table 4
Antimicrobial activities of different SFE extracts from *S. platensis*

| Sample | *E. coli* MBC[a] | *S. aureus* MBC | *C. albicans* MBC | *A. niger* MBC |
|---|---|---|---|---|
| *Exp. 2* | | | | |
| S1 | 15 | 20 | 10 | >35 |
| S2 | 20 | 20 | 15 | >35 |
| *Exp. 4* | | | | |
| S1 | 20 | 20 | 10 | >35 |
| S2 | 10 | 10 | 10 | 35 |
| *Exp. 5* | | | | |
| S1 | 25 | 25 | 15 | >35 |
| S2 | 15 | 15 | 10 | 35 |
| *Exp. 8* | | | | |
| S1 | 25 | 25 | 15 | >35 |
| S2 | 20 | 20 | 10 | >35 |
| *Exp. 10* | | | | |
| S1 | 30 | 25 | 15 | >35 |
| S2 | 20 | 20 | 10 | >35 |
| Exp. 12, S2 | 25 | 20 | 10 | >35 |
| Exp. 14, S2 | 25 | 20 | 15 | >35 |
| Exp. 15, S2 | 20 | 15 | 10 | >35 |
| Exp. 18, S2 | 25 | 20 | 10 | >35 |
| Exp. 20, S2 | 15 | 15 | 10 | >35 |
| Reference antibiotic | 10 | 10 | 100 | 150 |

[a] MBC, minimum bactericidal concentration. MBC values given as mg/ml for samples and μg/ml for antibiotic.

Table 5
GC-FID identification, peak and area contribution of fatty acid found in the SF extract (experiment 4) of *S. platensis*

| Fatty acid | %area (sep. 1) | %area (sep. 2) | $t_R$ (min) |
|---|---|---|---|
| Lauric acid, C12 | 3.53 | 19.2 | 5.84 |
| Myristic acid, C14 | 2.89 | 1.38 | 7.95 |
| Palmitic acid, C16 | 44.4 | 36.6 | 10 |
| Palmitoleic acid, C16:1 | 5.92 | 5.80 | 10.3 |
| Stearic acid, C18 | 7.22 | 8.93 | 14.0 |
| Oleic acid, C18:1 | 30.6 | 25.06 | 14.6 |
| Linoleic acid, C18:2 | 0.92 | 0.66 | 15.1 |

tions, with the lowest MBC values (15–10 mg/ml), whereas the least susceptible was the fungus *A. niger*. In fact, only two of the extracts tested were active against this fungus. *E. coli* and *S. aureus* were also sensitive to all *Spirulina* SFE fractions Table 5.

In the SFE extracts obtained using 10% ethanol as modifier, a higher antimicrobial activity (once ethanol was evaporated) was found in the fractions collected in the second separator (S2), with the exception of experiment 2, where the pressure employed for the extraction was the lowest. Comparing the results obtained with the S2 from the different experiments, the most active one, for all the microorganisms examined, was that of experiment 4 (220 bar, 27 °C), followed by fractions from experiment 5 (220 bar, 55 °C) and from experiments 8 (320 bar, 35 °C) and 10 (361 bar, 55 °C). These results showed that, when employing extraction pressures above 120 bar, the compounds responsible for the antimicrobial activity are selectively collected in the second separator. Moreover, an extraction pressure equal to 220 bar seemed to be optimal to extract substances with antimicrobial activity, since higher pressure values gave less active fractions. Besides, as can be seen from a comparison between experiment 4 and 5, the increase of extraction temperature produced fractions with a lower antimicrobial activity.

In the SFE extracts performed without ethanol as modifier, the S2 from experiment 20 (361 bar, 55 °C) showed the highest antimicrobial activity, against all the microorganisms tested, followed by S2 from experiment 15 (220 bar, 55 °C). These data indicated that, when pure $CO_2$ was used as extractant, the best results in terms of antimicrobial activity were obtained by working at intermediate temperatures (55 °C) and medium-high pressures.

Cyanobacteria have been screened for potential antimicrobial activity, which have been attributed to different compounds belonging to a diverse range of chemical classes (Borowitzka, 1995; Kreitlow, Mundt, & Lindequist, 1999; Ozdemir et al., 2004). Specifically, the antimicrobial activity of a methanolic extract of the *S. platensis* was explained by the presence of γ-linolenic acid (Demule et al., 1996), an antibiotically-active fatty acid present in high concentration in this alga (Xue et al., 2002). In that way, a detailed study of FA content of the two fractions obtained from experiment 4, that provided the best antimicrobial activity, was performed by using GC-FID analysis (Table 5). Besides, Fig. 5 shows the GC chromatogram obtained for these two fractions. The data obtained showed that the percentage of linolenic acid found in both S1 and S2 were very low, and, consequently, the antimicrobial activity found in SF extracts could not be attributed to this fatty acid. However, since the fatty acid analysis indicated the presence of other fatty acids that have been also reported to have some antimicrobial activity, specifically lauric, palmitoleic and oleic acids (Benkendorff, Davis, Rogers, & Bremner, 2005; Ouattara, Simard, Holley, Piette, & Bégin, 1997), the antimicrobial activity found in SF extracts could be linked to a synergic effect of all these fatty acids. Comparing the analysis of fatty acids obtained in both fractions (S1 and S2), it must be noted that the percentage of lauric acid found in fraction 2 (S2) was much higher than that in fraction 1 (S1), a fact that could be related to the higher antimicrobial activity found in this fraction.

## 4. Conclusions

Data presented in this study illustrate that supercritical fluid extracts from *S. platensis* show antioxidant and antimicrobial activity. Consequently, *Spirulina* SF extracts present a promising potential as an accessible and safe alternative to synthetic antioxidants and antimicrobials. Besides, the process developed involves the use of an environmentally clean technology, i.e, supercritical fluid extraction.

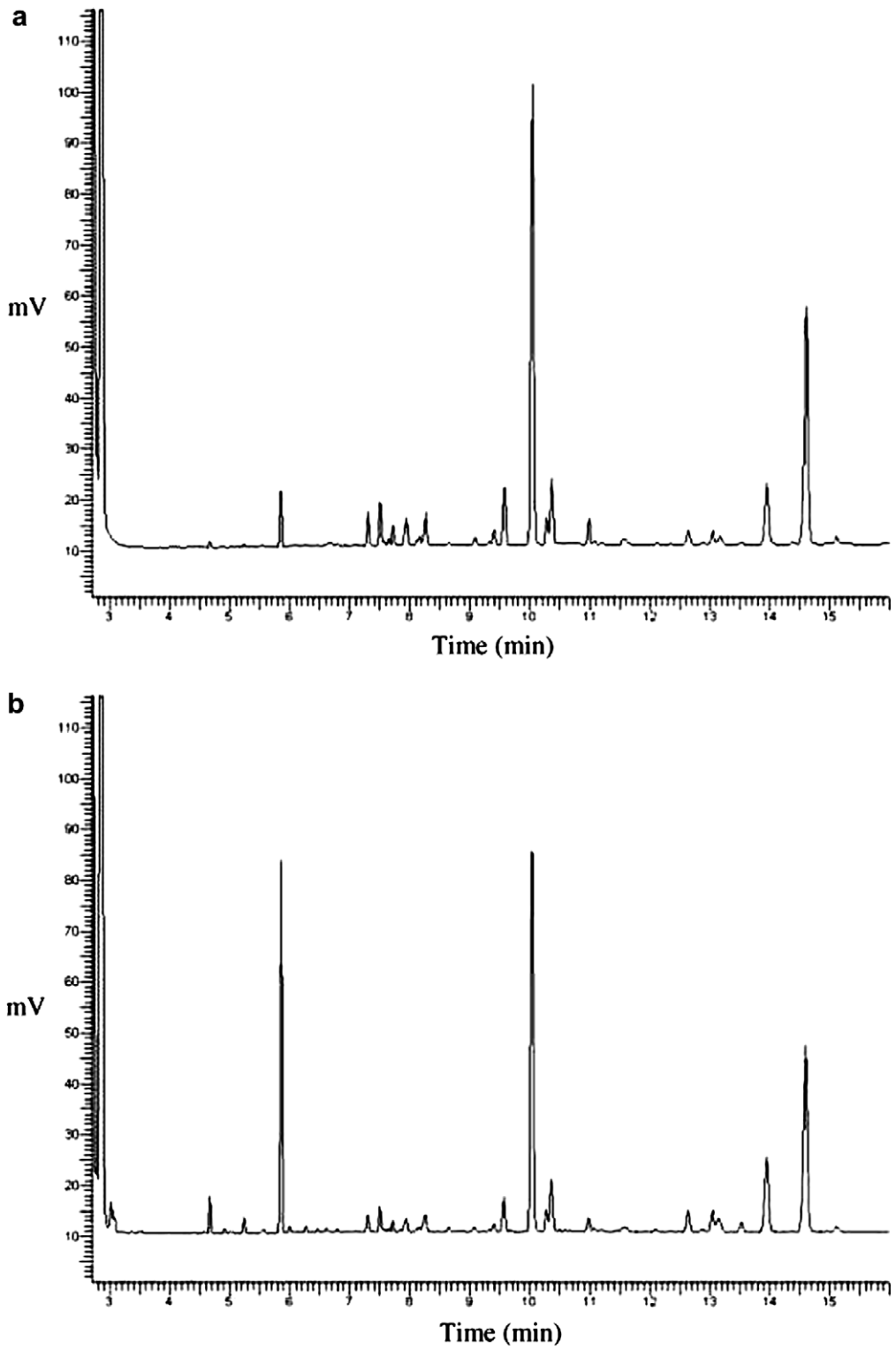


Fig. 5. GC-FID chromatogram of the supercritical $CO_2$ extract obtained at 220 atm and 27 °C (extract 4): (a) Separator 1 and (b) separator 2.

The optimal SFE conditions in terms of antioxidant activity were achieved in the first separator when using intermediate pressures and temperatures (220–320 bar, 55 °C) in the extraction with $CO_2$ plus 10% ethanol as cosolvent, whereas higher pressures and temperatures (320 bar, 75 °C) were needed to obtain the highest antioxidant extract when pure $CO_2$ was used. For the antimicrobial activity, the most active fraction against all the microorganisms tested, was the one collected in the second separator in the experiment performed at 220 bar and 26.7 °C with 10% of ethanol.

In general, the extractions performed with 10% of ethanol as a cosolvent presented a higher antioxidant and antimicrobial activities than did those the ones obtained using pure $CO_2$. This fact, along with the higher yields obtained with the binary mixture, make this solvent optimal for the extraction of functional compounds from microalgae. From the present study, it can also be inferred that extraction at 220 bar and 55 °C yields the best extracts in terms of antioxidant and antimicrobial activities. Under these conditions, a good fractionation was also achieved, with preferential recovery of antioxidant compounds in separator 1 and antimicrobial components in separator 2.

## Acknowledgements

J.A.M thanks the Spanish Ministry of Education and Science (MEC) for a FPI grant. The authors thank the Ministerio de Ciencia y Tecnología (Projects AGL2002-04621-C02-02 and AGL2005-06726-C04-01/ALI) for financial support. Project S-505/AGR-0153 (ALI-BIRD) granted by the Comunidad Autonoma de Madrid (Spain) is also acknowledged.

## References

Ayehunie, S., Belay, A., Baba, T. W., & Ruprecht, R. M. (1998). Inhibition of HIV-1 replication by an aqueous extract of *Spirulina platensis* (*Arthrospira platensis*). *Journal of Acquired Immune Deficiency Syndromes and Human Retrovirology, 18*, 7–12.

Becker, E. W., & Venkataraman, L. V. (1984). Production and utilization of the blue-green alga *Spirulina* in India. *Biomass, 4*(2), 105–125.

Benkendorff, K., Davis, A. R., Rogers, C. N., & Bremner, J. B. (2005). Free fatty acids and sterols in the benthic spawn of aquatic molluscs, and their associated antimicrobial properties. *Journal of Experimental Marine Biology and Ecology, 316*(1), 29–44.

Bhat, V. B., & Madyastha, K. M. (2000). C-Phycocyanin: a potent peroxyl radical scavenger in vivo and in vitro. *Biochemical and Biophysical Research Communication, 275*, 20–25.

Bohm, V., Puspitasari-Nienaber, N. L., Ferruzzi, M. G., & Schwartz, S. J. (2002). Trolox equivalent antioxidant capacity of different geometrical isomers of α-carotene, β-carotene, lycopene and zeaxanthin. *Journal of Agricultural and Food Chemistry, 50*, 221–226.

Borowitzka, M. A. (1995). Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology, 7*, 3–15.

Brand-Willians, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *Lebensmittel–Wisschenschaft und Technology, 28*, 25–30.

Careri, M., Furlattini, L., Mangia, A., Musci, M., Anklam, E., Theobald, A., et al. (2001). Supercritical fluid extraction for liquid chromatographic determination of carotenoids in *Spirulina pacifica* algae: a chemometric approach. *Journal of Chromatography A, 912*, 61–71.

Cocero, M. J., González, S., Perez, S., & Alonso, E. (2000). Supercritical extraction of unsaturated products. Degradation of β-carotene in supercritical extraction processes. *Journal of Supercritical Fluids, 19*(1), 39–44.

Demule, M. C. Z., Decaire, G. Z., & Decano, M. S. (1996). Bioactive substances from *Spirulina platensis* (cianobacteria). *International Journal of Experimental Botany, 58*, 93–96.

Dillon, J. C., Phuc, A. P., & Dubacq, J. P. (1995). Nutritional value of the alga *Spirulina*. *World Review of Nutrition and Dietetics, 77*, 32–46.

Gireesh, T., Jayadeep, A., Rajasekharan, K. N., Menon, V. P., Vairamany, M., Tang, G., et al. (2001). Production of deuterated β-carotene by metabolic labelling of *Spirulina platensis*. *Biotechnology Letters, 23*, 447–449.

Hawthorne, S. B., Krieger, M. S., & Miller, D. J. (1988). Analysis of flavor and fragrance compounds using supercritical fluid extraction coupled with gas chromatography. *Analytical Chemistry, 60*(5), 472–477.

Hernández-Corona, A., Nieves, I., Meckes, M., Chamorro, G., & Barron, B. L. (2002). Antiviral activity of *Spirulina maxima* against herpes simplex virus type 2. *Antiviral research, 56*, 279–285.

Hirahashi, T., Matsumoto, M., Hazeki, K., Saeki, Y., Ui, M., & Seya, T. (2002). Activation of the human innate immune system by *Spirulina*: augmentation of interferon production and NK cytotoxicity by oral administration of hot water extract of *Spirulina platensis*. *International Immunopharmacology, 2*, 423–434.

Jaime, L., Mendiola, J. A., Herrero, M., Señorans, F. J., Soler-Rivas, C., Santoyo, S., et al. (2005). Separation and characterization of antioxidant compounds from *Spirulina platensis* microalga combining pressurized liquid extraction, TLC and HPLC-DAD. *Journal of Separation Science, 28*, 2111–2119.

Kreitlow, S., Mundt, S., & Lindequist, U. (1999). Cianobacteria-a potential source of new biologically active substances. *Journal of Biotechnology, 70*, 61–63.

Marsili, R., & Callahan, D. (1993). Comparison of a liquid solvent extraction technique and supercritical fluid extraction for the determination of α- and β-carotene in vegetables. *Journal of Chromatographic Science, 31*(10), 422–428.

Mendes, R. L., Fernandes, H. L., Coelho, J. P., Reis, E. C., Cabral, J. M. S., Novais, J. M., et al. (1995). Supercritical $CO_2$ extraction of carotenoids and other lipids from *Chlorella vulgaris*. *Food Chemistry, 53*, 99–103.

Mendes, R. L., Nobre, B. P., Cardoso, M. T., Pereira, A. P., & Palavra, A. F. (2003). Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae. *Inorganica Chimica Acta, 356*, 328–334.

Mendiola, J. A., Marín, F. R., Hernández, S. F., Arredondo, B. O., Señoráns, F. J., Ibáñez, E., et al. (2005). Characterization via liquid chromatography coupled to diode array detector and tandem mass spectrometry of supercritical fluid antioxidant extracts of *Spirulina platensis* microalga. *Journal of Separation Sciences, 28*, 1031–1038.

Miranda, M. S., Cintra, R. G., Barros, S. B., & Manchini, F. J. (1998). Antioxidant activity of the microalga *Spirulina maxima*. *Brazilian Journal of Medical Biology Research, 31*, 1075–1079.

NCCLS (National Committee for Clinical Laboratory Standards). (1999). Performance standards for antimicrobial susceptibility testing (9th Int. Suppl.) M 100-S9, Wayne, PA.

Ouattara, B., Simard, R. E., Holley, R. A., Piette, G. J. P., & Bégin, A. (1997). Antibacterial activity of selected fatty acids and essential oils against six meat spoliage organisms. *International Journal of Food Microbiology, 37*, 155–162.

Ozdemir, G., Karabay, N. U., Dalay, M. C., & Pazarbasi, B. (2004). Antibacterial activity of volatile components and various extracts of *Spirulina platensis*. *Phytotherapy Research, 18*, 754–757.

Piñeiro Estrada, J. E., Bermejo Bescós, P., & Villar del Fresno, A. M. (2001). Antioxidant activity of different fractions of *Spirulina platensis* protean extract. *Il Farmaco, 59*, 497–500.

Quach, H. T., Steeper, R. L., & Griffin, G. W. (2004). An improved method for the extraction and thin-layer chromatography of chlorophyll a and b from spinach. *Journal of Chemical Education, 81*, 385–387.

Robles Medina, A., Molina Grima, E., Giménez Giménez, A., & Ibañez Gonzalez, M. J. (1998). Downstream processing of microalgal polyunsaturated fatty acids. *Biotechnological Advances, 16*, 517–580.

Señorans, F. J., Ibáñez, E., Cavero, S., Tabera, J., & Reglero, G. (2000). LC/MS analysis of supercritical fluid extracts from rosemary plants. *Journal of Chromatography A, 870*, 491–499.

Subhashini, J., Mahipal, S. V. K., Reddy, M. C., Reddy, M. M., Rachamallu, A., & Reddanna, P. (2004). Molecular mechanisms in C-

Phycocyanin induced apoptosis in human chronic myeloid leukemia cell line-K562. *Biochemical Pharmacology, 68*, 453–462.

Subra, P., Castellani, S., Jestin, P., & Aoufi, A. (1998). Extraction of β-carotene with supercritical fluids. Experiments and modelling. *Journal of Supercritical Fluids, 12*, 261–269.

Van den Hoek, C., Mann, D. G., & Jahns, H. M. (1995). *Algae: An introduction to phycology* (12–13 ed.). Cambridge University Press.

Velioglu, Y. S., Mazza, G., Gao, L., & Oomah, B. D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *Journal of Agricultural and Food Chemistry, 46*, 4113–4117.

Xue, C., Hu, Y., Saito, H., Zhang, Z., Li, Z., Cai, Y., et al. (2002). Molecular species composition of glycolipids from *Spirulina platensis*. *Food Chemistry, 77*, 9–13.