

SUPERCRITICAL FLUID EXTRACTION OF BIOACTIVE COMPOUNDS FROM *CYTOSEIRA ABIES-MARINA* ALGAE

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Abstract

Supercritical carbon dioxide with ethanol as modifier was employed in the extraction of functional food ingredients from *Cystoseira abies-marine* algae. Three different types of pre-treatment were employed to prepare the vegetal raw material producing an important effect on extraction yield. The experimental assays were carried out in a semi-continuous extraction cell at 290 bar, 40 °C and using 10% v/v of ethanol as modifier. Extraction yields of ca. 1% were obtained. Antioxidant and antimicrobial activity of the supercritical extracts was determined. In general, all extracts presented high antioxidant activity and high antimicrobial activity against bacteria, meanwhile against yeast or fungus these activities were too much lower.

1. Introduction

Algae and microalgae are being widely studied not only for their high nutritional value, but also for their potential therapeutic properties¹. They are a natural source of proteins, high digestible carbohydrates, lipids, essential vitamins and antioxidants. Thus, they are being employed to enhance the nutritional content of conventional food preparations.

Commercial applications are dominated by *Arthrospira*, *Chlorella*, *D. salina* and *Aphanizomenon flos-aquae* microalgae. They have interesting qualities, which can positively affect human health. For example, *Arthrospira* microalgae has various health-promoting effects, such as suppression of hypertension, protection against renal failure and growth promotion of intestinal *Lactobacillus*². The most important substance in *Chlorella* is β -1,3-glucan, which is an active immunostimulator, a freeradical scavenger and a reducer of blood lipids³. *D. salina* is exploited for its β -carotene content⁴ and *Aphanizomenon flos-aquae* was proposed as a nutraceutical promoting good overall health⁵.

Supercritical fluid extraction (SFE) of vegetal materials has a great potential in the food industry as a clean technology to produce natural food ingredients. In comparison with traditional extraction processes, SFE can provide higher selectivity and shorter extraction times. Additionally, extracts of better quality and higher functional properties are usually obtained, since no degradation reactions take place during SFE.

The SFE of algae and microalgae using supercritical carbon dioxide (SC-CO₂) is being widely studied in the literature⁶⁻⁸. Usually, ethanol is employed as modifier in order to enhance the extraction of polar substances and high extraction pressures are required (300 - 400 bar) to attain a suitable yield.

Many species of the genus *Cystoseira* have been identify as a potential source of substances with antioxidant and antimicrobial activity to be employed as natural food ingredients⁹⁻¹¹. In this respect

meroterpenoids, hydroquinones derivatives, showed a potent antioxidant capacity comparable to that of α -tocopherol^{11, 12}.

In this work the SFE of *Cystoseira abies-marine* algae and the functional properties of the extracts obtained have been studied. SC-CO₂ with ethanol as modifier was used as extraction solvent. Also, the effect of extraction conditions (pressure, temperature and amount of modifier) on yield was preliminary explored.

2. Experimental

2.1 Samples and chemicals

Cystoseira abies-marina was collected from Tagliarte beach in Las Palmas de Gran Canaria. Algae sample was washed, air dried, and stored in plastic bags at room temperature.

Ethanol was obtained from Scharlau Chemie S.A. (Barcelona, Spain). 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and potassium persulfate were obtained from Sigma (Madrid, Spain), whereas Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) standard were purchased to Fluka Chemie AG (Buchs, Switzerland).

Algae samples were treated in three different ways: Type A, without any treatment; Type B, algae sample was ground and particle size below 1000 μm was obtained by sieving the ground material to the appropriate size; Type C, algae sample was ground under cryogenic conditions and particle size below 1000 μm was also used.

2.2 Supercritical fluid extraction

The SFE pilot plant (Iberfluid, Spain) employed in this study is shown in Figure 1 and comprises an extraction cell of 290 cm³ capacity, two separator cells (270 cm³ capacity each) where a cascade decompression takes place and a cryogenic trap at atmospheric pressure. CO₂ and ethanol are mixed at the exit of the respective pumps (Dosapro Milton Roy) and preheated to achieve the extraction temperature before introduction into the extraction cell. All vessels are electrically heated. The plant has computerized PLC-based instrumentation and a control system, with several safety devices including valves and alarms.

Each experiment was carried out using 100 grams of solid sample. This amount completely filled the extraction cell for samples without milling treatment (Type A), resulting an apparent density is 0.4 g/cm³. Samples with treatment Type B or C were mixed with 180 grams of sea sand in order to maintain in these cases the same apparent density.

The SFE assays were carried out using the following procedure: once the extraction temperature was stabilized in the extraction cell, a constant flow of CO₂ (3 L/h, pumping conditions) was driven into the extraction device until the desired pressure was attained; then, a constant flow of modifier (ethanol) was pumped and mixed with the CO₂ during 40 minutes, the modifier pump was stopped and pure CO₂ was allowed to flow into the extraction system for another 10 minutes. The samples collected in separators S1 and S2 (typically, less than 5% of the extracted material was collected in S1) were mixed, ethanol was evaporated and the material remained was weighted. Yield was calculated in reference of the total amount of algae sample placed into the extraction cell (yield = grams of material extracted / 100 g).

2.3 Antioxidant activity determination

The TEAC (Trolox Equivalent Antioxidant Capacity) assay described by Re et al.¹³ was used to measure the antioxidant activity of the SFE extracts from *Cystoseira abies-marina*. Briefly, ABTS^{•+} radical cation was generated by reacting 7 mm ABTS with 2.45 mM potassium persulfate after incubation at room temperature for 16 h in the dark. The ABTS^{•+} radical solution was diluted with ethanol to an absorbance of 0.70 ± 0.20 at 734 nm. 10 μL of *C. abies-marina* at four different

concentrations extract was added to 0.990 μL of diluted ABTS^{•+} radical solution. The reaction was measured until the absorbance reached a plateau. Trolox was used as reference standard, and results were expressed as TEAC values (mmol Trolox/ g extract). All analyses were done at least in triplicate.

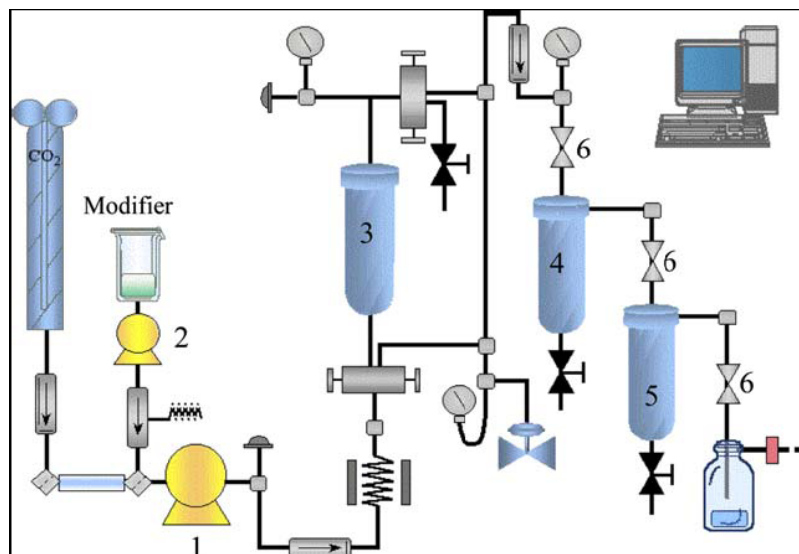


Figure 1. SFE laboratory scale equipment. (1) CO₂ pump; (2) modifier pump; (3) extraction cell; (4) and (5) separator cells; (6) valves; (7) cryogenic trap.

2.4 Antimicrobial activity determination

Microbial strains. The supercritical 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.

Determination of minimum inhibitory concentration (MIC) and minimal bactericidal (MBC) and fungicidal (MFC) concentration. A broth microdilution method was used, as recommended by NCCLS (National Committee for Clinical Laboratory Standards, 1999)¹⁴, for determination of the minimum inhibitory concentration. 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 10⁷ cfu/ml. Different quantities of *Cystoseira* extracts were dissolved in DMSO in order to obtain extracts concentrations ranging from 10 mg/ml to 250 mg/ml.

The 96 microwell plates were prepared by dispensing into each well 165 μL of culture broth, 5 μL of the inoculums and 30 μL of the different extracts 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 fungi. Negative controls were prepared adding 30 μL of DMSO, the solvent used to dissolve the microalgae extracts, to the culture broth containing 0.5% tween 20. After incubation, the MIC of each extract was determined by visual inspection of the wells bottom, 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.

3. Results and discussion

3.1 Supercritical fluid extraction

Table 1 show the extraction conditions employed together with the extraction yield obtained in the different experimental assays.

Very low extraction yields resulted with samples Type A; when the vegetal material is not milled, cell walls are not broken and thus the amount of material accessible to be extract is very low. Then, yield could not be improved although high pressure and high amounts of ethanol were employed (see Exp. 1 and 2 in Table 1) or large extraction times were applied (internal diffusion limitation). Milling of the vegetable sample can produce a considerably increase of yield. By comparison of experiments 2, 3 and 4 in Table 1, it can be deduced that treatment Type C is the most appropriate in order to increase extraction yield. Additionally, at 290 bar and 40 °C, a 50% reduction of the amount of ethanol employed produce only a 0.1% reduction in extraction yield (Exp. 4 and 5 in Table 1). A slight increase of yield was also observed by increasing extraction temperature to 70 °C.

Table 1. SFE extraction of *Cystoseira abies-marine* algae.

Exp.	Sample	Pressure (bar)	Temperature (°C)	% ethanol (v/v)	Extracted material (mg)	Yield (%)
1	Type A	180	40	3.5	161.2	0.16
2	Type A	290	40	10	182.6	0.18
3	Type B	290	40	10	683.7	0.68
4	Type C	290	40	10	842.1	0.84
5	Type C	290	40	5.0	762.5	0.76
6	Type C	260	70	10	910.7	0.91
7	Type C	290	70	10	899.7	0.90

3.2. Antioxidant activity

TEAC values (mmol trolox/g extract) of SFE extracts from *Cystoseira abies-marina* are shown in Table 2. The extracts obtained from samples Type C using similar extraction conditions (290 bar, 10% of ethanol modifier but two different extraction temperatures, 40 and 70°C) were employed to study the antioxidant activity of the supercritical extracts.

Both extracts showed a high antioxidant activity, nevertheless, antioxidant capacity decreased as extraction temperature enhanced. In this respect, an extraction temperature increase from 40 to 70°C caused ca. 11% decrease of TEAC values. This fall in antioxidant activity might be related to an increase in the extraction yield. Therefore, as extraction temperature increase a higher extraction of less active compounds is caused.

Table 2. TEAC values (mmol Trolox/g extracto) for SFE extracts from *Cystoseira abies-marina*.

Exp.	Sample	Extraction pressure (bar)	Extraction temperature (°C)	% ethanol (v/v)	Antioxidant activity
4	Type C	290	40	10	1.374 ± 0.056
7	Type C	290	70	10	1.219 ± 0.017

3.3 Antimicrobial activity

Four different microbial species, including a gram negative bacteria (*Escherichia coli*), a gram positive bacteria (*Staphylococcus aureus*), a yeast (*Candida albicans*) and a fungi (*Aspergillus niger*), were used to screen the potential antimicrobial activity of the best supercritical extracts (those with the highest yield) from *Cystoseira abies-marina*. The results obtained for minimal bactericidal and fungicidal concentrations are shown in Table 3. Both extracts showed a potent antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus*, meanwhile these extracts presented a very small activity against *Candida albicans* and the fungi *Aspergillus niger*.

The analysis of the antimicrobial activity of these extracts, as a function of extraction temperature, indicated that an increase in the extraction temperature normally produce a decrease in their antimicrobial activity. These data could indicate that at 70°C some of the compounds responsible for the antimicrobial activity might be degraded.

Table 3. Antimicrobial activities of the different extracts from *Cystoseira abies-marina*. MBC (minimal bactericidal concentration) and MFC (minimal fungicidal concentration) values are expressed in mg/ml.

Exp.	<i>Escherichia coli</i> MBC ¹	<i>Staphylococcus aureus</i> MBC	<i>Candida albicans</i> MFC ²	<i>Aspergillus niger</i> MFC
4	1.5 ± 0.2	1.5 ± 0.1	7.0 ± 0.7	12.1 ± 0.6
7	2.1 ± 0.3	2.2 ± 0.4	7.2 ± 0.5	12.5 ± 0.3

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