Study on the microalgal pigments extraction process: Performance of microwave assisted extraction

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ABSTRACT

The performance of microwaves irradiation (MAE and VMAE) to extract pigments from two marine microalgae was compared to conventional processes (cold and hot soaking and ultrasound-assisted extraction). Pigments were quantified by RP-HPLC and extraction performance was assessed regarding rapidity, reproducibility and extraction yields. Scanning electron microscopy was used at all extraction steps to assess the impact of the process on microalgal cell integrity. Freeze-drying and pigments extraction preserved microalgae cell integrity (except sonication) and evoked agglutination in superposed cells layers. All processes performed on *Dunaliella tertiolecta* (chlorophyte) lead to rapid pigments extraction, and equivalent pigments extraction yields, the absence of frustule allowing immediate solvent penetration in microalgae cells. In contrast, presence of the frustule in the diatom *Cylindrotheca closterium* (bacillariophyte) constituted a mechanical barrier to pigment extraction. MAE was identified as the best extraction process for CC pigments as it combined rapidity, reproducibility, homogeneous heating and high extraction yields.

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1. Introduction

Marine microalgae constitute one of the most diverse group of oceanic microorganisms, with an estimated 2×10^5 to several millions species [1], from which only 35,000 are described. Extensive screening of cultivated species has lead to the isolation and chemical determination of over 15,000 compounds, including fatty acids, sterols, phenolic compounds, terpenes, enzymes, polysaccharides, alkaloids, toxins and pigments [2]. Because of their high biodiversity and huge productivity, microalgae represent an untapped resource offering great possibilities for the isolation of original natural substances of interest for food, health or biotechnological applications [3,4]. Their interest also lies in their convenient use as a biotechnological biomass, as they can easily be grown in controlled conditions, handled as conventional lab microorganisms, and genetically modified without any risk of dissemination.

Lipids and pigments extraction processes applied to microalgae are mainly derived from phytochemical techniques developed on superior plants and macroalgae. The main parameters driving selection of an extraction technology are biochemical characteristics of extracted molecules, rapidity, limitation of solvent use, reproducibility, extraction yield, selectivity, protection of extracted molecules against chemical transformation, dimension, cost and easiness [5,6]. Classical organic solvent extraction techniques, including maceration (soaking), percolation, countercurrent extraction, pressurized liquid extraction, and soxhlet are widespread technologies described to extract lipids and pigments. These processes are reproducible, allow the rapid extraction of chemicals, but usually imply the use of large amounts of solvents, and the risk of thermal denaturation or transformation of molecules of interest [5]. Coupling steam distillation or hot water extraction with maceration in solvent increases extraction yields for plant essential oils and bioactive compounds [7], but thermolabile molecules are damaged using this technology. The use of enzymes, such as xylanases, pectinases or cellulases, to enhance pigments extractability rates was proposed and validated for superior plants tissues [8,9] and macroalgae [10], and

could be of interest for unfrustulated microalgae. Various techniques limiting chemical transformation of pigments have been proposed. Samples can be frozen (−80 °C, liquid nitrogen), freeze-dried, dessicated, or stored in water vapour saturated atmospheres [11] to avoid oxidation and thermal denaturation. Maceration in liquid nitrogen followed by buffer extraction, consisting in pigment precipitation in 50% ammonium sulphate, was described for the cyanobacterial blue pigment phycocyanin. However subsequent dialysis and gel filtration chromatography steps are needed to desalt pigments solutions [12]. Extraction in aqueous solutions implies a strict control of the pH as many porphyrin pigments can undergo chemical transformations in acidic or alkaline conditions. For instance, chlorophylls can undergo epimerization, dephytylation and demetallation (pheophytination) [13]. The presence of chlorophyllases in microalgae extracts also explains the rapid degradation of porphyrin pigments [14,15]. Changing water to selected solvents, such as sec-butanol, allows a rapid and efficient extraction from plants and microalgae with a good stability of pigments [16]. This solvent can also be favourably used to concentrate pigments extracted with acetone [16]. Hejazi and colleagues proposed a biocompatible strategy to extract carotenoids from *Dunaliella salina* without killing the cells, using solvents having a Log *P* (octanol) > 6 [17]. Other cell disruption techniques based on mechanical or osmotic shocks, such as bead beating and ultrasound assisted extraction have been proposed [18]. Mechanical treatments are very drastic, and usually induce thermal denaturation of molecules of interest, especially when using an ultrasonic sharp probe [13]. Osmotic shock may not be efficient when working with frustulated species or armoured dinoflagellates. The use of supercritical carbon dioxide (SCF-CO$_2$) or combination of solid-phase extraction with SCF-CO$_2$ to extract lipids, pigments or bioactive compounds from plants [19,20] or microalgae [21–27] was also described as a very efficient technology, in spite of a high cost. For instance, paprika [28] and *Lithospermum* pigments are easily and efficiently extracted and purified using SCF-CO$_2$, in optimized conditions favouring differential solubilization in CO$_2$ [19].

In the last 10 years, there has been an increased interest in using extraction techniques involving microwaves. MAE and VMAE have been proposed as efficient and rapid processes to extract antioxidants [29] or pigments from plants [30] or spices [19], oils from vegetables [31], allowing reduced solvent consumption and shorter extraction times, with equivalent or higher extraction yields. MAE and VMAE have been applied to marine microalgae to extract lipids [32], and allowed the highest recovery for all tested species compared to autoclaving, bead-beating, sonication or maceration in 10% NaCl solution. Choi and colleagues [33] reported the microwave-assisted extraction of astaxanthin from the red yeast *Xanthophyllomyces dendrorhous* and showed that combining a microwave irradiation to destroy cell walls with a solvent extraction allows a good pigment recovery [33]. Paprika pigments were also efficiently extracted with high yields using microwave irradiation, with a selectivity and efficacy depending on the dielectric constant of the extraction mixture [34]. The combination of sonication and microwaves was studied to extract lipids from vegetables and microalgae sources. Ultrasonication alone, microwave irradiation alone or combination of both techniques gave excellent extraction efficiencies in term of yields and time, with a 10-fold reduction in the time needed with conventional methods, and yields increased from 50 to 500% [31]. The different studies mentioned above suggested that microwaves could be of interest to extract pigments from microalgae. We report here data obtained on the utility and performance of microwave-assisted extraction of pigments from the microalgae *Cylindrotheca closterium* and *Dunaliella tertiolecta*, compared to soaking in solvents and ultrasonic extraction.

2. Experimental

2.1. Selection of microalgae species for pigments extraction

Two model species were studied. *C. closterium* (*CC*) belongs to the bacillariophyceae class (diatoms), which covers the most abundant and best known unicellular planktonic algae species. The characteristic feature of diatoms is the presence of a box-like silica wall or *frustule* surrounding the cell. The frustule is composed of two main components: an outer organic coat, and a complex inner wall made of polymerized hydrated silica [13]. *CC* is a pennate (pen-shaped) diatom, exhibiting a fusiform shape, with long spines on both ends and a total length of 40–90 μm [35]. The two main pigments described in *CC* are chl *a* and fucoxanthin. *D. tertiolecta* (*DT*) belongs to the chlorophyceae class (green microalgae). Species from the genus *Dunaliella* exhibit a small ovoid unfrustulated cell (<20 μm), covered of cellulose, xylans, mannans and/or glycoproteins, with two equal flagella inserted at the apexes of the cell [13]. Chl *a*, chl *b* and β,β-carotene are the three major pigments described in *DT*.

2.2. Microalgae culture, collection and storage

Microalgae were grown at IFREMER PBA, Nantes. *CC* strain AC170 (Algo-banque, France) was cultivated in 2.2 l tubular photobioreactors under constant illumination (120 μmol m^{-2} s^{-1}), at 21 °C in continuous culture maintained in exponential growth phase, with pH regulation at 7.6, in Walne (Conway) medium diluted in 0.22 μm sterile-filtered natural seawater. The *CC* cell suspension was harvested and cells were separated from culture medium by sedimentation and soft centrifugation (3500 g, 15 min, 4 °C). *DT* strain CCMP364 (CCMP, USA) was grown in 10 L flasks under a photoperiode of 16 h illumination and 8 h darkness at an average light intensity of 180 μmol m^{-2} s^{-1}. Growth was performed at 20 °C, in pH unregulated batch culture, in Walne (Conway) medium diluted in 0.22 μm sterile-filtered natural seawater. The *DT* cell suspension was harvested at the end of the exponential growth phase, and cells were separated from culture medium by soft centrifugation (4000 g, 20 min, 10 °C). Cells were frozen at −20 °C and sent to lab LIENSs, La Rochelle. Microalgae were freeze-dried at −55 °C and P < 1 hPa, on a freeze-dryer equipped with an HetoLyoPro 3000 condenser and Heto cooling trap (Therma Electron Corporation, France). All extractions were performed on freeze-dried biomass to calculate extraction yields in micrograms of pigments per mg of dry microalgae.

2.3. Pigments extraction

Extractions were performed in triplicate independent assays on 50 mg freeze-dried cells suspended in 30 ml acetone, in a 50 ml Erlenmeyer flask (room temperature (Rt) soaking and UAE) or in a 100 ml round bottom glass flask (hot soaking, MAE and VMAE). All extractions were performed in obscurity and under argon atmosphere (except VMAE) to limit chlorophyll photo-oxidation. Rt soaking extractions were performed for 10–120 min, at 20 °C, under magnetic stirring. Hot soaking extractions were performed in the same conditions at acetone boiling temperature at atmospheric pressure (56 °C), under reflux. Microwave assisted extraction (MAE) and vacuum microwave assisted extraction (VMAE) were performed using a CEM Discovery Microwaves Reactor. For VMAE, the flask was placed in the reactor, connected to a vacuum pump via a condenser (median pressure during VMAE = 26,697 ± 1144 Pa) and microalgae were irradiated for 3–15 min, at 25–100 W, under magnetic stirring. Temperature was stabilized at the acetone boiling temperature at VMAE pressure. For MAE the procedure was the same as VMAE, but under atmospheric pressure, at 56 °C. UAE was performed in usual conditions described in most extracting processes, on samples thermostated in an icewater bath, to limit overheating due to the local hot temperature at the tip of the ultrasonic probe. Ultrasounds were continuously applied for 3–15 min at 4.3–12.2 W (maximal power supplied by the sonicator). Median sample temperature measured at the end of the UAE process was 8.5 °C.

2.4. Pigments concentration, identification and dosage

Extracts were filtered onto 0.2 μm PVDF membrane filters to remove cells and cell debris that were further characterized by scanning electron microscopy. Pigments solutions were dried using a rotary evaporator under vacuum at 40 °C. The dry extracts were solubilised in 5 ml acetone for immediate RP-HPLC analysis. The RP-HPLC system was composed of a binary pump (Waters, W600), an autosampler (Waters, W717), a thermostated (20 °C) column compartment, and a photodiode-array detector (Waters, W486). Pigments were separated in triplicate independent assays on a Phenomenex Luna C18 (2) column (250 mm × 4.6 mm, 10 μm), the mobile phase consisting of a ternary gradient of solvent A (methanol/water (80/20)); solvent B (acetonitrile/water (90/10)) and solvent C (ethyl acetate). The gradient flow program was adapted from Jeffrey and collaborators [13], as follows: 0 min – 100% A, 3 min – 100% B, 35 min – 30% B and 70% C, 38 min – 100% C, 41 min – 100% C, 43 min – 100% B, 45 min – 100% A. The flow rate was 1.0 ml min^{-1} and pigments elution was monitored at 435 nm. The injection volume was 1–50 μl for standard pigments and 5 and 10 μl for extracts. Pigments were identified on the basis of their retention time compared with standards. The extraction yield for each pigment was calculated using the calibration curve obtained with standards and expressed in
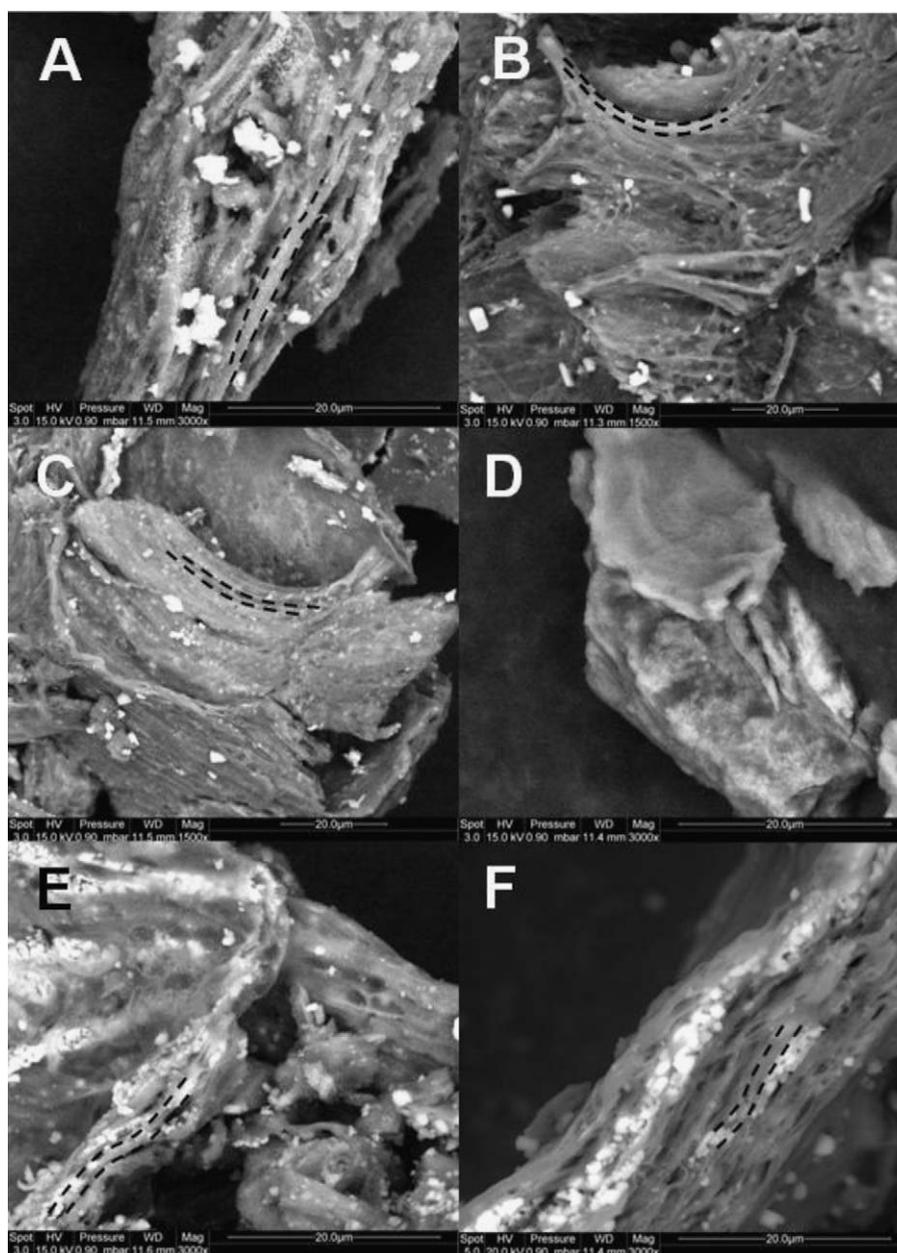
Fig. 1. SEM observation of *CC* after freeze-drying (A) or freeze-drying followed by hot soaking (B), Rt soaking (C), UAE (D), MAE (E), or VMAE (F). Salt crystals formed during pH regulation of the cell culture medium and precipitated during freeze-drying and pigment extractions were observed in most *CC* samples. Black hyphens underline the layers formed by *CC* cells accumulation.

µg of pigment per mg of dry biomass. Performance of the extraction process was assessed calculating pigments extraction yields (chl *a* and fucoxanthin for *CC*; chl *a*, chl *b* and β,β-carotene for *DT*). As a control to assess the impact of sterilizing filtration on pigment extraction, 50 mg of freeze-dried *CC* or *DT* cells were placed on the filtration membranes and pigments were extracted filtrating 30 ml acetone onto the cells. Samples were dried, solubilized in 5 ml acetone, and considered as soaking extracts at 0 min and 20 °C.

2.5. Scanning electron microscopy (SEM)

Microalgal cell integrity was studied on freeze-dried cells and after each filtration step following the extraction processes. Cells were placed on a conductive double layer carbon support and examined by scanning electron microscopy using a Philips-FEI Quanta 200 ESEM/FEG microscope (environmental mode) equipped with a FEG canon delivering 1–30 kV beam current.

2.6. Chemicals and standards

Acetone, methanol, acetonitrile and ethyl acetate were purchased from Carlo-Erba, France. All solvents used in this study were HPLC grade. β,β-carotene, chl *a* and chl *b* were obtained from Sigma Aldrich, France. Fucoxanthin and pheophytin *a* were obtained from DHI Lab Product, Denmark. Ultra-pure water was obtained using a Milli-Q system (Millipore, France).

3. Results

3.1. Microalgal cell integrity

SEM observation of *CC* revealed that cells stuck together and deposited in superposed layers during freeze-drying (Fig. 1A, black hyphens). Salt contained in the culture medium also precipitated as white clusters associated to cells layers (Fig. 1A). Pigments extraction steps had no effect on cell integrity, except in the case of UAE, that destroyed cells and induced the formation of a homogeneous cell paste (Fig. 1D). SEM observation of *DT* cells revealed that freeze-drying conserved the cell shape and induced flattening into confluent homogeneous layers (Fig. 2A). Pigments extraction
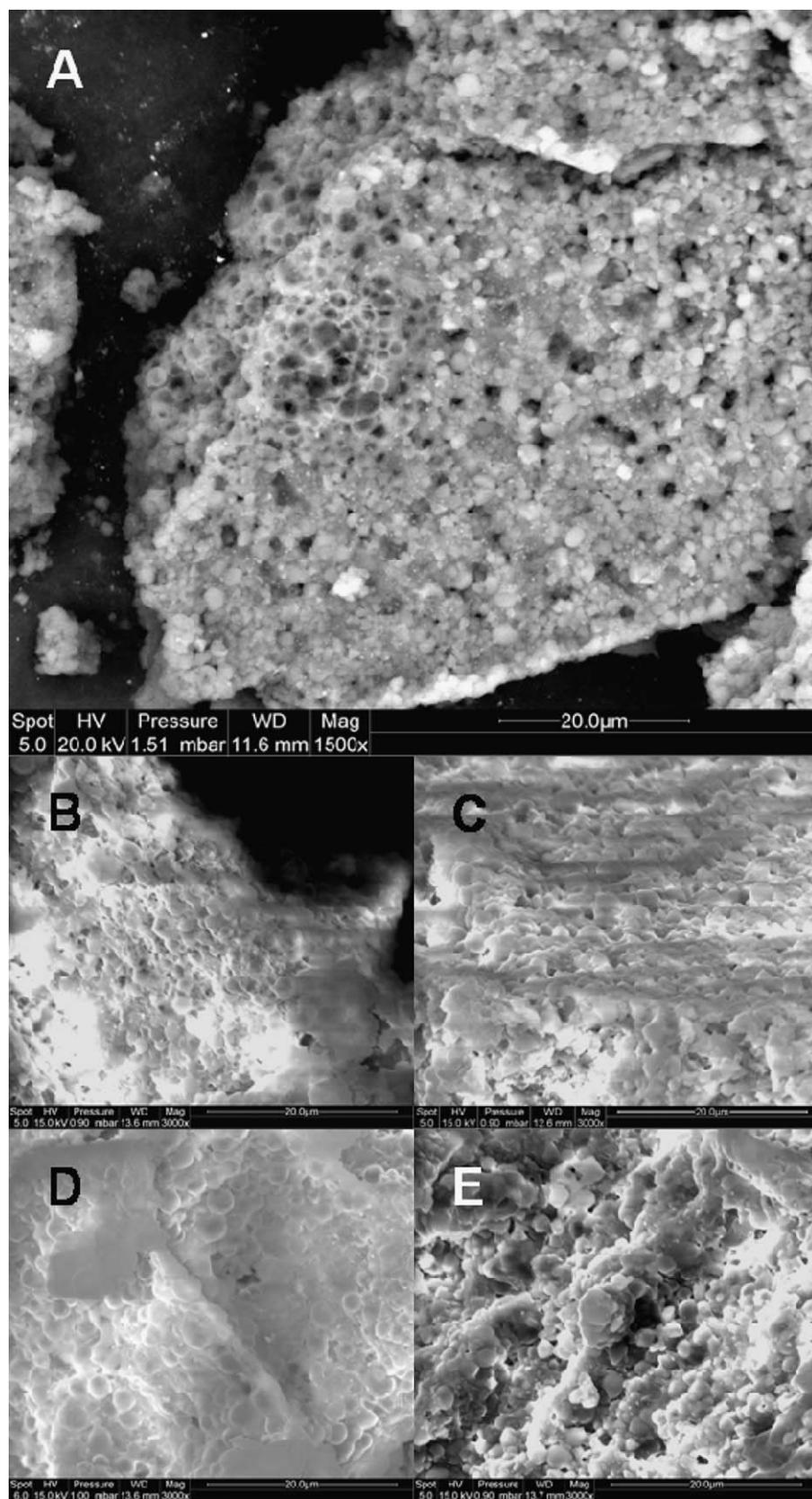
Fig. 2. SEM observation of *DT* after freeze-drying (A) or freeze-drying followed by Rt soaking (B), UAE (C), MAE (D), or VMAE (E). Cells flattening into confluent homogeneous layers were observed in all samples.

processes had no influence on cell shape, except sometimes junction of neighbour cell membranes or conglomeration of cell debris between cell membranes.

3.2. Pigments content

Fig. 3 presents the chromatograms obtained after *CC* and *DT* pigments extraction by Rt soaking in acetone. As expected, chl *a* and fucoxanthin were the two major pigments detected in *CC* extracts at 435 nm. β,β-carotene and pheophytin *a* were present in a too weak concentration to be dosed accurately by peak integration on chromatograms. Chl *a*, chl *b* and β,β-carotene were identified as the three major pigments in *DT* extracts. As expected, no fucoxanthin was detected in *DT* chromatograms. The peaks identified by a retention time between 17 and 23 min corresponded to minor xanthophylls described in *DT* (neoxanthin, violaxanthin and lutein).

3.3. Pigments extraction yields and kinetics, pigments degradation

3.3.1. Cold and hot soaking

Pigment extraction from *CC* was time-dependent at Rt and the yield increased steadily (Fig. 4A). The best yields were obtained after 60 min for chl *a* (7.48 ± 0.21 µg mg^{-1}) and 120 min for fucoxanthin (5.34 ± 0.06 µg mg^{-1}). Degradation of chl *a* after 60 min was obvious as the yield of chl *a* recovered after 120 min was lower than that at 60 min. In contrast, no fucoxanthin degradation was measured, indicating a good stability in the extraction conditions. *DT* soaking in acetone at Rt allowed a rapid extraction of chl *a*, chl *b* and β,β-carotene (Fig. 4C). The maximal extraction yields were reached instantaneously for chl *b* and β,β-carotene (1.50 and 1.20 µg mg^{-1}, respectively), and after 10 min for chl *a* (4.60 µg mg^{-1}). Heating the soaking suspension accelerated pigments extraction as most *CC* pigments were extracted in 30 min, with a higher extraction yield for chl *a* in twice as less time (9.31 ± 0.44 µg mg^{-1} in 30 min), and an equivalent yield in twice as less time for fucoxanthin (5.23 ± 0.06 µg mg^{-1} in 60 min) (Fig. 4B). However, heating the soaking suspension evoked chl *a* degradation in *CC* and *DT* beyond 30 min as demonstrated by the decrease of extraction yields (*CC*, Fig. 4B; *DT*, data not shown).

3.3.2. Microwaves-assisted extraction at atmospheric pressure (MAE)

MAE of *CC* at 50 W (Fig. 5A) allowed total extraction of fucoxanthin in 3–5 min, with a maximal extraction yield (4.24 ± 0.09 µg mg^{-1}), equivalent to the yield obtained after 60 min Rt soaking (4.68 ± 0.10 µg mg^{-1}) or hot soaking (5.23 ±
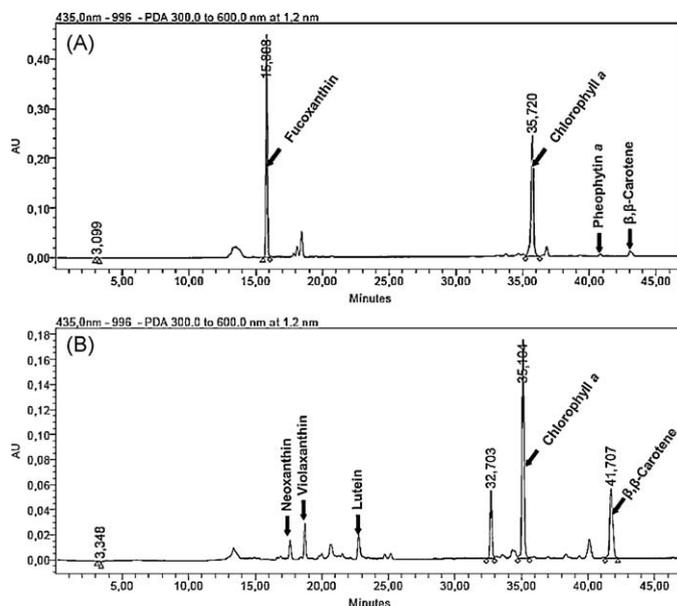

Fig. 3. RP-HPLC chromatograms at 435 nm of acetone extracts of *CC* (A) and *DT* (B). For each species, identical chromatograms were obtained, whatever the pigment extraction process used (data not shown).

0.06 µg mg^{-1}). Increasing irradiation time or power had no impact on the fucoxanthin extraction yield, demonstrating that all extractable fucoxanthin was recovered in 5 min and not damaged by microwave irradiation. The highest chl *a* extraction yield was reached after only 5 min of MAE (8.65 ± 0.29 µg mg^{-1}) when 60 min and 30 min were respectively necessary for Rt soaking (7.48 ± 0.21 µg mg^{-1}) and hot soaking (9.31 ± 0.44 µg mg^{-1}). Increasing irradiation time beyond 5 min, as well as increasing irradiation power to values superior to 50 W, lead to chl *a* degradation as indicated by the drastic decrease of extraction yields (Fig. 5B). As expected, MAE performed at 50 W on *DT* allowed instantaneous extraction of pigments (Fig. 6).

3.3.3. Vacuum microwaves-assisted extraction (VMAE)

Fig. 7 present the VMAE of chl *a* and fucoxanthin from *CC*. VMAE allowed a rapid extraction of fucoxanthin and chl *a* (best extraction yields measured after 5 min irradiation). As observed with MAE, increasing the irradiation power beyond 75 W resulted in chl *a* degradation. VMAE was less efficient than MAE to extract chl *a* and fucoxanthin, as increasing the irradiation power to 75 W did not allow to obtain equiva-
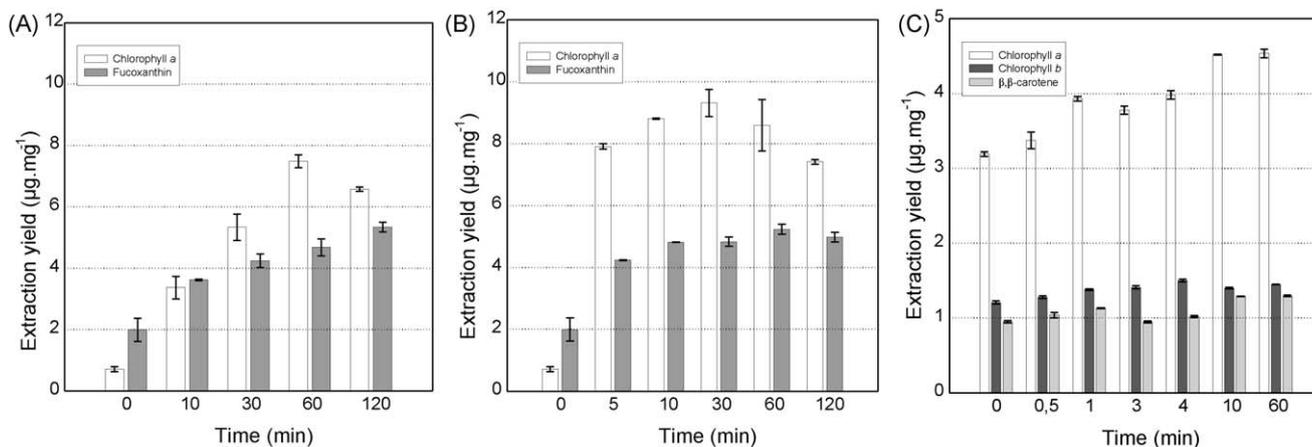

Fig. 4. Pigments extraction kinetics. Rt soaking of *CC* cells (A), hot soaking of *CC* cells (B) and Rt soaking of *DT* cells (C).
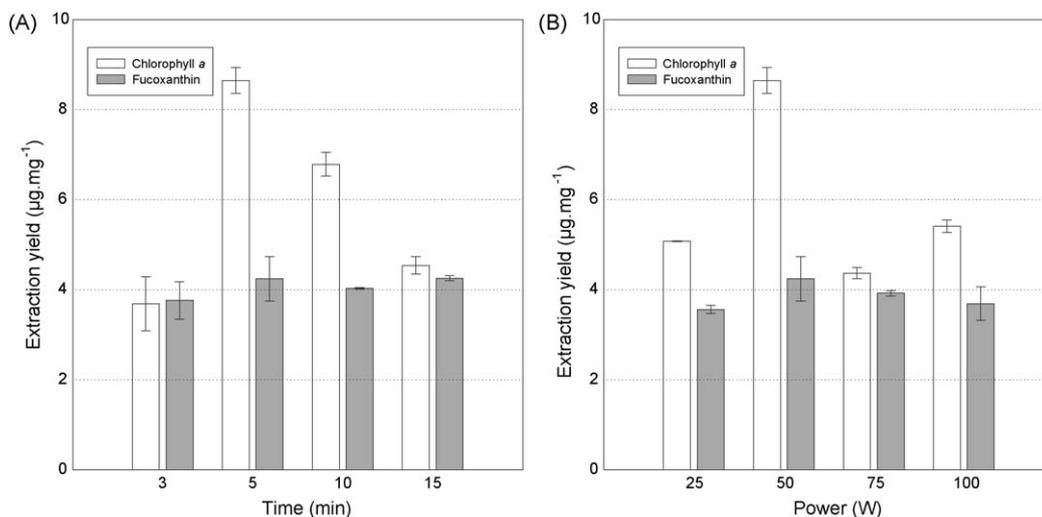
Fig. 5. (A and B) Influence of MAE duration (power = 50 W) and irradiation power (duration = 5 min) on the extraction yields of *CC* pigments.

lent extraction yields (chl *a*: $5.25 \pm 0.04\,\mu g\,mg^{-1}$ for VMAE and $8.65 \pm 0.29\,\mu g\,mg^{-1}$ for MAE; fucoxanthin: $3.68 \pm 0.05\,\mu g\,mg^{-1}$ for VMAE and $4.24 \pm 0.09\,\mu g\,mg^{-1}$ for MAE). VMAE performed at 50 W induced instantaneous extraction of *DT* pigments (Fig. 6).

3.3.4. UAE

Figs. 8 and 6 present the UAE performed on *CC* and *DT*, respectively. Sonication of *CC* at 12.2 W (maximal power supplied by the sonicator) and 8.5 °C under atmospheric pressure allowed a maximal extraction of fucoxanthin in 3–10 min, with an extraction yield ($4.49 \pm 0.08\,\mu g\,mg^{-1}$) in the range of values obtained by Rt soaking for 60 min ($4.68 \pm 0.10\,\mu g\,mg^{-1}$) or MAE for 5 min ($4.24 \pm 0.09\,\mu g\,mg^{-1}$). Extraction of chlorophyll *a* was also maximal after 3–10 min sonication, but with

an extraction yield of $4.95 \pm 0.27\,\mu g\,mg^{-1}$, much lower than the values obtained by the other processes (Rt soaking in acetone for 60 min: $7.48 \pm 0.21\,\mu g\,mg^{-1}$; hot soaking in acetone for 30 min: $9.31 \pm 0.44\,\mu g\,mg^{-1}$; MAE: $8.65 \pm 0.29\,\mu g\,mg^{-1}$; VMAE: $5.25 \pm 0.04\,\mu g\,mg^{-1}$). Variability of the extraction yields of fucoxanthin and chlorophyll *a* was important between each extraction assay, as demonstrated by important errors bars in Fig. 8. UAE performed on *DT* cells induced instantaneous extraction of *DT* pigments with yields equivalent to the other processes (Fig. 6).

4. Discussion

In this study, acetone was chosen as a model extraction solvent as it extracts most photosynthetic pigments, in a wide range of polarity, and acetone 90% is recommended for phytoplankton pigment analysis [13,36,37]. However, pigment extraction was performed in acetone 100% to limit chl *a* hydrolysis by the thylakoid-bound enzyme chlorophyllase, present in most diatom species and chlorophyceae [38], activated during extraction even before solvent extraction (cell harvesting and filtration), and still active to a small extent in 90% acetone [14,39]. Chlorophyllase activity was previously reported to be low in *CC*, with 0–5% chlorophyllide *a* formed as percentage of total chl *a* when pigment extraction was performed in 50% acetone at 20 °C, and high in *DT*, with 12–79% chlorophyllide *a* formed in the same conditions [38]. The good extraction yields obtained in acetone allowed a relevant comparison of the processes performances, in spite of absence of selectivity. In order to improve selectivity of the process towards a selected pigment, MAE and VMAE could be performed in others solvents allowing a more selective solubilization of apolar (chlorophylls, carotens) or polar (xanthophylls, phycobiliproteins) pigments. Also, MAE and VMAE could be performed in mixtures of different solvents, in solvents authorized by regulations to extract pigments for nutritional purposes (e.g. carotenoids), or in apolar solvents such as hexane mixed with weflon®.

This study suggests that microwaves-assisted processes have weak utility for pigments extraction from species lacking frustule and thick outer exopolysaccharide envelope, as conventional techniques exhibit equivalent efficiency in term of yields, rapidity and protection of extracted molecules against chemical transformation. It seems indeed that freeze-drying is sufficient to weaken the cell membrane and access pigments for solvent solubilization in these species. In contrast, MAE is very efficient when a
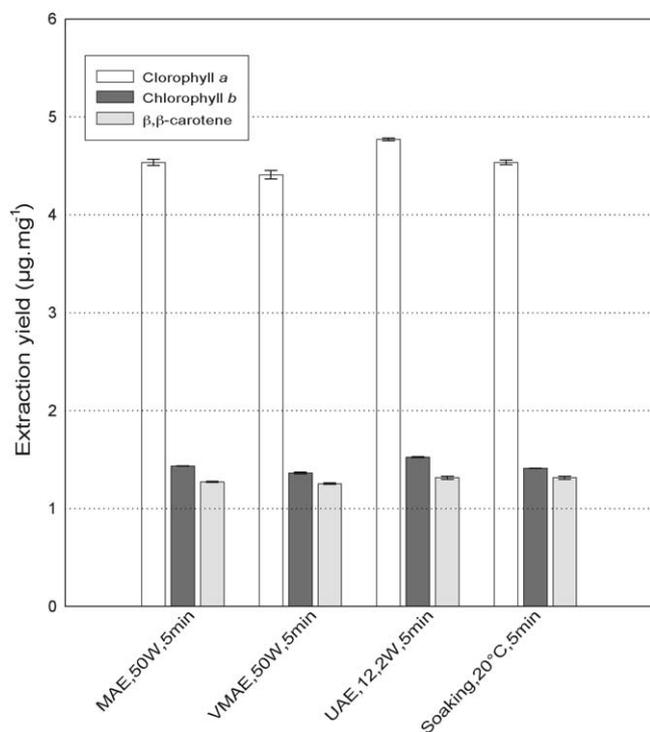

Fig. 6. Comparison of *DT* pigments extraction yields obtained with MAE, VMAE, UAE and cold soaking.
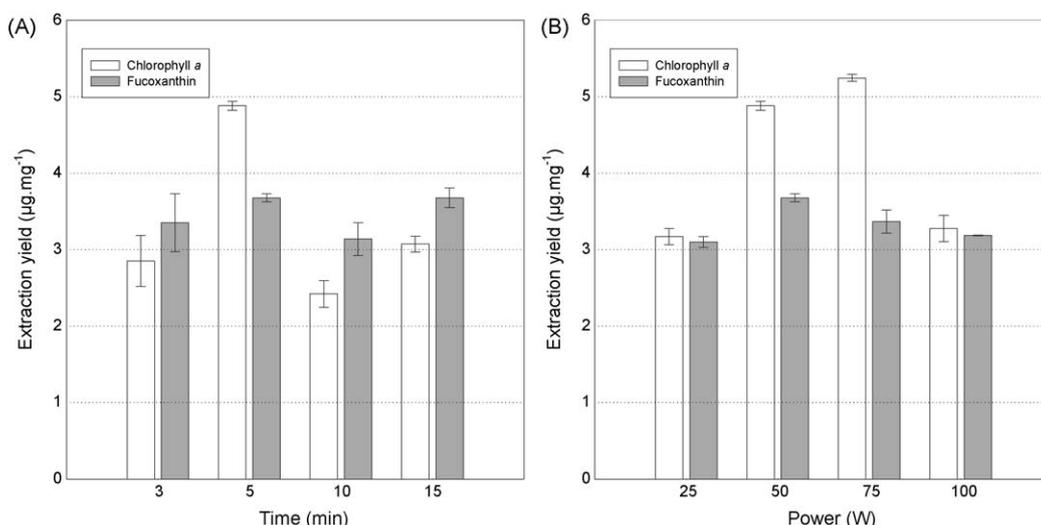
Fig. 7. (A and B) Influence of VMAE duration (power = 50 W) and irradiation power (duration = 5 min) on the extraction yields of *CC* pigments.

mechanical resistance such as frustule in diatoms limits solvent access into the cells. In this view, MAE could also be useful to extract pigments from unfrustulated microalgae synthesizing a thick exopolysaccharide envelope, such as species from the genus *Porphyridium*, or from armoured species dinoflagellates for which pigments extraction is very difficult [13]. Compared to conventional extraction methods, the use of microwaves accelerated diatom pigments extraction kinetics, as extraction yields usually reached after 30–60 min using Rt and hot soaking were obtained in a few minutes using MAE. This excellent result suggests that high extraction yields could still be obtained increasing the ratio biomass/solvent, thus limiting the volume of solvent to be handled. The fact that MAE and VMAE are very fast extraction processes performed in highly controlled temperature conditions probably accounts for pigments protection from chemical transformation. Also, we observed that pigments extraction yields and reproducibility of the process were strongly dependent upon temperature regulation in the extraction medium. Compared to conventional processes, microwaves-assisted extraction processes offer the advantage of a homogeneous thermoregulation of the medium, as no heat transfers are required to heat cells located in the center of the flask, and no hot spots exist as in UAE. Microalgal organites are also homo-

geneously heated, allowing synchronous pigments extraction, whatever their subcellular localization (cytoplasmic or chloroplastic). In this study, no degradation of fucoxanthin was observed during microwave irradiation or hot extraction at 56 °C, and the degradation of other minor xanthophylls was not studied. Degradation of carotenoids during microwave heating usually starts at temperatures superior to 60 °C for the most thermosensitive molecules (violaxanthin and antheraxanthin) while provitamin A carotenoids (β-carotenes, α-carotenes and β-cryptoxanthin) as well as lutein are more heat-stable [40–42]. It can thus be considered that MAE temperature should not exceed 60 °C to ensure carotenoids extraction in non-denaturing conditions. Assessment of the frustule integrity after MAE and VMAE demonstrated that microwave irradiation did not evoke frustule disruption as observed in UAE. Microwaves-assisted processes can thus be considered as a "clean" processes inducing frustule permabilization but avoiding frustule explosion. MAE presents the advantage of allowing fast and efficient pigment extraction without the need of high pressures to obtain important yields, as in SCF processes, which however have also the high interest of allowing pigments extraction in toxic solvent-free medium. Considering that efficiency of a pigment extraction process depends upon its selectivity, rapid-
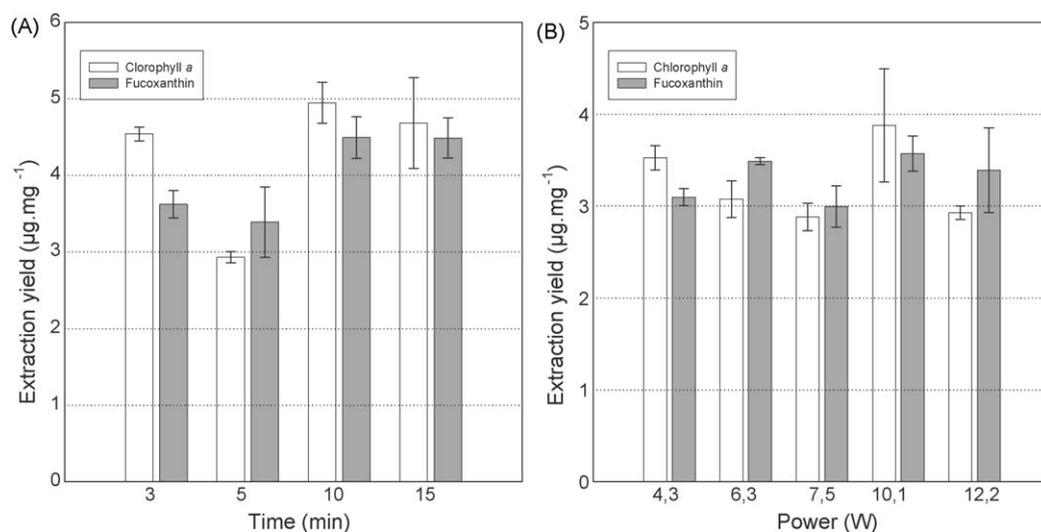

Fig. 8. (A and B) Influence of UAE duration (power = 12.2 W) and irradiation power (duration = 5 min) on the extraction yields of *CC* pigments.

ity, yield, reproducibility, and protection of extracted molecules against chemical transformation, MAE appears as a relevant and innovative process to extract pigments in labs studying photosynthetic pigments or performing routine spectrofluorimetric or spectrophotometric pigments dosages. Also, the development of microwaves pilot units and industrial microwaves reactors allows to consider a possible use of MAE to extract pigments of interest at industrial scale or in particular conditions (e.g. oceanographic ships sampling seawaters), overcoming the limits of traditional techniques in term of quality or profitability of the extracts. This first study on the performance of microwaves-assisted processes to extract major pigments from frustulated and unfrustulated microalgae suggests that optimizing the extraction parameters may be necessary to efficiently purify selected pigments present in a particular species. It will be of interest to establish whether microwaves-assisted processes have also interest to extract minor pigments present in microalgae and other pigments present in various marine organisms.

5. Conclusive remarks

The freeze-drying process and pigments extraction steps preserved microalgae cell integrity and evoked agglutination in superposed layers, except UAE. Subsequently the success of pigments extraction from freeze-dried microalgae may mostly depend on solvent diffusion across the cells, pigments solubility, and thermodynamical parameters. Acetone extracted all major and minor pigments described in the two microalgae species, in a wide range of polarity. Cold soaking was a time-consuming process to extract pigments from the model diatom CC, did not allow a total extraction and induced chl a degradation. In contrast, cold soaking was efficient, simple and rapid to extract pigments from the model chlorophyte DT, demonstrating that the use of more sophisticated processes are unnecessary. All other assays performed on DT confirmed that the absence of frustule allowed a fast and efficient solvent penetration and pigment extraction, whatever the extraction process studied. The absence of colour of DT cells and cell debris after filtration on PVDF membranes, confirmed that DT pigments extraction in acetone was total. Heating the soaking suspension accelerated CC pigments extraction, allowed a total extraction but was damaging for chl a after 30 min. The differences observed in between Rt and hot soaking extraction yields demonstrate that temperature is a crucial parameter determining the yield of microalgal pigment extraction. It is then important to accurately define the highest extraction temperature allowing a rapid extraction with high yields, without however damaging thermolabile pigments such as chl a. MAE drastically accelerated CC pigments extraction, and did not damage chl a if a short (≤5 min) and weak irradiation (≤50 W) was applied. In our conditions, MAE was considered as the best extraction process, as it combined rapidity, efficiency and protection against thermal denaturation. VMAE allowed a rapid and efficient extraction of chl a and fucoxanthin from CC, at moderate irradiation power (75 W), and was not degradative for chl a in soft conditions. However, it did not allow to reach extraction yields obtained with MAE, and was technically more complex than MAE as vacuum was needed. Except pressure, the main difference between VMAE and MAE was temperature, VMAE being performed at 22 °C and MAE at 56 °C. It is then obvious that a higher temperature favours pigments extraction, whatever the process considered, as long as pigments are not damaged. In spite of high extraction yields, the repeatability of UAE was insufficient to define a relevant and reproducible pigment extraction process. The weak chl a extraction yield obtained by UAE of CC was probably due to an insufficient extraction power in our conditions, as the high chl a extraction yield obtained after 5 min sonication of DT invalidated the hypothesis of chl a thermal degradation at the tip of the sonication probe.

Acknowledgements

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