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Adsorbent-assisted supercritical CO_2 extraction of carotenoids from *Neochloris oleoabundans* paste

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ABSTRACT

Neochloris oleoabundans emerges as alternative source of bioactives that complies with the algae-based biorefinery concept, which consists of a platform that offers a multitude of algae bioproducts. The development of an integrated extractive processes in line with the green chemistry principles have motivated the use of Supercritical CO_2 ($scCO_2$), as an alternative to toxic organic solvents, for the extraction of bioactives. However, process integration and optimization is challenging because microalgae are grown in liquid cultures, therefore is often necessary a drying step prior to $scCO_2$ extraction. Moreover, this step is usually energy intensive and risks damaging the compound's bioactivity. An alternative is the simultaneous extraction process of the microalgae paste (containing around 70–80% water), nevertheless little information is available that explores this type of extraction. This work aims to explore the direct extraction of microalgae paste and to evaluate the effect of water on carotenoid extractions of *N. oleoabundans*. To study the extraction under a batch-wise system, an indirect extraction system was developed by mixing the microalgae paste with low cost adsorbents as support medium. Two types of silica gels, two different chitosans and active carbon were tested as adsorbents; sea sand was used as inert control. All of the materials showed different adsorbent capacity, being chitosan adsorbents those with higher capacity. However, oleoresin yield and recovery was negligible in a system with only $scCO_2$ as a solvent and ethanol as co-solvent was required to improve the extraction yield. Although the overall oleoresin recoveries were low for all adsorbents (ranging from 2 to 10%), chitosan-assisted extraction showed the highest carotenoid recoveries (60–140% g/g) surpassing acetone benchmark extraction in case of chitosan microspheres. These results are interesting for the development of low energy consumption processes, since there is no need to dry the microalgal paste.

1. Introduction

Microalgae consist of a wide range of autotrophic organisms, which grow through photosynthesis similar to land based plants. Their unique unicellular structure allows them to easily convert solar energy into chemical energy and a broad range of high-value bioactives with important applications in food, cosmetics, pharmaceutical, and biofuels [1–3]. Particularly, recent consumer trends for natural and healthy products have shifted the food industry to develop new food products enriched with functional ingredients such as carotenoids, which have been recognized for preventing human diseases and maintaining good health [4,5].

In light of the algae-based biorefinery concept, which consist in the development of a platform that offers a multitude of different algae derived bioproducts (bulk chemicals, food products and ingredients, fertilizers, biofuels, etc.), from an optimized and integrated process, the microalgae *Neochloris oleoabundans* emerges as an attractive and alternative source of bioactives [6]. This microalgae has a high-lipid productivity, for biofuels applications, and is a potential source of the carotenoids lutein and astaxanthin, for functional food applications [7]. The development of an integrated processes in line with the Green Chemistry Principles [8] poses significant challenges to researchers in the design of new extraction processes to recover high-value compounds from natural sources. These requirements have motivated the use of supercritical (sc) carbon dioxide (CO_2) as an alternative to organic solvents because it is nontoxic, sustainable, and has versatile qualities [9].

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The development of $scCO_2$ extraction technology of high-value compounds for commercial application is a demanding task that involves process optimization (operating conditions, co-solvent use, and scale-up), substrate optimization (mainly drying processes and particle size adjustments by grinding and optional agglomeration), economic assessment of capital cost (purchase, installation, and start-up of a pilot to industrial supercritical fluid plant) and operational cost as extensively described by de Melo et al. [10] and del Valle [11]. In the case of microalgae, since they are grown in liquid cultures, it is often necessary a drying step prior to $scCO_2$ extraction. This step is usually energy intensive and it can be an important economic bottleneck for further process development [12–14]. Moreover, drying may also have a negative impact in the bioactivity of the compounds due to its vulnerability to medium-to-high temperature [15].

We are interested in exploring the concept of simultaneous extraction process, as described by Machmudah et al. [16], of microalgae paste. This concept involves the extraction and separation process, where both water and $scCO_2$ extraction flow counter-currently or only $scCO_2$ flows while the aqueous suspension is static in the extractor. Since water is a polar substance, partitioning of compounds will depend on polarity; mass transfer mechanism of bioactives to $scCO_2$ will need to consider the presence of water during extraction.

Previous experiments done in the "Extraction Laboratory of Biological Material" (LEMaB acronym from the Spanish name) of $scCO_2$ extraction of *Haematococcus pluvialis* paste showed that temperature and the interaction between temperature and pressure were significant in astaxanthin extraction. As temperature increase from 40 to 70 °C so did the astaxanthin yield under low (35 MPa) to medium (45 MPa) pressures. However, under high pressure (55 MPa) this relationship reversed. Pressure by itself played no significant role, while during extraction of dried *H. pluvialis* powder it did [17]. Although water affected negatively the solubility of oleoresin and astaxanthin in $scCO_2$, it was possible to achieve higher purities (~20% more at high pressure conditions) [17]. Kinetic studies showed a sigmoidal extraction curve in which water was extracted from the extractor during the first 4 h; after this time, the extraction started behaving similar to dry powder extraction. To fully understand the extraction dynamics under the presence of water it is necessary to keep the water inside the extractor chamber. Since this phenomenon was inevitable in a batch-wise direct extraction system, an alternative is to use an indirect liquid extraction system which involves mixing the microalgae paste with support media prior $scCO_2$ extraction [18]. We propose the use of adsorbents susceptible to trap the water and some components of the CO_2 + solutes flux, inducing thus a difference in the total extracted amount, and in the purity of the bioactives isolated. It is expected that by increasing the adsorbents' trapping capacity, a lower recovery is achieved. But, considering that water and oleoresin/carotenoids sorption can be competitive, a complex behavior is foreseen that needs an in depth study and explanation.

Thus, the objective of this work was to assess the use of adsorbents during $scCO_2$ extraction of bioactives from *N. oleoabundans* paste. Particularly, we are interested in extraction of carotenoids and its selectivity toward different adsorbents. In light of a potential industrial or analytical application, the adsorbent selection criteria will consider to be low cost, easily available, and non-toxic.

2. Materials and methods

2.1. Samples and pretreatment

N. oleoabundans (UTEX#1185) was obtained from the culture collection of algae at the University of Texas (Austin, TX, USA). Batch cultures were grown in 20-cm-wide glass reactors containing 35 L of modified Bold's basal medium [25] supplemented with 6 mM of KNO_3 and subjected to continuous stirring by bubbling air through the mixture at a constant flow rate. Pure CO_2 was supplied every 30 s at 10-min intervals to the air stream to provide inorganic carbon and to maintain the pH at 8. This was achieved using an electronic gas-control valve (Wilkerson R03-C2). Reactors were maintained in a culture chamber at 25 ± 2 °C, with a 16:8 h light:dark photoperiod using fluorescent light (Philips TLD 58 W) at a photosynthetic photon flux density of 300 μmol photons per square meter per second. After the cells had reached the late exponential phase, biomass was harvested by centrifugation (7000 rpm for 5 min at 10 °C), frozen at −20 °C and stored under dry and dark conditions until further use.

Frozen *N. oleoabundans* paste was thawed at room temperature and cryogenically ground as described by Castro-Puyana et al. [19] with minor modifications: 28 g of microalgae paste was treated with three cycles of cryogenic grinding using a Retsch CryoMill mixer mill (Haan, Germany). Each consecutive cycle consisted of a precooling step (frequency 5/s for 2 min), grinding (frequency 20/s for 3 min), and intermediate cooling (frequency 5/s for 1 min). The sample was withdrawn and stored at −20 °C under dry and dark conditions until further use.

2.2. Analytical methods

As benchmark, conventional acetone extraction was carried out to determine the total extractable compounds in *N. oleoabundans* paste. The method used was the one previously reported by Castro-Puyana et al. [19], with minor modifications. A sample of 200 mg equivalent microalgae substrate (0.7 cm^3 of microalgae paste) was mixed with 20 cm^3 of HPLC-grade acetone (LabScan, Gliwice, Poland) containing 0.1% (w/v) butylated hydroxytoluene (Sigma–Aldrich, Saint Louis, MO, USA) in a 50-cm^3 Falcon protected from light using aluminum foil. The mixture was shaken for 24 h in an agitated thermostatic bath (Selecta, Barcelona, Spain) operating at 500 rpm and 20 °C. After extraction, the exhausted substrate was precipitated out in a refrigerated centrifuge (Hettich, Tuttlingen Germany) operating at 10,000 rpm and 4 °C for 10 min. Following centrifugation, the supernatant was collected, and solvent was removed by a gentle blow of technical N_2 as done for the extracts in $scCO_2$ extractions. Dry acetone extracts were weighed in a SI-234 analytical balance (Denver Instruments, Bohemia, NY, USA, 0.1 mg accuracy) and stored at −18 °C until further use. Benchmark extractions were quadruplicated.

HPLC analysis was performed in an Agilent HP 1100 Series (Palo Alto, CA, USA) apparatus equipped with a diode array detector (DAD) using the method of Castro-Puyana et al. [19]. Carotenoids separation was performed in a YMC-C30 reversed-phase column 250 mm length × 4.6 mm (inner diameter), 5-μm particle size (YMC Europe, Schermbeck, Germany). The mobile phase was a mixture of methanol, methyl *terc*-butyl ether (MTBE) both from LabScan (Gliwice, Poland), and water purified using a Milli-Q system (Millipore Corporation, Billerica, MA). Solvent A, which was a mixture of methanol–MTBE–water (90:7:3 v/v/v), and solvent B, a mixture of methanol–MTBE (10:90 v/v), were eluted according to the following gradient: 0 min, 0% solvent B; 20 min, 30% solvent B; 35 min, 50% solvent B; 45 min, 80% solvent B; 50 min, 100% solvent B; 52 min, 0% solvent B. The flow rate was adjusted to 0.8 cm^3/min and the injection volume was 20 μL. Detection was at 450 and 660 nm with a recorded spectra between 240 and 770 nm by diode-array detection (DAD). Calibration curves were prepared using solutions in acetone of lutein (ranging from 2.5×10^{-5} to 0.1 mg/cm^3), cantaxanthin (1.25×10^{-3}–0.02 mg/cm^3) from Sigma–Aldrich (Saint Louis, MO), and astaxanthin monopalmitate (9.4×10^{-4}–7.5×10^{-3} mg/cm^3)