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Supercritical fluid extraction from a brown alga by stagewise pressure increase

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ABSTRACT

The use of supercritical carbon dioxide as an extractant for biological materials is described. Extractions from a mediterranean brown alga were conducted with commercial micro-scale equipment, improved by introducing a sapphire extraction cell, and coupled to a high-pressure vessel in order to collect the extracts. By applying a controlled stagewise pressure increase one obtains separate fractions, the colours of which range from colourless to green, depending on the temperature of the extraction. The results, in terms of weight, colour and analysis by high-performance liquid chromatography and by high-performance thin-layer chromatography, are discussed.

INTRODUCTION

Recent concern about the hazardous nature of many commonly used solvents as well as the costs and environmental dangers of waste solvent disposal have led to the development of alternative sample extraction methods. The limitations of conventional methods such as liquid-liquid or liquid-solid extractions, which are time and solvent consuming, have fuelled interest in the development of high-pressure extraction by supercritical gases, especially for compounds intended for human consumption. Many research groups have reported extractions of a variety of compounds from natural products, including flavouring materials from leaves and needles [1,2] and from citrus and orange peels [3–5], food colours from dried grass and turmeric roots [6,7] and flavours from milk fat [8], but no reports on marine materials, especially algae, have been published.

Algae are widely exploitated owing on the one hand to their technological and nutritional properties (asiatic nutritional algae industry), and on the other to their content of specific polysaccharides (colloids industry), with a world production of 1 600 000 tons of collected algae [9]. There are also many prospects for new developments, as described in a recent review [10], including the use of algae in the medical field, where numerous studies have confirmed the antifungal, anticoagulative and antimicrobial activities of algae extracts [11,12]. Screenings of such activities have been obtained from crude extracts, but only a few active molecules have so far yet been isolated [13–15].

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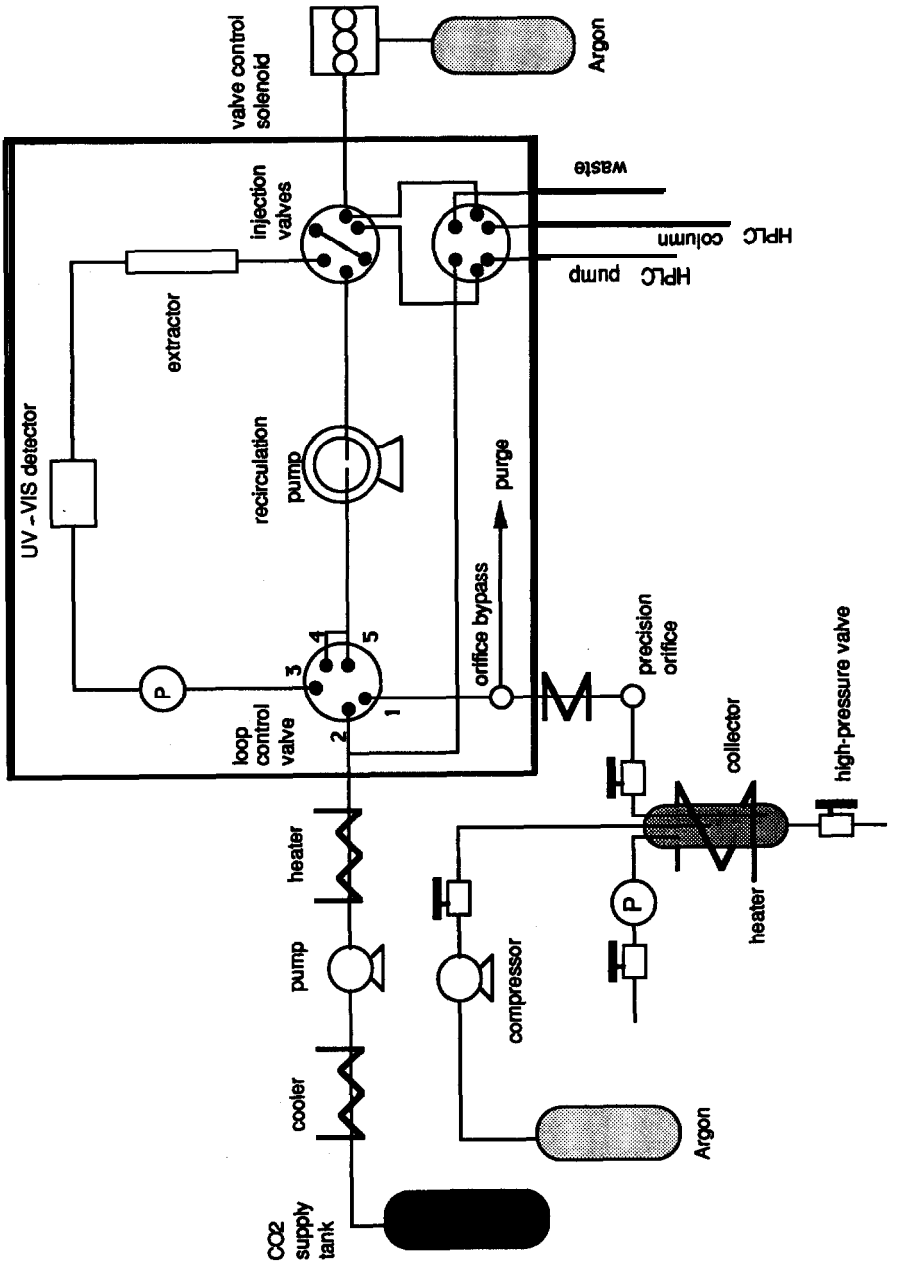

Fig. 1. Supercritical fluid extraction apparatus.

Classical purifications often require numerous steps, such as liquid extraction and flash chromatographic techniques [16], including evaporations and sample transfers which may induce losses and contamination risks. For such purification of active molecules from complex natural matrices, supercritical fluids may offer some advantages over liquid organic solvents; from a brown mediterranean alga, supercritical carbon dioxide leads to extracts similar to those obtained by liquid extraction in terms of antifungal properties, but they contain interfering compounds with a narrower range of polarity, and they are obtained in a shorter analysis time [17]. Selectivity may be adjusted by controlling the solvent strength of the fluid, depending on the pressure and the temperature of the extraction; at a constant temperature, extraction at lower pressures will favour less polar analytes, while higher pressures will favour more polar and higher molecular weight analytes [18].

In this paper, we present the results of supercritical carbon dioxide extraction from a brown mediterranean alga, *Dilophus ligulatus*, which exhibits a wide range of antifungal activities. By applying a stagewise pressure increase, from 8 to 25 MPa, differents fractions, in terms of colour and composition are obtained.

EXPERIMENTAL

Supercritical fluid extraction (SFE) is conceptually simple to perform, particularly on an analytical scale. A micro-scale extractor offers many advantages: (1) ease of construction with components available for liquid or gas chromatography, (2) low cost of the optimization because of the small sizes of samples and fluids, (3) on-line monitoring of extract by UV during the extraction step and (4) through the use of switching valves, on-line analysis of extracts by chromatography. The sample preparation accessory (SPA) from LDC Analytical (Thermo Instrument Division) combines most of these advantages.

SFE can be performed in either a dynamic or a static mode. In the dynamic mode, the fluid is constantly flowing through the cell containing the raw material, while a flow restrictor maintains pressure in the system and allows the fluid to depressurize into the collection device (most of the published examples are performed in such a mode). The static mode is performed by pressurizing the cell and extracting the sample with no outflow of the fluid. Specific criteria for selecting dynamic or static modes are not yet clear, and both methods have been used for quantitative SFE of a variety of samples [19]; the commercial extractor we used combines both techniques, as described below.

Micro-SFE apparatus

The SPA is shown in Fig. 1. Carbon dioxide (N 45 grade, Air Liquide) from the supply tank was cooled to the liquid state and then compressed to the desired pressure, up to 35 MPa, by a constant-pressure pump. The compressed carbon dioxide was preheated to the desired temperature, up to 333 K, before being introduced into the oven. The oven houses the extraction loop, which is made up of the loop control valve, recirculating pump, switching valves, extractor and high-pressure UV detection cell. Two modifications were carried out: the original extraction cell, which was a 5-ml 3 16 stainless-steel cup with a porous bottom and lid with fiits, was replaced with an empty column, because an amount of cosolvent always remained in the bottom of

the cell; and a sapphire cell [20] was introduced into the extraction loop in order to observe various phenomena.

The original purpose of this extraction system is to provide both modes of extraction (dynamic or static), depending on the position of the loop control valve. When 34, and therefore l-2, are connected, the extraction loop is isolated from the supply tank and becomes a closed system, filled with a known volume of carbon dioxide of density precisely controlled by the pressure and the temperature parameters. When the recirculating pump is activated, the fluid continuously cycles though the closed loop until extraction of the desired analytes has occurred, as indicated by the W-VIS detector. After a set period of time, the loop control valve is switched, so that 2-5 and therefore 3-1, are connected. A constant flow-rate of fresh carbon dioxide delivered by the main pump, enters the loop to sweep out the carbon dioxide enriched with analytes through a flow restrictor into a collection device; this orifice is temperature controlled to prevent ice build-up due to rapid depressurization.

Collection device

A quickly and easily dismantled device, made of 316 stainless steel, is shown in Fig. 2. The vessel head is securely fastened to the body by two half-circle nuts and sealed with anti-extrusion O-rings; the nuts themselves are confined in a lid. The lid, nuts and head can be removed by hand, without using any tools.

Three tubes of 1/8 in. O.D. pass through the vessel head; the compressor tube (not drawn in Fig. 2) is connected to an air compressor, which pressurizes the vessel with argon above the set pressure. Through the inlet tube, connected to the extractor,

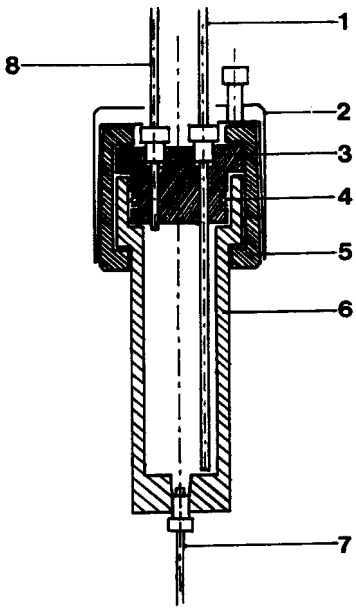

Fig. 2. Collection device. I = Tubing of 1/8 in. O.D. connected to the extractor; 2 = lid; 3 = vessel head; 4 = anti-extrusion O-rings; 5 = half-circle nuts; 6 = body of the collector; 7 = tubing of 1/8 in. O.D. connected to the collection valve; 8 = outlet port.

supercritical carbon dioxide enriched with analytes is introduced into the vessel, which generally contains a few milliliters of hexane. Extracts are dissolved in the organic solvent and carbon dioxide flows out from the outlet port.

Analysis

High-pressure thin-layer chromatography (HPTLC) , Extracts are dissolved in 100 μl of the developing solvent [hexane–ethyl acetate (80:20, v/v)]; 5 μl are applied to a glass plate (5 cm × 5 cm) precoated with silica (Silica gel 60 F_{254}; Merck, Nogent sur Marne, France). Separated substances are revealed using a spray reagent (sulphuric acid-methanol).

High-performance liquid chromatography (HPLC). Extracted substances are separated, after dilution in 3 ml of ethyl acetate, on a Spherisorb ODS-2 column (15 cm × 0.46 cm I.D.). Pumps, column oven, single-wavelength UV detector and integrator were obtained from LDC Analytical.

Sample matrix

A brown alga, ***Dilophus ligulatus***, was collected from the Mediterranean at Villefranche sur Mer, France, during May and June 1989. If the algae were then stored in ethanol, the weights of the supercritical extracts were too low for subsequent quantitative analysis. However, if fresh algae were stored in a dark room until they were dry, repeated samplings showed that storage did not alter the antifungal properties. Algae were therefore stored at 255 K in a freezer.

For each experiment, 2.7 g of the alga were crushed into pieces of about 0.5 mm, mixed with an equal volume of 50-μm glass beads and introduced into an empty column (8.3 cm × 1.05 cm, I.D.).

Procedure

The extraction cell was connected to the extraction loop and the carbon dioxide was then pressurized to the set pressure of 8 MPa. The system was allowed to equilibrate for 30 min before the recirculating pump was activated for 30 min. This time corresponds to the altainment of a constant UV detector response, indicating that the maximum amount of dissolved compounds had been reached. Subsequently, a constant flow-rate of fresh carbon dioxide swept the extract into the collector, previously pressurized to the extraction pressure. The collection device was then isolated by shutting off the two high-pressure regulating valves, while the extraction loop was pressurized to the second set pressure value. The collector was depressurized by opening the collection valve and the hexane containing solutes flowed out. The organic solvent was gently evaporated under a stream of nitrogen; extracts were weighed and analysed by both types of chromatography.

Pressure values investigated during the stagewise pressure increase were 8, 10, 15, 20 and 25 MPa; three temperatures of extraction were also investigated, 308, 318 and 328 K.

RESULTS AND DISCUSSION

The densities and amounts of carbon dioxide used in the experiment are listed in Table I. Because the SPA extractor consists mainly of a closed loop of finite

TABLE I

VALUES OF DENSITY AND MASS OF CARBON DIOXIDE AS A FUNCTION OF PRESSURE AND TEMPERATURE

Density (p) is expressed in g cm^{-3} and mass of CO, (m_f) in g.

T (K)	Pressure (MPa)									
	8		10		15		20		25	
	ρ	m_f	ρ	m_f	ρ	m_f	ρ	m_f	ρ	m_f
308	0.505	9.52	0.678	12.78	0.816	15.39	0.856	16.14	0.895	18.80
318	0.294	5.55	0.490	9.24	0.740	13.97	0.800	15.08	0.857	16.16
328	0.205	3.88	0.338	6.37	0.655	12.35	0.756	14.26	0.802	15.46

volume, each density corresponds to a precise amount of fluid. This point depends on the fact that density cannot be dissociated from the mass of the extraction fluid; therefore, the effect of the density parameter on the solubilization has to be counterbalanced by the effect of the mass of carbon dioxide used for the extraction.

Colours and weights of extracts

As indicated in Table II, the colours of the extracts range from colourless to green, depending on the carbon dioxide density; for densities less than 0.74 g cm^{-3}, most extracts are colourless excepted that at 15 MPa and 328 K, which is slightly orange; up to a density of 0.8 g cm^{-3} the extracts are yellow and at higher densities green pigments are extracted.

As noted in Table III, the total amount of fluid used for each isotherm decreases

TABLE II

COLOURS OF EXTRACTS AS A FUNCTION OF PRESSURE AND TEMPERATURE

T (K)	Pressure (MPa)				
	8	10	15	20	25
308	Colourless	Colourless	Yellow	Light green	Green
318	Colourless	Colourless	Colourless	Yellow	Green
328	Colourless	Colourless	Orange	Yellow	Green

TABLE III

TOTAL AMOUNTS OF EXTRACTS (M_e, mg) AND CO, (M_f g), AND RATIO OF THESE TWO VALUES FOR EACH ISOTHERM

T (K)	M_e	M_f	M_e/M_f
308	36.1	70.72	0.510
318	74.5	60.01	1.241
328	88.5	52.33	1.691

TABLE IV

WEIGHTS OF EXTRACTS (mg) COLLECTED FOR EACH PRESSURE INCREASE

T (K)	Pressure (MPa)				
	8	10	15	20	25
308	1.4	1.4	21.3	1.5	4.5
318	2.9	13.5	38.4	12.8	6.9
328	1.1	2.6	44.3	22.4	18.1

with increasing temperature, while the total weight of extracted material increases. Therefore, a temperature of 328 K was chosen in order to obtain a greater amount of extract with a smaller consumption of carbon dioxide.

Weights of the extracts are listed in Table IV. For a constant temperature, an increasing pressure up to 15 MPa leads to an increase in the weight of the extracts, owing to the general influence of pressure (and density) on the solubility of compounds in a supercritical fluid. For higher pressure, the amounts decrease; this may be due to the depletion of the algae in soluble analytes, which does not overcome the possible increase in solubility of more polar compounds. This depletion in soluble analytes is also observed when several extractions are performed on a same sample, with a constant density of carbon dioxide.

By representing the distribution of extraction yields with pressure in the form of fractions (Fig. 3), one observes that the fraction corresponding to a pressure of 15 MPa (and also to an extraction time of 3 h), contains about 50% of the total amount, whatever the temperature. Hence temperature seems to be a parameter influencing

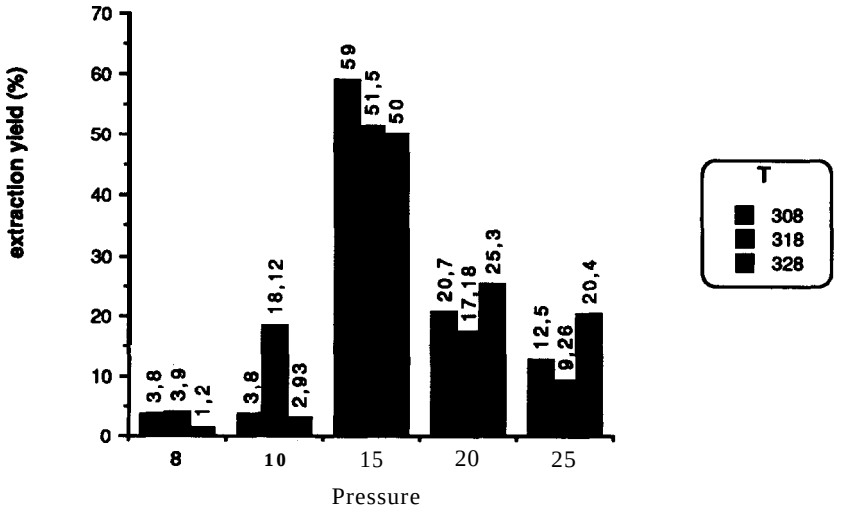

Fig. 3. Distribution of extraction yields by fractions depending on extraction temperature. Yield is defined by the ratio between the extracted amount in a fraction *i* and the sum of amounts obtained at constant temperature. Temperature in K, pressure in MPa.

mainly the amount of extractable material, but not the distribution of yields by pressure. The HPTLC and HPLC results confirm these tendencies.

HPTLC

TLC plates of extracts are shown schematically in Fig. 4. For a pressure of 8 MPa, only a few compounds are revealed; because of their migration zone, these species are fairly non-polar or moderately polar analytes; for example, compounds 9 and 5 are eluted as standards of b-carotene and cholesterol respectively. By increasing the pressure, compounds with a wider range of polarity are extracted by carbon dioxide; the third fraction contains the nine solutes, in increasing concentration with temperature. For higher pressures, the concentration of each solute generally decreases, except for a green compound eluted between 2 and 3 and which appears in the fourth and the fifth fractions depending on temperature. Refering to Table III, the presence of this compound may be compared with the green colour of extracts.

The influence of temperature is not clearly defined: it modulates the distribution of solutes by fractions, but generally it does not induce important changes in selectivities.

HPLC

Four chromatograms corresponding to fractionation along the isotherm at 3 18 K are shown in Fig. 5. Whatever the pressure, there is a lack of polar compounds which might be eluted rapidly under such conditions. When the pressure is increasing, several modifications in terms of concentration and selectivity are observed: a pressure of 15 MPa leads to the extraction of compounds with retention times less than 45 min, whereas materials collected at higher pressures show fewer compounds present. The variations also depend on the isotherm considered. As shown in Fig. 6, the first fraction obtained at 8 MPa and 308 K contains compounds with a wider range of retention times than the corresponding fraction obtained at 3 18 K. For a quantitative description, peaks have been collected into groups numbered from I to VII; these groups are determined by their responses to a change in extraction pressure or tem-

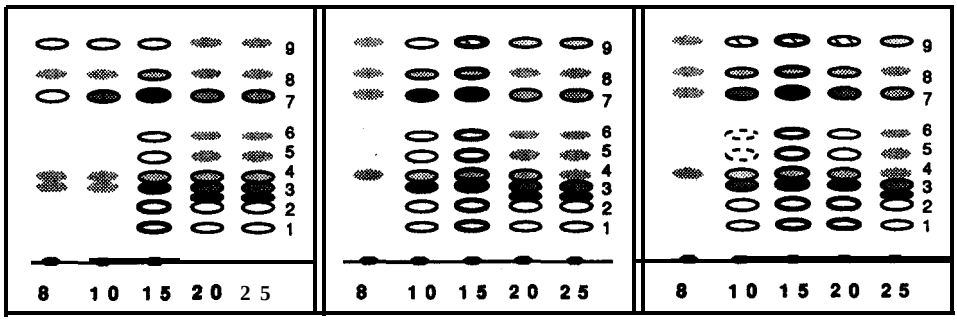

Fig. 4. HPTLC plates of fractions obtained for each isotherm (fractions are identified by pressure, in MPa). Experimental conditions are described in the text.

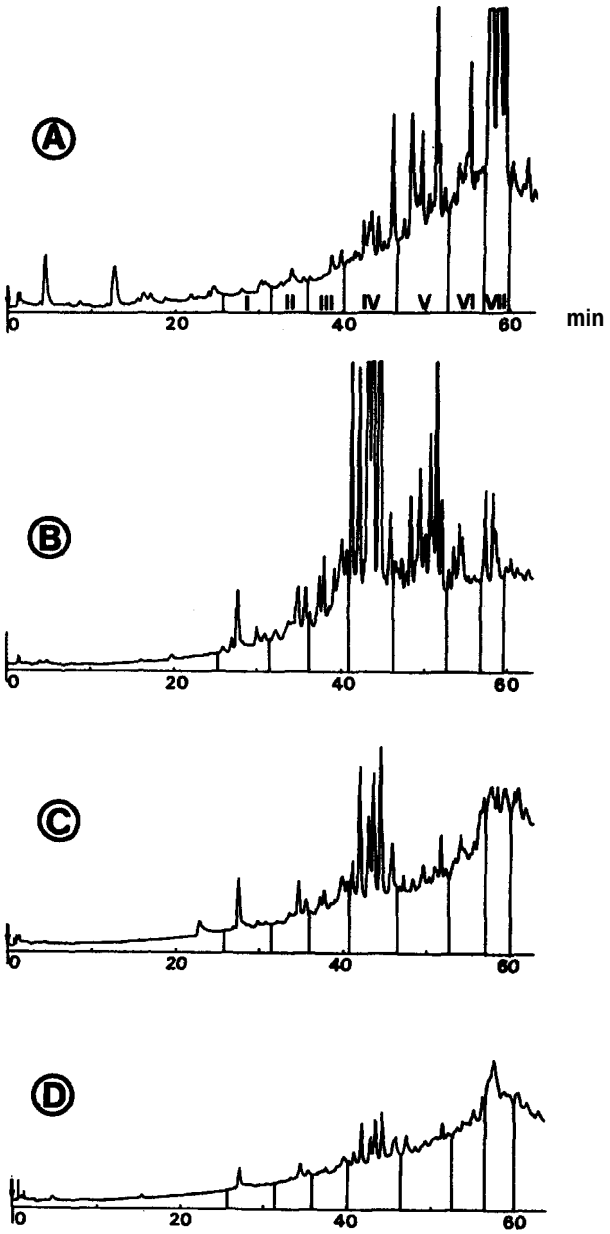

Fig. 5. HPLC of fractions obtained for the isotherm 318 K. Corresponding pressures: (A) 8 MPa; (B) 15 MPa; (C) 20 MPa; (D) 25 MPa. Conditions: column, 15 × 0.46 cm I.D.; stationary phase, Spherisorb ODS-2 d_p 5 μm; mobile phase: methanol-water with a methanol content of initially 44% for 5 min, reaching 84% in 50 min and then remaining constant for 10 min; flow-rate 1.5 ml min^{-1}; UV detection wavelength, 254 nm; attenuation, 64.

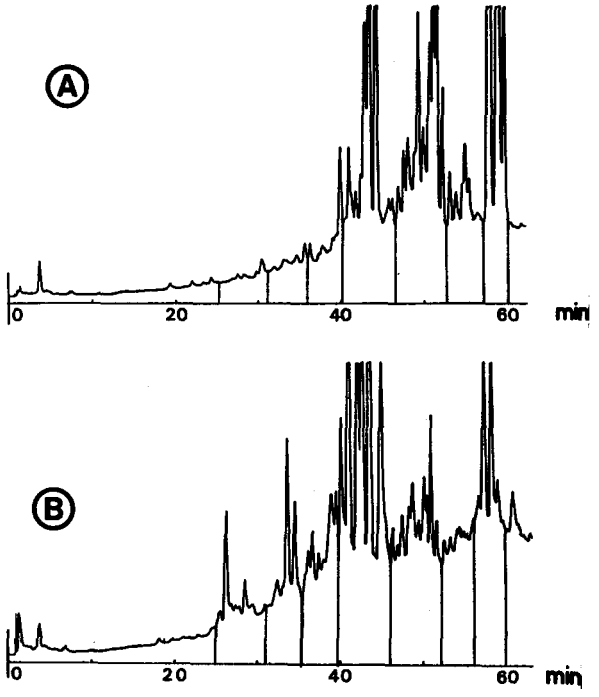

Fig. 6. HPLC of fractions corresponding to a pressure of (A) 8 MPa and (B) 15 MPa and to a temperature of 308 K. Conditions are as in Fig. 5.

TABLE V

DISTRIBUTION (%) OF GROUPS FOR EACH SUPERCRITICAL FRACTION, FOR A CRUDE SAMPLE AND FOR A PURIFIED EXTRACT AND SELECTIVITY OF GROUP V

Selectivity is the % of group V divided by the sum of % of other groups.

Group number	Supercritical fractions								Crude sample	Purified extract
	T = 308 K				T = 318 K					
	8 MPa	15 MPa	20 MPa	25 MPa	8 MPa	15 MPa	20 MPa	25 MPa		
I	1	4	4	3	1	2	4	3	2	0
II	3	9	9	2	1	5	4	5	3	3
III	4	10	3	3	4	9	14	16	11	0
IV	22	39	35	18	7	42	45	58	37	7
V	27	18	15	16	15	28	8	7	10	65
VI	1	1	10	5	11	3	13	8	30	14
VII	42	19	24	53	61	11	12	3	7	11
Selectivity	0.37	0.22	0.18	0.19	0.18	0.39	0.09	0.07	0.11	1.86

perature. The distributions of groups for each extracted fraction are listed in Table V and may be correlated with the selectivity; results for a crude sample extracted by a conventional method (two organic solvents, hexane and dichloromethane) and for a purified fraction (the isolation of which involved selective elutions by flash chromatography [17]) are also given. With the supercritical extracts, most of compounds are distributed mainly among three groups, IV, V and VII, whereas the content of the crude sample is distributed among more groups. A further purification of this crude sample led to a narrow distribution, with a high content of 65% for the fifth group; the presence of compounds in this fraction has to be correlated with the antifungal activities of this extract. Hence terms of selectivity between this group and the six others (Table V), supercritical extraction does not allow the production of highly purified fraction, but it may compete favourably in a first step to the achievement of a crude extract of better quality than is obtainable with organic solvents.

CONCLUSION

The small amounts of sample and fluids used in this modified commercial extractor were useful in optimizing the carbon dioxide extraction of compounds from a complex matrix such as an alga. By increasing the pressure stagewise, extracts of different contents in terms of selectivity and amount can be obtained; the choice of operating conditions will depend on the purpose and on the demands of the extraction process. Therefore, these results indicate that an extraction process with a supercritical fluid in order to obtain different active compounds from algae is practicable and that this process will compete favourably with classical processes such as liquid extraction.

Further experiments will be performed in order to increase the selectivity of the extraction. We have already used a modifier such as ethyl acetate in addition to carbon dioxide, but it does not induce noticeable modifications. Future studies will concern the use of packed beds to fractionate the extract during the collection step.

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