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Supercritical fluid extraction as a tool to valorize underexploited freshwater green algae

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ABSTRACT

Water reservoirs in Poland such as rivers, lakes and ponds are a rich source of freshwater green macroalgae that can be exploited as a good source of biologically active compounds, once an appropriate process has been developed and the metabolites and biological activity of the different species have been demonstrated. With this goal, in the present work a supercritical fluid extraction process (SFE) has been studied to extract carotenoids and phenolic compounds from *Cladophora glomerata*, *Ulva flexuosa* and *Chara fragilis*. Optimization of the parameters involved in the SFE process (temperature, pressure and % ethanol as co-solvent) has been carried out by using a 3-level factorial experimental design and different responses have been evaluated simultaneously (extraction yield, total carotenoids, total phenols and antioxidant activity). Optimized conditions include the use of 40 °C, 300 bar and 11.4% ethanol as co-solvent. Under these conditions, *C. fragilis* extracts were the richest in total carotenoids and total phenols (24.90 mg fucoxanthin equivalents g^{-1} extract and 30.20 mg gallic acid equivalents g^{-1} extract, respectively), whereas those produced from *U. flexuosa* possessed the highest antioxidant activity (0.944 mmol trolox equivalents g^{-1} extract). The chemical characterization of the different extracts, carried out by HPLC-DAD-MS, allowed the tentative assignment of diverse carotenoids, chlorophylls and related compounds in the studied algae; among them some described for the first time on these species. Hence, extracts obtained from the studied algae using SFE can be considered of potential interest in cosmetic, food and pharmaceutical industry as a way to valorize these underexploited materials.

1. Introduction

Nowadays, algae have found a good number of applications in various industries, as cosmetic, pharmaceutical, food and agriculture, due to the variety of bioactive compounds these organisms possess [1–5]. Literature shows plenty of data regarding marine algae, their chemical composition and applications [2–4,6–8]. However, as far as freshwater algae are concerned, their properties are poorly understood because just a few investigations related to analysis of bioactive compounds in these algae have been reported [9–13]. Water reservoirs in Poland such as rivers, lakes and ponds are a rich source of freshwater green macroalgae, with an important growing rate which can be revalorized if a proper identification of metabolites and biological activity is carried out. This is especially interesting for freshwater algae such as *Cladophora glomerata*, *Ulva flexuosa* and *Chara fragilis*.

Cladophora glomerata (L.) Kütz. (*Chlorophyta*, *Ulvophyceae*) is filamentous green macroalga commonly occurring in freshwater ecosystems [14]. Freshwater *C. glomerata* has been shown to be a natural source of carotenoids [15–20] including β-carotene, lutein, zeaxanthin, astaxanthin, violaxanthin, loroxanthin, antheraxanthin and 9-*cis* neoxanthin, as well as of phenolic compounds [15,16,21]. *Ulva flexuosa* subsp. *pilifera* (Kütz.) (*Chlorophyta, Ulvophyceae*) is a green tubular macroalgae occurring mostly in marine, but also in freshwater reservoirs [22]. So far, research has been focused on marine species from *Ulva* genera and most of them concerning *Ulva lactuca* [23–26]. Indeed, the knowledge on the composition and activity of *U. flexuosa* remains scarce. Marine *Ulva* species are well known sources of characteristic carbohydrates, called ulvans [26] which have been related to a number of bioactivities [27–29]. Our recent studies have shown the presence of ulvans also in freshwater *Ulva* species [30]. Regarding *Chara fragilis* A.N. Desvaux (*Charophyta, Charophyceae*), this is a small macroalga of highly branched green or light grey thallus, which was found both in marine and freshwater habitats [31]. Biochemical characteristic of algae from freshwater *Charophyta* phylum is hardly known. Some researches related to fatty acids and sterols composition in *Chara* species were conducted [32,33]. Also carotenoids such as lutein, neoxanthin, zeaxanthin, violaxanthin, fucoxanthin, β-, α- and γ-carotene [34] have been described, although this species remains largely unknown from a chemical composition perspective.

Nowadays, different advanced green extraction techniques, such as supercritical fluid extraction (SFE) are widely used to isolate bioactive

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compounds from natural sources. In SFE, solvents above their critical pressures and temperatures are employed resulting in an easier diffusion through solid materials and therefore faster extraction yields [7,35]. Usually supercritical CO_2 (SC-CO_2) is used as a solvent in SFE due to its mild critical conditions, non-toxicity and low-cost [8]. SC-CO_2 is suitable for isolation of non-polar compounds, for this reason, in the case of extraction of medium polarity natural bioactives, the addition of a co-solvent such as ethanol is necessary [36]. As a result, SFE has found numerous applications to recover biologically active components from algae, allowing the extraction of labile or easily oxidizable compounds [8,37]. Predominantly, SFE has been used for carotenoids isolation from marine macroalgae species, for instance for the recovery of fucoxanthin [38–41]. Some phenolic compounds were also extracted from seaweeds using SFE [42]. In contrast to marine algae, literature shows a few examples of SFE applications to isolate bioactive compounds from freshwater algae [11].

Bearing this in mind, the main aim of this work was to develop SFE-based processes as a green and environmentally friendly approach to revalorize different freshwater green macroalgae species from Poland such as *Cladophora glomerata*, *Ulva flexuosa* and *Chara fragilis* and to proceed to their chemical and functional characterization. For this purpose, extraction conditions were optimized to maximize extraction yield, as well as amount of isolated bioactive compounds (carotenoids, phenols) and antioxidant activity in the obtained extracts. Afterwards, *in vitro* assays including total carotenoids, chlorophylls and phenols, as well as antioxidant activity (TEAC) were carried out for extracts obtained under optimum conditions to determine their antioxidant properties. Furthermore, the evaluation of carotenoids and chlorophylls in the extracts using HPLC-DAD-MS was attempted to define the chemical composition of studied freshwater green macroalgae species from Poland for the first time.

2. Materials and methods

2.1. Algae material and chemicals

Three species of freshwater green algae were used in the study: *Cladophora glomerata*, *Ulva flexuosa* and *Chara fragilis*. Samples were collected manually from freshwater reservoirs located in Greater Poland region (Poland). Specifically, *C. glomerata* and *U. flexuosa* were harvested from Nielba river, in July 2014 and August 2014, respectively, whereas *C. fragilis* was collected from Wielkowiejskie Lake in January 2015. After harvesting, fresh algal biomass was weighed and dried in a drying chamber FD with forced air (Binder) under the temperature of 35 °C until a dry matter having a water content of <15% was obtained. Then, dry algae were milled using a laboratory mill with grinding tank.

Carbon dioxide (99% purity), purchased from Carburos Metálicos (X50S, Barcelona, Spain), and ethanol (99.5%), provided by VWR Chemicals (Fontenay-sous-Bois, France), were used for supercritical fluid extraction (SFE). Ultrapure water was obtained from a Millipore system (Billerica, MA, USA). Fucoxanthin, chlorophyll *a*, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, ≥97%) and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS, ≥99%) were purchased from Sigma-Aldrich (Madrid, Spain). Folin–Ciocalteu phenol reagent was provided by Merck (Darmstadt, Germany). Methanol and methyl tert-butyl ether (MTBE) provided by LabScan (Gliwice, Poland), were used for HPLC-DAD-MS analysis.

2.2. Supercritical fluid extraction

The extractions were carried out using home-made SFE instrument. Fig. 1 shows the scheme of the apparatus. CO_2 from a cylinder (1) is compressed with a pump (2), preheated inside a tube with the proper dimensions (8) placed in the oven (7), and through the extraction cell (9) containing the algae material. CO_2 pressure and flow rate are controlled by a micrometering valve (3) and a back pressure regulator

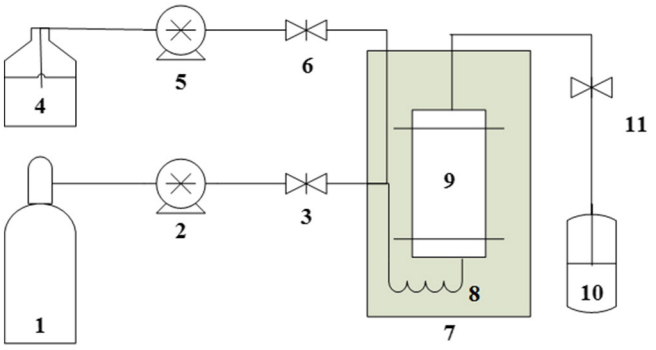

Fig. 1. Schematic diagram of supercritical fluid extraction apparatus: 1 – CO_2 cylinder; 2 – CO_2 pump; 3 – micrometering valve; 4 – co-solvent reservoir; 5 – co-solvent pump; 6 – micrometering valve; 7 – oven; 8 – preheating tube; 9 – extraction cell; 10 – collection vial; 11 – back pressure regulator.

(11). Co-solvent is added by pumping it from a reservoir (4) to the extraction cell using an HPLC pump (5); co-solvent flow is regulated through a micrometering valve (6). Afterwards, the obtained extract is collected in a collection vial (10) by depressurization of the system. For each extraction, 0.5 g of dried algae biomass was used with a CO_2 flow rate of 2 ml min^{-1}. Each extraction was carried out for 2 h in triplicate. Extraction conditions for all algae were selected based on an experimental design conducted for *C. glomerata*. After extraction, the resulting extracts were collected in vials and the residual ethanol was evaporated under vacuum to calculate extraction yield. Then, dried extracts were diluted with ethanol to concentrations from 10 to 20 mg ml^{-1} and stored at −20 °C and protected from light until analysis.

2.3. Experimental design

To optimize extraction conditions a 3-level factorial experimental design 3^3 (including three central points) was used based on three factors: temperature (40–60 °C), pressure (100–300 bar) and percentage of ethanol as co-solvent (0–15%). The effect of the factors on different responses, including extraction yield (%), total carotenoids content, total phenolic content (TPC) and antioxidant activity (TEAC assay) was studied. A total of 15 experiments were conducted in a randomized order (as shown in Table 1) for *C. glomerata*. The experimental design and data analysis were carried out using response surface methodology (RSM) with Statgraphics Centurion XVI® (StatPoint Technologies, Inc., Warrenton, VA, USA) software. The effects of the independent factors on the response variables in the separation process were assessed using the pure error, considering a level of confidence of 95% for all the variables. The effect of each factor and its statistical significance, for each of the response variables, was analyzed from the standardized Pareto chart. The response surfaces of the respective mathematical models were also obtained, and the significances were accepted at $p \leq 0.05$. A multiple response optimization was carried out by the combination of experimental factors, looking for maximizing the desirability function for the responses in the extracts. Afterwards, the optimum extraction conditions obtained for *C. glomerata* were used for the extraction of the rest of the algal species, *i.e.*, *Ulva flexuosa* and *Chara fragilis*.

2.4. In vitro assays

2.4.1. Total carotenoids and chlorophylls determination

Total carotenoids and chlorophylls content in the algal extracts was evaluated spectrophotometrically following the method described by Liechtenthaler [43] with some modifications. Briefly, ethanolic extracts were diluted with methanol to obtain concentrations in the range from 0.1 to 0.5 mg ml^{-1}. Then, 300 μl of each extracts were transferred to a 96-well microplate. The absorbance was measured at 470 nm, 652 nm and 665 nm in a microplate spectrophotometer reader