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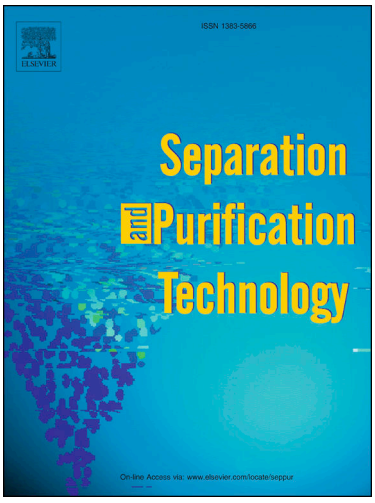


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# Recovery of pigments from *Ulva rigida*

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**Abstract**

Pigments, such as chlorophylls and carotenoids, have important applications in various fields, such as colorants in food, cosmetic or textile industries and in biomedical applications. Both pigments have an important role in the photosynthetic process and can be found in the marine green macroalgae genus *Ulva*. In this work, an integrated downstream process was developed to extract chlorophylls and carotenoids from those macroalgae. The solid-liquid extraction was optimized. For that, several conditions were tested, namely the use of different mechanical processes (maceration, microwave- or ultrasound- assisted extraction), type of solvent, number of consecutive extractions, solid-liquid ratio, and the design of the extraction process using a mechanical treatment. Using the extract obtained, a liquid-liquid extraction system composed of ethanol, hexane, and water was then studied. Different mixture points within the biphasic region were tested in terms of their ability to selectively separate chlorophylls and xanthophylls to opposite phases in a single step. The optimization and implementation of a simple, fast and efficient downstream process to separate both classes of pigments such as chlorophylls and xanthophylls from green macroalgae is reported. It could be applied to fractionate other extracts with similar compositions obtained from other natural sources.

**Keywords:** Green macroalgae, *Ulva rigida*, solid-liquid extraction, liquid-liquid extraction, chlorophyll, xanthophyll.

**Introduction**

Oceans contain nearly 200,000 identified species, but the actual number may be in the order of millions. Included in the organisms identified are the macro and microalgae. Although macroalgae are still an under-explored resource (1), over the past few years, the commercial application of algae-based products is gaining relevance in different fields. Seaweeds have been used since long as food (2), however, the markets are expanding and other commercial applications are envisioned (1,3,4). Included in the most valuable bioactive compounds are the pigments, namely phycobiliproteins, chlorophylls and carotenoids, with the latest two classes of pigments being the focus of this work.

Chlorophylls are photosynthetic pigments used by photoautotrophic organisms, such as plants and algae, to absorb light and to produce, in combination with the fixation of carbon dioxide, the carbohydrates needed for the growth of plants and algae (5). In green macroalgae, the most common types of chlorophylls present are the *a* and *b* (6). Structurally, chlorophylls are composed by a reduced porphyrin ring, with a central magnesium atom, and a long hydrophobic tail (phytol), which confers them low solubility in water (7). However these macrocycles can not be considered entirely hydrophobic due to the presence of ester and carbonyl polar functions in their structures (8). Chlorophylls and derivatives are used in the food industry as natural colorants in foods and beverages (9), however other important features are being reported, namely their antioxidant (10), anti-tumoral (11,12), and antimicrobial activities. A recent study proved the possibility of chlorophylls to be used as precursors of photosensitizers for photodynamic therapy, namely for cancer treatment and inactivation of microorganisms (13).

Carotenoids, divided into xanthophylls and carotenes (14), are another group of photo-pigments present in brown macroalgae and green algae, although in lower amounts (1,15). These photo-pigments absorb light at different wavelengths than those absorbed by chlorophylls, allowing the supplementation of the light captured by algae and in this way helping them to survive even at low (sun)light intensities (1). Among the carotenoids, xanthophylls due to the presence of polar functional groups (e.g. hydroxyl, carbonyl, carboxyl or epoxide) in the polyunsaturated hydrocarbon chain are less hydrophobic molecules than carotenes with no oxygen function. These pigments are even less hydrophobic than chlorophylls (miLog*P* around 9.8, whereas for fucoxanthin is around 8.5) (16).

Species from the genus *Ulva*, besides several other interesting compounds (3,17,18), present a significant content of chlorophylls and carotenoids, as recently found by Abd El-Baki et al. (19). However, despite their high commercial value and the increased consumer demand for natural products, few studies report simple and efficient purification processes able to recover both classes of pigments at high purity levels, as required by some of their applications.

Various studies have been reported considering the extraction and purification of pigments from macroalgae. In case of the pigments fractionation, paper (20), thin layer (21), and liquid chromatographic (22) techniques are often used. Nevertheless, some are delicate, expensive, and difficult to apply at an industrial scale. A US patent from 1946 (23) proposed a procedure to purify carotene from chlorophyll extracted from green leaves by the saponification of chlorophyll at high temperature after their extraction using organic solvents and an alkali salt. Then, by the addition of water a two-phase system is formed, in which the saponified chlorophyll was no longer soluble in

organic solvents but in the aqueous phase, promoting thus their separation from the carotene concentrated in the organic phase. Although this procedure is quite simple, the chlorophyll content obtained at the end of the process is not ready to be used. Moreover, if in one hand, the heating step increases the cost of the overall procedure, on the other hand, it may cause pigment degradation (23).

In this work, the extraction and separation of carotenoids and chlorophylls from the green macroalgae *Ulva rigida* was studied. A process in two steps was designed and adequately optimized considering: (i) the solid-liquid extraction of pigments, with the maximization of the chlorophylls yield, from the fresh biomass, followed by (ii) the purification of both classes of pigments using a liquid-liquid extraction system. In step (i), the parameters under optimization were the application or not of mechanical processes (maceration, microwave- or ultrasound- assisted extraction), type of organic solvent, the effect of consecutive extractions, the solid-liquid ratio, and the combination of mechanical-assisted methods to improve the action of the most efficient organic solvent. Step (ii) comprised the application of a liquid-liquid extraction system composed of two common organic solvents and water, in which the mixture point was the main parameter studied. After optimization, a low-cost downstream process, simple, efficient, and easily scaled-up, was designed. The process here proposed (patent CI-19-006) could have a crucial role in the improvement of aquaculture infrastructures by the transfer of marine technology, following the demands of Sustainable Development Goals to Oceans (*Conserve and sustainably use the oceans, seas and marine resources for sustainable development* - Goal 14).

**Experimental Section**

**Materials**

Fresh *Ulva rigida* was collected from March to June of 2016 (different batches) at a land-based integrated aquaculture system by ALGAplus Ltda, a company specialized in the production of marine macroalgae, located in Ílhavo, Portugal. ALGAplus farms *Ulva* sp. biomass at Ria de Aveiro lagoon (40°36'44.7" N, 8°40'27.0" W) in coastal Portugal under the EU organic aquaculture standards (EC710/2009). This aquaculture is performed in a land-based integrated multi-trophic aquaculture system (meaning that the nitrogen input is higher than in the outside natural lagoon due to the use of effluent water from fish production).

Several organic solvents were used on the extraction of pigments from the biomass. Ethanol, hexane, dimethyl sulfoxide, and acetonitrile were acquired from Fisher Scientific. Cyclohexane and dodecane were purchased from Sigma-Aldrich, while acetone was purchased from VWR™. Heptane and methanol were acquired from Labsolve and Chem Lab, respectively. All the mentioned chemicals used are HPLC-grade.

**Methodology**

**Standard methodology of solid-liquid extraction:** After the harvesting of the macroalgae, the samples were washed and stored in a freezer at -20°C until utilization. Frozen macroalgae samples were firstly grounded in liquid nitrogen (particle size < 0.5 mm) and homogenized in different pure organic solvents, in triplicate and with a solid-liquid ratio (SLR) of 0.01 $g_{algae}.mL_{solvent}^{-1}$. The extraction was performed in an incubator shaker (IKA KS 4000 ic control) at 250 rpm, for 30 minutes at 20 °C and protected from light exposure, being these initial conditions adopted from Martins et al.

(24). At the end of the solid-liquid extraction, the obtained green organic-based extract was centrifuged in a Thermo Scientific Heraeus Megafuge 16R Centrifuge at 4620 *g* for 30 minutes at 4 °C. The pellet was discarded while the green supernatant was collected. The absorption spectra were determined for each extract in the interval between 200-700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) and the chlorophyll concentration was calculated using a calibration curve previously determined.

**Microwave- and ultrasonic-assisted extractions:** Ungrounded algae samples were used to study the microwave- and ultrasonic-assisted extraction using a SLR of 0.01 $g_{algae}.mL_{solvent}^{-1}$ for different organic solvents. The microwave-assisted extraction was performed using a Milestone Microsynth MLS Ethos 1600 microwave at 300 W for 1 minute. The ultrasonic-assisted extraction was performed using a ultrasonic bath Sonorex Digitec DT 100 for 10 minutes. The mentioned conditions of extraction were adopted from Picot et al. (25) and adapted to avoid overheating of each system. Other times of extraction for microwave and ultrasonic-assisted extraction were not studied in this work. In future works this variable should be consider, but with small extraction times to avoid overheating. After both mechanical treatments, in which temperatures were carefully controlled to avoid passing 40 °C, the green solution was centrifuged in a Thermo Scientific Heraeus Megafuge 16R Centrifuge at 4620 *g* for 30 minutes at 4 °C. The pellet was discarded while the green supernatant was collected. The absorption spectra were determined for each extract in the interval between 200-700 nm in a UV-Vis microplate reader (Synergy HT microplate reader – BioTek) and the chlorophyll concentration was calculated using a calibration curve previously determined ($R^2$ = 0.9805).

**Pigments fractionation by a liquid-liquid extraction:** A liquid-liquid extraction was performed at room temperature until the equilibrium is achieved using a system composed of water + hexane + pigment-rich ethanolic extract. Different mixture points, covering the biphasic region (previously described by Moriyoshi et al. (26)) were studied. The content in pigments was determined for each phase by ultra-performance liquid chromatography - tandem mass spectrometer.

**Ultra-performance liquid chromatography - tandem mass spectrometer (UHPLC-MS) analysis:** The UHPLC-MS was performed in a Thermo Scientific LC-MS Ultimate 3000RSLC. The separation of the compounds was carried with a gradient elution program at a flow rate of 0.3 $mL.min^{-1}$, at 30 °C, by using a Hypersil Gold C18 column (150x2.1 mm; 5 µm, Thermo Fisher). The injection volume in the UHPLC system was 3 µL and the mobile phase consisted in formic acid 0.1 % (A) and acetonitrile (30):methanol (70) (B).

**Results and discussion**

In a first part of this section a solid-liquid extraction procedure to recover the pigments from *Ulva rigida* is proposed. Additionally, an effective and easy to scale-up liquid-liquid extraction is suggested to separate the chlorophylls and xanthophylls present on the extract.

**Solid-liquid extraction**

A screening of several organic solvents was performed to optimize the solid-liquid extraction of pigments and, particularly, chlorophylls from *Ulva rigida*. Included in the list of organic solvents tested are methanol, ethanol, hexane, dimethyl sulfoxide, acetonitrile, cyclohexane, dodecane, acetone, and heptane. These solvents were selected due to their different polarities and following some previous literature results obtained for the extraction of chlorophylls from spinach leaves (27).

Despite the interest in both classes of pigments, carotenoids and chlorophylls, the latest class was focused considering the solid-liquid extraction. Thus, the first step in this work was the application of the various organic solvents in the extraction of chlorophylls from the fresh biomass, being the yields of extraction depicted in Figure 1. UV-Vis spectra of the extracts obtained in the screening of organic solvents are depicted in Figure S1 in ESI. Concentration of chlorophylls and respective yields of extraction are presented in Table S1 in ESI.

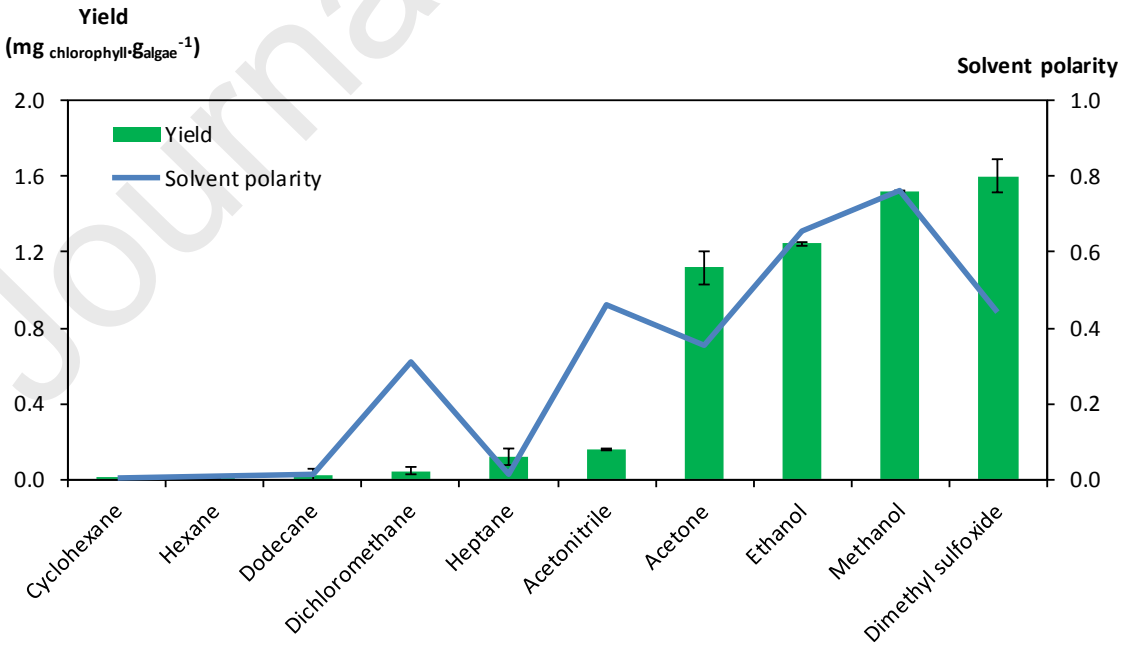


**Figure 1.** Yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) of chlorophyll obtained by the application of different organic solvents (green bars). The solvents polarity (blue lines) is also shown (28).

According to the results obtained, the highest yields of extraction of chlorophyll were obtained using acetone, ethanol, methanol, and dimethyl sulfoxide. Contrarily, hexane and cyclohexane were not able to extract efficiently the pigments. This higher affinity to more polar organic solvents can be justified taking in account that both chlorophylls and xanthophylls are not completely hydrophobic due to the presence of polar functionalities as mentioned above. Moreover, the enhancement in the extraction yields with the polar solvents can also be associated to their increased capacity to disrupt the algae cells allowing the release of the target compounds. Indeed, solvents such as acetone, methanol, and ethanol) are known for dissolving cell wall membranes, a mechanism that can strongly favor the yield of extraction (29,30).

The set of the four solvents identified as the most efficient was used in further studies. Firstly, consecutive extractions were conducted to investigate the eventual solvent saturation (Figure 2). In this context, after the first solid-liquid extraction, the remaining biomass was recovered, homogenized and reused in a new cycle of extraction, with the results presented in Figure 2.

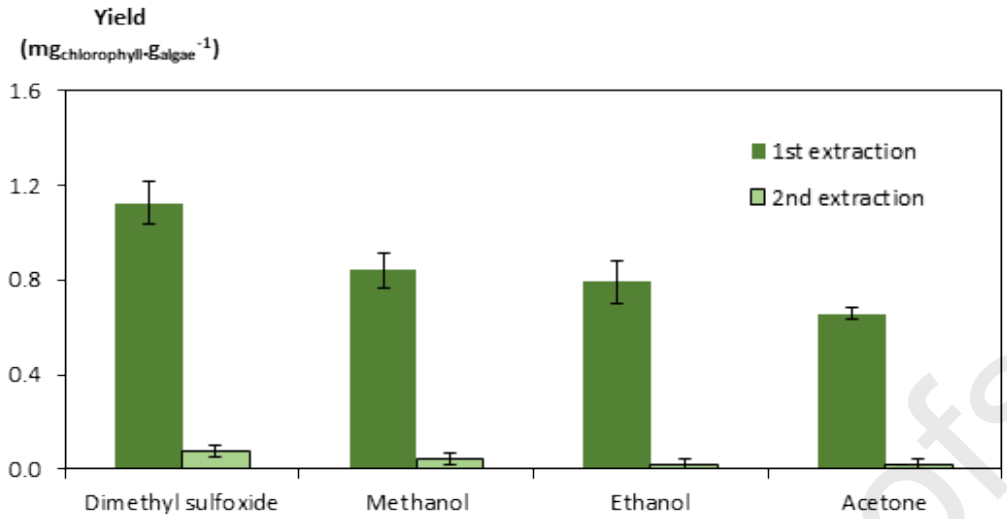



**Figure 2.** Yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) of two consecutive extractions done for the same biomass using different solvents.

For all solvents, only a residual amount of chlorophylls was extracted in the second cycle. Thus, the solvent saturation does not seem to be happening in the first extraction, being the second extraction step not significant. However, it is important to notice that not all chlorophylls is being extracted, indicating the need for further optimization of the operational conditions in order to remove the highest possible chlorophylls content in a single extraction step. At the same time, some organic solvents are known for their toxic character and their safety issues at an industrial scale. For instance, the exceptionally high boiling temperature of dimethyl sulfoxide (189 °C) (31) makes it tricky to remove. Given these concerns and the insignificant difference among the yields of extraction provided by methanol and ethanol, ethanol was selected for further process optimization due to its green and sustainable nature (32). In this sense, the SLR effect was studied between 0.05 and 0.015 $g_{algae}.mL_{solvent}^{-1}$, being the results presented in

Figure 3. The yield of extraction increased with the SLR until 0.01. Higher SLR do not seem to provide better results, being this parameter fixed at 0.01.

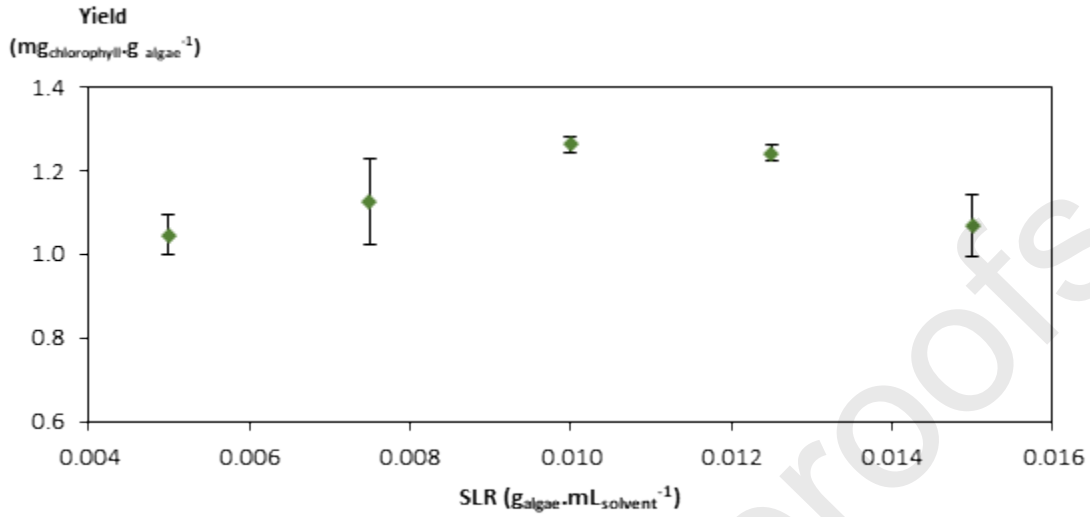



**Figure 3.** SLR effect on the yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$).

As described in the experimental part, in the standard methodology, liquid nitrogen was used to freeze the macroalgae samples, to facilitate the biomass milling and the cell wall breakage. Nevertheless, the associated costs with the use of liquid nitrogen, especially at industrial scale, makes this process less attractive.

Microwave- and ultrasonic-assisted extractions were performed using intact biomass (which did not suffer any milling or maceration, and without the use of liquid nitrogen) and under the best conditions previously found for the appropriate solvent (ethanol) and SLR (0.01) - Figure 4. UV-Vis spectra of the extracts are depicted in Figure S2 in ESI. Concentration of chlorophyll and respective yields of extraction are presented in Table S2 in ESI.

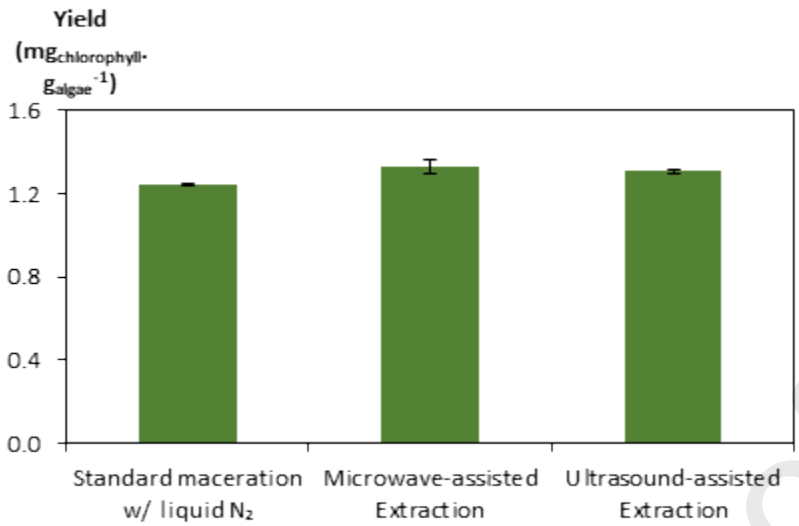



**Figure 4.** Yield of extraction ($mg_{chlorophyll}$·$g_{algae}^{-1}$) of the optimized standard methodology using biomass previously grounded with liquid nitrogen and without mechanical treatment, in comparison with the microwave- and ultrasonic-assisted extractions using intact biomass.

Similar yields of extraction were achieved for all procedures, indicating that radiation treatments might also be promoting cell wall breakage, allowing the complete solubilization of chlorophyll in the solvent. It should be highlighted that according to the procedures used, the time of extraction went from 30 minutes with the conventional extraction, down to 10 and to 1 minute with ultrasound and microwave treatments, respectively, being these approaches more appropriate to be applied from an industrial point of view. However, microwave-assisted extraction lead to an increase in temperature, which can compromise the viability of the pigments. In this sense, ultrasonic-assisted extraction using ethanol as solvent was preferred.

After the complete optimization of the solvent and process conditions, the ultrasonic-assisted ethanolic extract rich in pigments (particularly chlorophyll) was characterized

by UHPLC-MS. The identification was based on a direct comparison of their retention times, UV–Vis spectra, and mass spectra data with reference standards and data reported in literature. The data and molecular structures of the proposed compounds obtained are depicted in Table 1 and Figure S3 in ESI, respectively.

**Table 1.** Compounds present in the ultrasonic-assisted ethanolic extract from *Ulva rigida* and their molecular ions species (*m/z*) data.

| Compound | Retention time (min) | UV-Vis (nm) | Mass (*m/z*) | Molecular structure |
|---|---|---|---|---|
| Chlorophyll *a* | 12.02 | 416, 430, 663 | $[M + H]^+$ 893 | Figure S3 (i) |
| Xanthophyll | 12.57 | 422, 444, 472 | 569 | --- |
| Chlorophyll *b* | 18.46 | 450, 650 | $[M + CH_3OH + H]^+$ 939 | Figure S3 (ii) |
| Chlorophyll *b* derivative | 19.45 | 460, 651 | $[M + H]^+$ 923 | Figure S3 (iii) |
| Chlorophyll *b* | 20.60 | 452, 552, 586, 635 | $[M + HCO_2H + H]^+$ 953 | Figure S3 (ii) |
| Chlorophyll *b* derivative | 21.99 | 453, 636 | $[M + 2Na]^+$ 967 | Figure S3 (iv) |

Different compounds were found in the raw extract, mainly chlorophyll *b*, xanthophyll, chlorophyll *b* derivatives, and chlorophyll *a* contributing with abundances of 52, 24, 18, and 6 %, respectively. The ionic species are a result of the UHPLC-MS analysis conditions, which are in positive mode so ions such as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ are usually the detected species. The chlorophyll derivatives can be formed during the extraction

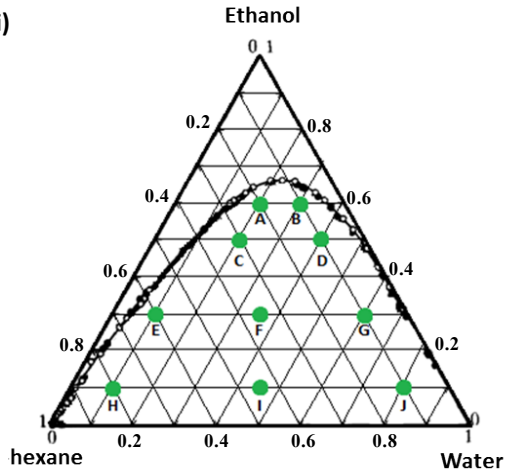
process due to the solvent, light and/or oxygen exposure. Since $MS^2$ was not performed at this stage, xanthophylls is not precisely identified, but considering the results found in literature (33), this should be lutein or zeaxanthin.

**Pigments separation by liquid-liquid extraction**

Taking into account the UHPLC-MS results shown in Table 1, it is clear the presence of different classes of pigments, namely chlorophylls and xanthophylls and some of their derivatives on the extract, which demands the development of a separation step in order to separate chlorophylls from xanthophylls.

Aiming to design a process of extraction and purification of pigments, a liquid-liquid extraction system was applied, considering the pigment-based ethanolic extract rich in pigments as the basis. Systems combining the pigment-based ethanolic extract, hexane and water were experimentally prepared. Ten different mixture points were tested (Figure 5). The biphasic region was studied, and for all systems the two-phase formation was confirmed, as shown in Figure 5(iii).

(i)




(ii)

| System | Water (%) | *n*-hexane (%) | Ethanolic extract (%) |
|---|---|---|---|
| A | 20.0 | 20.0 | 60.0 |
| B | 30.0 | 10.0 | 60.0 |
| C | 20.0 | 30.0 | 50.0 |
| D | 40.0 | 10.0 | 50.0 |
| E | 10.0 | 60.0 | 30.0 |
| F | 35.0 | 35.0 | 30.0 |
| G | 60.0 | 10.0 | 30.0 |
| H | 10.0 | 80.0 | 10.0 |
| I | 45.0 | 45.0 | 10.0 |
| J | 80.0 | 10.0 | 10.0 |

(iii)

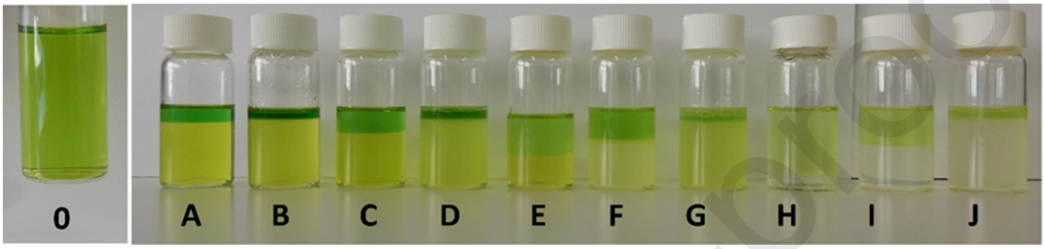



**Figure 5.** (i) and (ii) Mixture points tested for pigments fractionation based on the phase diagram (adapted from Moriyoshi et al. (26)) of water + ethanol + hexane. (iii) Photograph of the liquid-liquid extraction systems tested (from A to J) prepared with the pigment-based ethanolic extract (0) obtained from the ultrasonic-assisted extraction.

All systems studied differ essentially in the volume ratio and pigmentation content, being the top phases preferably greenish denoting, the presence of chlorophylls and the yellow color of the bottom phases representing the presence of xanthophylls (Figure 5). After the phase separation, both the top and bottom phases were recovered and analyzed by UHPLC-MS. The data is shown in Table 2 and Figure 6 and structures are identified in Figure S3 in ESI.

**Table 2.** Characterization of both top and bottom phases obtained after the fractionation of pigments, their molecular ions species and fragments (*m/z*) data. Green background means systems with complete separation of chlorophylls and xanthophylls, and red background means systems not completely pure.

| System | Fraction | Compound | Retention Time (min) | UV-Vis (nm) | Mass (*m/z*) | Molecular structure |
|---|---|---|---|---|---|---|
| **A** | TOP | Chlorophyll *a* | 12.85 | 431, 471, 663 | $[M + Na]^+$ 915 | Figure S3 (i) |
| | | Chlorophyll *b* | 19.26 | 420, 439, 463, 653 | $[M + K]^+$ 945 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 20.09 | 420, 439, 463, 653 | $[M + Na]^+$ 959 | Figure S3 (v) |
| | | Chlorophyll *b* derivative | 20.43 | 439, 483, 654 | $[M + Na]^+$ 959 | Figure S3 (v) |
| | | Chlorophyll *b* | 22.09 | 463, 600, 653 | $[M + Na]^+$ 929 | Figure S3 (ii) |
| | BOTTOM | Xanthophyll | 12.58 | 440, 472 | 568 | --- |
| **B** | TOP | Chlorophyll *a* | 12.59 | 431, 471, 663 | $[M + Na]^+$ 915 | Figure S3 (i) |
| | | Chlorophyll *b* | 21.71 | 463, 649 | $[M + Na]^+$ 929 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 26.50 | 428, 672 | $[M + Na]^+$ | Figure S3 (vii) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | 931 | |
| | BOTTOM | Xanthophyll | 12.57 | 444, 473 | 658 | --- |
| **C** | TOP | Chlorophyll *a* | 12.68 | 469, 664 | $[M + Na]^+$ 915 | Figure S3 (i) |
| | | Chlorophyll *b* | 14.20 | 462, 647 | $[M + Na]^+$ 929 | Figure S3 (ii) |
| | | Chlorophyll *a* | 15.85 | 430, 663 | $[M + H]^+$ 893 | Figure S3 (i) |
| | | Chlorophyll *b* derivative | 24.07 | 463, 649 | $[M + H_2O + 2K]^+$ 725 | Figure S3 (viii) |
| | BOTTOM | Xanthophyll | 8.61 | 460, 472 | 568 | --- |
| | | Xanthophyll | 12.53 | 440, 470 | 551 | --- |
| **D** | TOP | Chlorophyll *a* derivative | 12.63 | 442, 472, 663 | $[M + HCO_2H + K]^+$ 993 | Figure S3 (vi) |
| | | Chlorophyll *b* | 18.98 | 462, 649 | $[M + K]^+$ 945 | Figure S3 (ii) |
| | | Chlorophyll *b* | 21.71 | 463, 648 | $[M + Na]^+$ 929 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 26.50 | 429, 663 | $[M + Na]^+$ 931 | Figure S3 (vii) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | BOTTOM | Xanthophyll | 12.56 | 460, 472 | 568 | --- |
| **E** | TOP | Chlorophyll *a* | 11.80 | 429, 663 | $[M + Na]^+$ 915 | Figure S3 (i) |
| | | Chlorophyll *b* | 19.29 | 463, 647 | $[M + K]^+$ 945 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 20.11 | 462, 649 | $[M + Na]^+$ 959 | Figure S3 (v) |
| | | Chlorophyll *b* derivative | 20.44 | 460, 662 | $[M + Na]^+$ 959 | Figure S3 (v) |
| | | Chlorophyll *b* derivative | 27.04 | 460, 662 | $[M + Na]^+$ 931 | Figure S3 (vii) |
| | | Chlorophyll *b* | 28.91 | 410, 422, 662 | $[M + K]^+$ 945 | Figure S3 (ii) |
| | BOTTOM | Xanthophyll | 12.93 | 420, 442, 472 | 568 | --- |
| | | Xanthophyll | 22.52 | 419, 438, 455 | 585 | --- |
| **F** | TOP | Xanthophyll | 12.86 | 443, 471 | 551 | --- |
| | | Chlorophyll *b* | 19.28 | 461, 648 | $[M + K]^+$ 945 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 20.10 | 462, 649 | $[M + Na]^+$ 959 | Figure S3 (v) |
| | | Chlorophyll *b* derivative | 20.43 | 457, 645 | $[M + Na]^+$ 959 | Figure S3 (v) |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | Chlorophyll *b* derivative | 27.04 | 414, 429, 663 | $[M + Na]^+$ 931 | Figure S3 (vii) |
| | BOTTOM | Chlorophyll *a* | 12.19 | 430, 663 | $[M+HCO_2H+H]^+$ 940 | Figure S3 (i) |
| | | Chlorophyll *a* | 17.16 | 429, 480, 663 | $[M + H]^+$ 893 | Figure S3 (i) |
| **G** | TOP | Xanthophyll | 12.86 | 424, 442, 471 | 551 | --- |
| | | Chlorophyll *b* | 19.27 | 415, 462, 646 | [M + K]+ 945 | Figure S3 (ii) |
| | | Chlorophyll *b* | 22.09 | 462, 649 | [M + Na]+ 929 | Figure S3 (ii) |
| | | Chlorophyll *b* | 22.31 | 463, 649 | [M + Na]+ 929 | Figure S3 (ii) |
| | | Chlorophyll *b* derivative | 27.03 | 463, 652 | [M + Na]+ 931 | Figure S3 (vii) |

Due to the complexity of Table 2, the results were organized by mixture point (from A to G), considering the representation of their pigments composition and respective abundancies, for both top and bottom phases (Figure 6). To help the analysis, the green bars were defined as representing chlorophylls *a*, *b* and derivatives and in yellow are represented the xanthophylls.

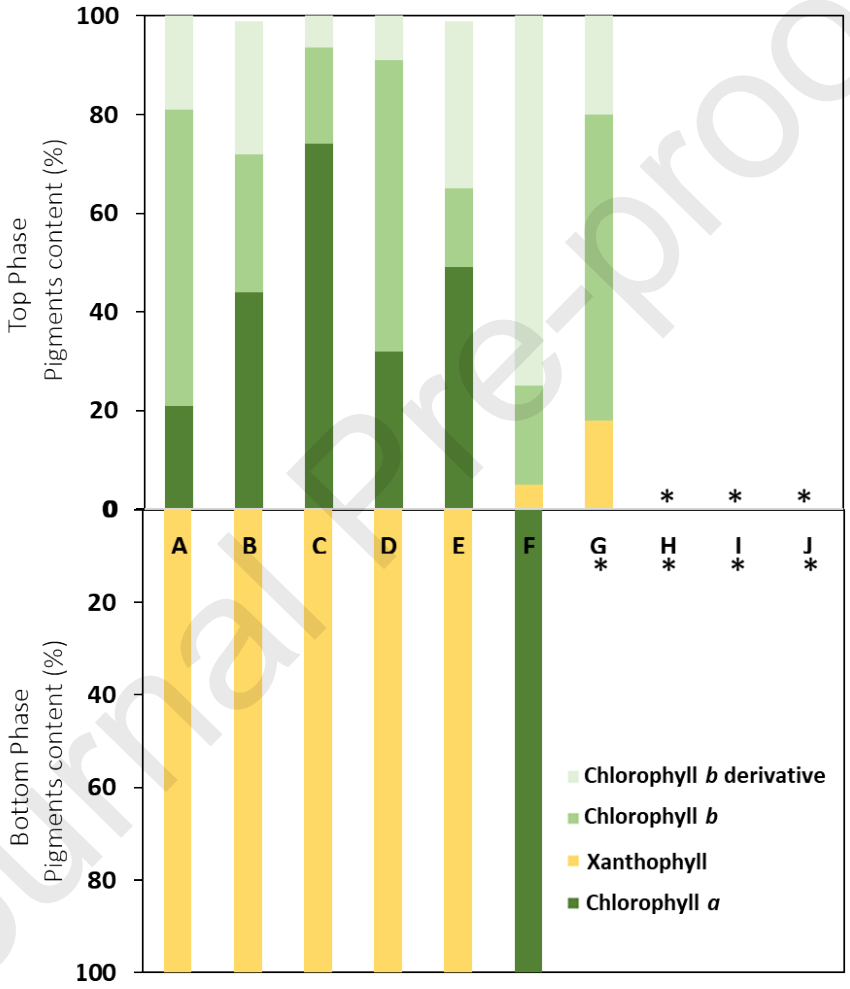



**Figure 6.** Total content of each pigment and derivatives identified for both top and bottom phases obtained after the application of the different liquid-liquid extraction systems. Presented results based on surface area peaks. Fractions identified with * were

not analyzed by UHPLC-MS due to their very low concentration in pigments, making these systems not particularly interesting for further application.

As already identified in the ultrasonic-assisted extract, the fractions obtained after the application of liquid-liquid extraction systems are essentially composed of xanthophyll, chlorophylls *a* and *b* and chlorophyll-derivatives. The results suggest the preferential partition of chlorophylls to the top phase, the hexane-rich phase, while the xanthophylls stay preferentially in the ethanol-rich (bottom) phase, as pointed out in Figure 6. This behavior does not follow the trend reported by Wall and Kelley (23) due to some significant differences. In their patent, the chlorophylls partition to the phase miscible in water due to their saponification. Chlorophylls, after alkaline saponification in which the ester groups are hydrolyzed and converted into salts, have then a higher affinity to the most hydrophilic phases. At the same time, the alkaline pH of this phase is forcing the partition of carotenoids to the most hydrophobic phase (23). The process presented in this work is much simpler as it does not require a saponification. This fractionation can be explained by the more hydrophobic nature of chlorophylls allowing them to partition preferentially to the less polar phase while xanthophylls, which are less hydrophobic, partition to ethanol/water phase, the layer with higher polarity.

Moreover, analyzing the systems A to E carefully, it is possible to identify the complete separation of chlorophylls and xanthophylls to distinct phases, purifying each class of pigments. Besides, in some cases (i.e. systems B and D) it is possible to concentrate the chlorophyll content in very small fractions without compromising their separation performance. Despite the efficient separation of chlorophylls and xanthophylls achieved by some liquid-liquid extraction systems, in some cases, some chlorophyll derivatives were formed. Chlorophylls have a chemically unstable molecule in the presence of

oxygen, light, temperature, and/or type of solvent. Systems A, C, and D have less than 20 % of chlorophyll-derivatives. However, the best system seems to be system C composed of 50 % of ethanolic extract + 30 % of hexane + 20 % of water, in which only a tiny percentage of chlorophyll derivative was identified (~ 6 %), meaning that the chlorophyll structures are pure and chemically intact.

**Final downstream process**

Considering the study on the optimization of both extraction and purification steps to obtain pure chlorophylls and xanthophylls, a final conceptual downstream process was designed, as depicted in Figure 7. It was achieved by the integration of three main tasks, starting by the (i) ultrasound-assisted solid-liquid extraction of pigments using ethanol (SLR of 0.01 $g_{algae}.mL_{solvent}^{-1}$), followed by the (ii) separation of chlorophylls and xanthophylls by applying a liquid-liquid extraction system composed of 50 % of ethanolic extract + 30 % of hexane + 20 % of water, and ending with the (iii) solvents recycle and reuse by using a vacuum dryer with low pressures and temperatures, being this last step a proposal of what can be done in large scales. With the process envisaged, pure fractions in xanthophylls and chlorophylls could be obtained at the end. In the polishing step of pigments from both top and bottom phases, evaporation units were not considered in this process, due to the high sensitivity of chlorophylls (34) and xanthophylls (35) to the range of temperatures required to evaporate hexane (68 °C) (36), ethanol (around 78 °C) (37), and water (100 °C). Instead, it was considered the application of a vacuum dryer chamber with low pressure and temperature (≈ 35 °C), allowing the recovery of the pigments as powder without compromising their stability.

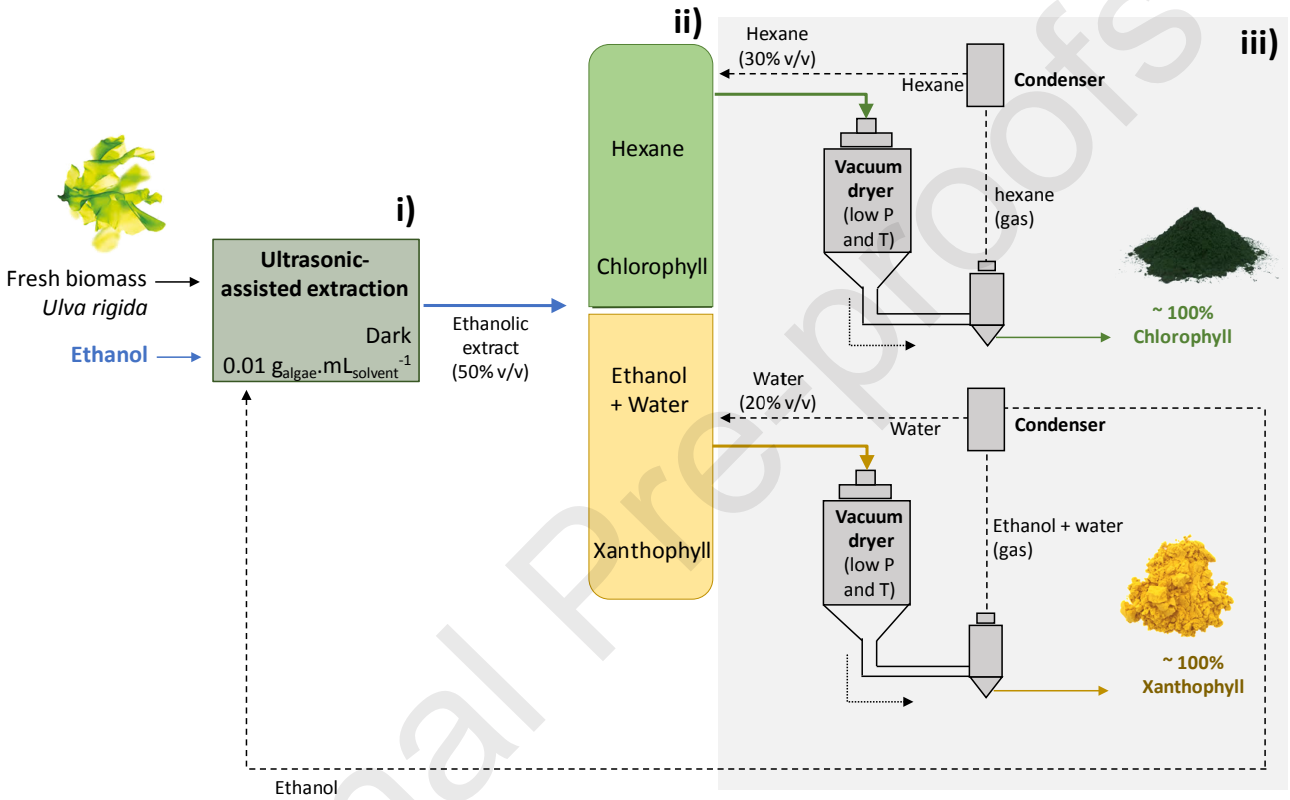



**Figure 7.** Downstream process diagram comprising the (i) solid-liquid extraction of pigments by ultrasound-assisted extraction with ethanol, (ii) pigments purification by applying liquid-liquid extraction, and (iii) pigments polishing and solvents recycle by vacuum drying using low pressure and temperature to avoid pigment degradation. The polishing of pigments and solvents (grey area in the figure) is just a proposal of what can be done industrially, not being tested in this work.

The ethanol/water mixture could be also separated by evaporation and each solvent reintroduced correctly in the process but only if demanded by the final application of each class of pigments. Both the spray dryer and evaporation are not only efficient in promoting the polishing of the pigments and reuse of the solvents, but they are also fast and feasible processes at both bench and industrial scales. This is a very simple, fast, and easy to scale-up process. Additionally, it can be used to extract and fractionate xanthophylls and chlorophylls from any natural source or raw material with similar pigment contents.

**Conclusions**

Pigments like chlorophylls and xanthophylls have an endless number of applications, for which a high purity level is demanded. In this work, an integrated downstream process was designed by the integration of three main tasks, starting by the (i) ultrasound-assisted solid-liquid extraction of pigments using ethanol (10 minutes, SLR of 0.01), followed by the (ii) separation of chlorophylls and xanthophylls by applying liquid-liquid extraction system composed of 50 % of ethanolic extract + 30 % of hexane + 20 % of water, and ending with the (iii) pigments polishing and solvents recycle and reuse by spray drying the phases. In the end and with the process envisaged, pure fractions in xanthophylls and chlorophylls were obtained. In summary, this work allowed the optimization and implementation of a simple, fast and efficient downstream process to separate hydrophobic classes of pigments, with industrial application and that could also be applied to the fractionation of other extracts with similar compositions from other natural sources and raw materials, like brown macroalgae, microalgae, and cyanobacteria.

**Acknowledgments**

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# Electronic Supporting Information

## Recovery of pigments from *Ulva rigida*

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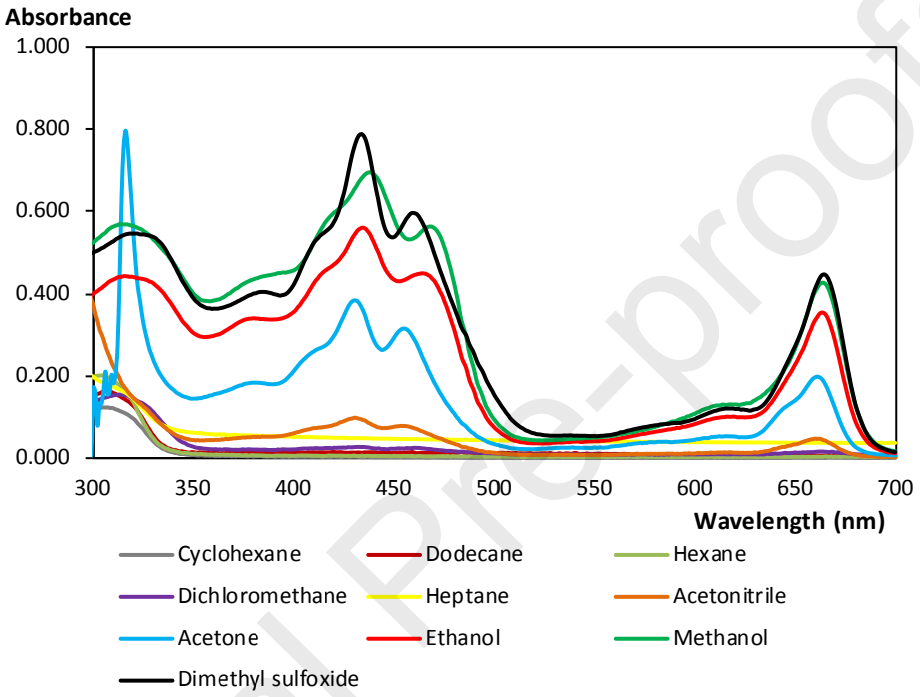


**Figure S1.** UV-Vis spectra of the extracts obtained in the screening of organic solvents for the extraction of chlorophyll from *Ulva rigida*.

**Table S1.** Chlorophyll concentration ($mg.L^{-1}$) and yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) obtained in the screening of organic solvents for the extraction of chlorophyll from *Ulva rigida*.

| | Chlorophyll concentration ($mg.L^{-1}$) | Standard deviation of chlorophyll concentration | Yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) | Standard deviation of the yield of extraction | % total chlorophyll extracted |
|---|---|---|---|---|---|
| Cyclohexane | 0.009 | 0.013 | 0.001 | 0.001 | < 0.1 |
| Hexane | 0.299 | 0.346 | 0.030 | 0.035 | 1.6 |

| | | | | | |
|---|---|---|---|---|---|
| Dodecane | 0.236 | 0.333 | 0.023 | 0.033 | 1.3 |
| Dichloromethane | 0.462 | 0.192 | 0.044 | 0.020 | 2.4 |
| Heptane | 1.377 | 0.205 | 0.136 | 0.021 | 7.4 |
| Acetonitrile | 6.463 | 0.865 | 0.637 | 0.077 | 34.4 |
| Acetone | 1.585 | 0.064 | 0.150 | 0.011 | 8.1 |
| Ethanol | 12.636 | 0.192 | 1.243 | 0.006 | 67.1 |
| Methanol | 15.380 | 0.128 | 1.518 | 0.007 | 82.0 |
| Dimethyl sulfoxide | 16.178 | 0.589 | 1.599 | 0.086 | 86.3 |

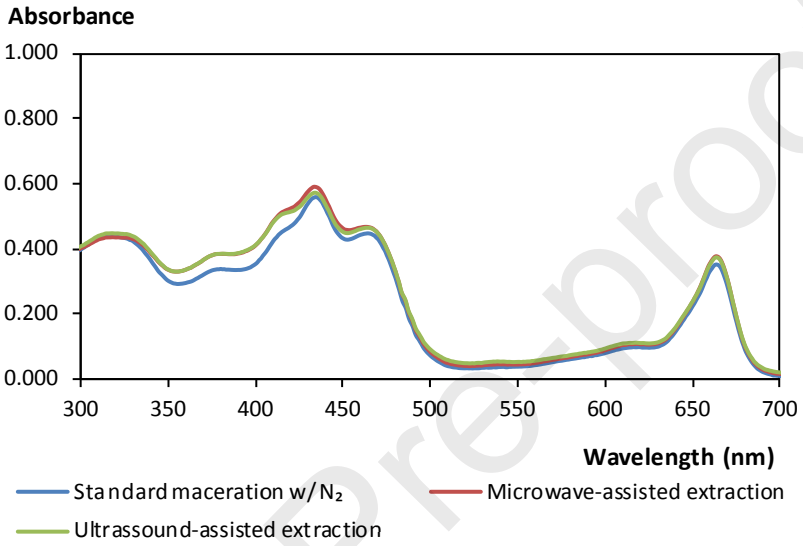


**Figure S2.** UV-Vis spectra of the extracts obtained using ethanol in different approaches of extraction of chlorophyll from *Ulva rigida*.

**Table S2.** Chlorophyll concentration ($mg.L^{-1}$) and yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) obtained using ethanol in different approaches of extraction of chlorophyll from *Ulva rigida*.

| | Chlorophyll concentration ($mg.L^{-1}$) | Standard deviation of chlorophyll concentration | Yield of extraction ($mg_{chlorophyll}.g_{algae}^{-1}$) | Standard deviation of the yield of extraction | % total chlorophyll extracted |
|---|---|---|---|---|---|
| Standard maceration w/ $N_2$ | 12.636 | 0.192 | 1.243 | 0.006 | 67.1 |

| | | | | | |
|---|---|---|---|---|---|
| Microwave-assisted extraction | 13.533 | 0.384 | 1.334 | 0.035 | 72.0 |
| Ultrassound-assisted extraction | 13.424 | 0.179 | 1.304 | 0.018 | 70.4 |

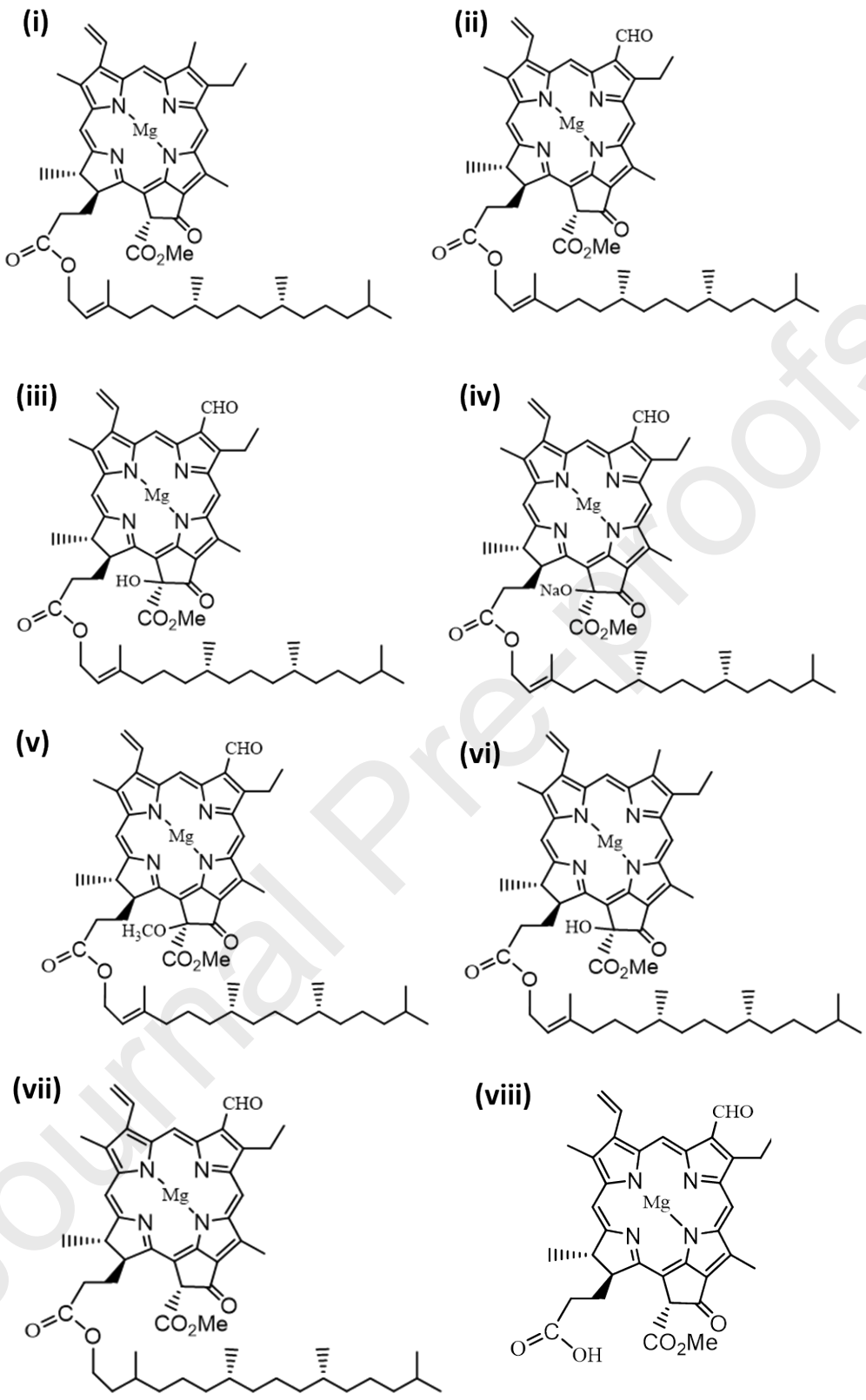


**Figure S3.** Molecular structures of the extracted chlorophyll *a* and *b* as well as the proposed structures for the derivatives detected.

**Highlights**

The recovery and separation of hydrophobic pigments was developed.

The process includes a first step of ultrasound-assisted solid-liquid extraction with ethanol.

A sustainable liquid-liquid extraction system was applied to separate the pigments.

In the end, the reuse of solvents was carried by spray drying.

**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

# CRediT author statement

**Margarida Martins:** Methodology, Validation, Investigation, formal analysis, writing – original draft

**Rui Oliveira:** Investigation

**João A. P. Coutinho:** writing – review & editing, supervision, funding acquisition

**M. Amparo F. Faustino:** Methodology, Formal analysis, Resources, writing – review & editing, supervision, Formal analysis

**M. Graça P. M. S. Neves:** Methodology, Formal analysis, Resources, writing – review & editing, supervision,

**Diana C. G. A. Pinto:** Methodology, Formal analysis, Resources, writing – review & editing, supervision,

**Sónia P.M. Ventura:** Conceptualization, methodology, resources, writing – review & editing, supervision, project administration, funding acquisition