Green Ultra-High Pressure Extraction of Bioactive Compounds from

*Haematococcus pluvialis* and *Porphyridium cruentum* Microalgae

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GRAPHICAL ABSTRACT

ABBREVIATIONS

ANOVA, analyzed by analysis of variance; APC, allophycocyanin; BHT, butylated hydroxytoluene;
B-PE, B-Phycoerythrin; DW, dry weight; EPA, eicosapentanoic acid; EtOAc, ethyl acetate; EtOH, ethanol; FAMEs, fatty acid methyl esters; FAs, fatty acids; GC-MS, gas chromatography-mass spectrometry; GXL, gas-expanded liquid extraction; HPLC−DAD-MS, high-performance liquid chromatography coupled to the diode array detector and mass spectrometry detector; MS/MS, tandem mass spectrometry; MTBE, methyl tert-butyl ether; MUFAs, monounsaturated fatty acids; PI, purity index; PLE, pressurized liquid extraction; PUFAs, polyunsaturated fatty acids; R-PC, R-phycocyanin; SEM, scanning electron microscopy; SFAs, saturated fatty acids; SFE, supercritical fluid extraction; GRAS, generally recognized as safe; UHPE, ultra-high pressure extraction.
ABSTRACT

Microalgae are considered prolific sources of bioactive compounds that can be useful for nutraceuticals. In this study, the potential of ultra-high pressure extraction (UHPE) for the simultaneous cell disruption and extraction of bioactives from two microalgae species, *Haematococcus pluvialis* and *Porphyridium cruentum*, was evaluated. The variables studied to extract carotenoids for *H. pluvialis* were pressure (100-600 MPa) and number of cycles (1 and 3 cycles) whereas the variables studied to obtain bioactives such as B-phycoerythrin, carotenoids, and PUFAs for *P. cruentum* were pressure (100-600 MPa) and different extraction solvents (water, ethanol, ethyl acetate or ethanol/D-limonene), generally recognized as safe (GRAS). The UHPE results showed significant increase on the extraction of carotenoids (109.74 – 119.34 mg per g extract) from *H. pluvialis* using 1 cycle of 20 min regardless of the pressure used. For *P. cruentum*, an UHPE with water provided extracts enriched in B-phycoerythrin (up to 144.43 mg per g extract), while subsequent UHPE using ethanol, ethyl acetate or ethanol/D-limonene 1:1 (v/v) provided extracts enriched in carotenoids (up to 65.05 mg per g extract) and polyunsaturated fatty acids (mainly eicosapentanoic acid and linoleic acid). Therefore, UHPE proved to be a viable green alternative for the recovery of bioactives from microalgae biomass.

Industrial relevance: Microalgae are promising sources of bioactives such as B-phycoerythrin, carotenoids and polyunsaturated fatty acids. The potential of ultra-high-pressure extraction (UHPE) has been demonstrated as a fast and viable eco-friendly alternative using GRAS solvents (water, ethanol, ethyl acetate or ethanol/D-limonene) for the simultaneous cell disruption and extraction of these bioactives from *Haematococcus pluvialis* and *Porphyridium cruentum*. The bioactives obtained with one step or two step-UHPE process can be used in an array of food, cosmetic and pharmaceutical applications.

Keywords: Bioactive compounds, biorefinery, GRAS solvents, microalgae, polyunsaturated fatty acids, phycoerythrin, ultra-high pressure extraction.
1. INTRODUCTION

In recent years, the economic potential of the microalgae industry has been accepted. Microalgae are promising microorganisms that can play a key role in the bio-based economy, since they may serve as a continuous and reliable source of safe natural products due to their wide application possibilities in the renewable energy, biopharmaceutical, nutraceutical, and food industries (Khan, Shin, & Kim, 2018). In the previous decade, bioactive properties from microalgae, such as immunomodulatory, anti-inflammatory, antioxidant or anti-hypertensive, among others, have been studied (Barkia, Saari, & Manning, 2019) and several microalgae extracts have been introduced to the market as food (Barkia, et al., 2019; Torres-Tiji, Fields, & Mayfield, 2020), cosmetic (Apone, Barbulova, & Colucci, 2019) or pharmaceutical supplements (Jha, Jain, Sharma, Kant, & Garlapati, 2017).

Valuable components present in *Haematococcus pluvialis* and *Porphyridium cruentum* microalgae include carotenoids, proteins and polyunsaturated fatty acids (PUFAs). Astaxanthin, fucoxanthin, β-carotene and zeaxanthin are the most commercially used carotenoids obtained from microalgae (Torregrosa-Crespo, et al., 2018) due to their health promoting properties such as anti-cancer (Rao, et al., 2013) or anti-diabetes (Naito, et al., 2004). Zeaxanthin and β-carotene are described as major carotenoids of *P. cruentum* (Di Lena, Casini, Lucarini, & Lombardi-Boccia, 2019; Gallego, Martinez, Cifuentes, Ibáñez, & Herrero, 2019b), whereas the largest known natural astaxanthin source is the microalgae *H. pluvialis*. Using certain optimized growing conditions, an accumulation of astaxanthin up to 5.5-5.8% dry weight (DW) (Chekanov, et al., 2014; Suh, Joo, & Lee, 2006) in *H. pluvialis* may be achieved. In terms of proteins, *P. cruentum* has a high content (34% DW), of which 2% DW is phycoerythrin (Rebollos-Fuentes, Acien-Fernandez, Sanchez-Perez, & Guil-Guerrero, 2000). This phycobiliprotein is commonly used as a fluorescent indicator in biomedical investigations and as a natural colorant in the food industry (Mysliwa-Kurdziel & Solymosi, 2017). Furthermore, other relevant biological activities have been associated to this protein such as antioxidant (Sonani, Singh, Kumar, Thakar, & Madamwar,
and anti-cancer (Pan, et al., 2013) activities. Moreover, *P. cruentum* has 4.6% DW of fatty acids (Rebollos-Fuentes, et al., 2000), including eicosapentaenoic acid (EPA), which has been associated with decreased mortality from cardiovascular diseases (Simopoulos, 2002) and enhancing anti-inflammatory properties of high-density lipoproteins (Tanaka, et al., 2014).

Supercritical fluid extraction (SFE), gas-expanded liquid extraction (GXL), and pressurized liquid extraction (PLE) are techniques frequently used for the extraction of bioactive compounds from microalgae (Gallego, Bueno, & Herrero, 2019a). Nevertheless, some microalgae species are characterized by the presence of a thick cell wall that may hamper to a certain extent the efficiency of the extraction. To overcome this problem, the combination of ultrasound, microwave or enzymatic pretreatments with compressed fluids-based extraction processes have been reported (Getachew & Chun, 2017; Krakowska, Rafinska, Walczak, & Buszewski, 2018; Tang, Qin, Wang, Li, & Tian, 2011), although these pretreatments often lack to provide positive results (Sanchez-Camargo, et al., 2016). Recently, the use of high pressure homogenization up to 250 MPa has demonstrated to weaken cell wall integrity by scanning electron microscopy (SEM) and fluorescence microscopy (Bernaerts, Gheysen, Foubert, Hendrickx, & Van Loey, 2019). Therefore, ultra-high pressure extraction (UHPE) could be a useful process for cell disruption and simultaneous extraction of different bioactives from natural matrices. For example, this approach has been recently used for the removal of proteins from *Galdiera phlegrea* microalgae using water as extraction solvent at 200 MPa (Imbimbo, et al., 2020; Jubeau, et al., 2013; Tran, et al., 2019a) or diterpenes from *Siegesbeckia orientalis* (Kim, Park, Woo, Lim, & Hwang, 2014), at 100-600 MPa, 3-20 min holding time, solid:solvent ratio (1:10–1:90 w/v), and ethanol concentration (0–100%) by mono-factor experiments. Furthermore, to maintain the ‘green character’ of the microalgae biomass process, high-value compounds should preferably be extracted using Generally Recognized As Safe (GRAS) solvents following a biorefinery-like approach. To the best of our knowledge, the use of other eco-friendly solvent alternatives besides water, at ultra-high pressures, has not been yet explored. Therefore, the
main goal of this study was to evaluate the potential of UHPE for the recovery of carotenoids from *H. pluvialis* and *P. cruentum* microalgae using ethanol. In addition, a sequential approach for the valorization of *P. cruentum* biomass was developed using UHPE in which the residue from each extraction step was used as the raw material for the next step using GRAS solvents (water, ethanol, ethyl acetate and D-limonene), targeting bioactives such as B-phycoerythrin, carotenoids, and PUFA. Finally, UHPE was compared to the PLE and conventional processes.

**2. MATERIALS AND METHODS**

**2.1. Samples and reagents**

Both microalgae strains, *Haematococcus pluvialis* and *Porphyridium cruentum*, consisted of a freeze-dry powder, kindly provided by Microphyt (Baillargues, France) and stored at 4 °C until further use. Microalgae growing conditions were the same as previously described (Tran, et al, 2019b). Briefly, each microalga was grown in 5000 L photobioreactor (PBR) consisting in 1.2 km of glass tubes, each, with co-circulation of liquid medium and CO₂ enriched air. The PBR was placed under a greenhouse, allowing the control of temperature between 22 and 28 °C and the intensity of natural light with curtains. The pH was set at 7.5 and was automatically controlled by CO₂ injection monitored by an inline pH probe (Fermprobe F-235, Broadley James, Silsoe, UK). Air was injected continuously at a rate of 35 L min⁻¹. The culture medium used for the initial microalgal grown in the PBR was a marine type medium, corresponding to a modified Hemerick’s medium by the addition of N and P up to 20 and 4 mmol L⁻¹, respectively. Also, salt-saturated brine was added to the culture medium (1:8 v/v) for *P. cruentum* growth. Cultivation was conducted in a semi-continuous condition enabling to maintain the exponential growth phase. In the case of *H. pluvialis*, part of the semi-continuous harvesting was inoculated to another PBR with sterilized water as the culture medium to grow the cells as red phase. When reaching a specific absorbance, cells (*P. cruentum* and *H. pluvialis* in red phase) were harvested by bowl centrifugation at 6000 rpm (KG 8006, GEA, Oelde, Germany) and concentrated to around 8-15%
of dry matter, frozen at -20 °C in polyethylene bags, heat sealed and stored at -20 °C before freeze drying.

HPLC-grade solvents, including methyl tert-butyl ether (MTBE), methanol, acetone, ethanol (EtOH) and ethyl acetate (EtOAc) were purchased from VWR (Leuven, Belgium), whereas DL-limonene and chloroform were acquired from Merck (Darmstadt, Germany). Sea sand (0.25–0.30 mm diameter) was purchased from Panreac (Castellar del Vallés, Spain).

Butylated hydroxytoluene (BHT), acetyl chloride, canthaxanthin and β-carotene from Anacystis nidulans algae were obtained from Sigma-Aldrich (St Louis, MO, USA). Lutein from Echinacea purpurea was purchased from Chengdu Biopurify Phytochemicals Ltd. (Chengdu, China). Astaxanthin was acquired from Acros Organics (Geel, Belgium), whereas zeaxanthin was purchased from Carbosynth Ltd. (Berkshire, UK). Ultrapure water was purified in a Milli-Q system from Millipore 18.2 MΩ·cm (Billerica, MA, USA).

2.2. Proposed UHPE process for the valorization of microalgae

As the aim of this study was to recover key bioactive compounds from microalgae using UHPE with GRAS solvents, two different approaches, one for each microalgae strain, were used. Figure 1 illustrates a scheme of the processes proposed for the valorization of both microalgae biomasses.

For H. pluvialis, a one-step process for carotenoid extraction using ethanol as a solvent at 50 °C was proposed. The extraction parameters studied were pressure (100, 300 and 600 MPa) and number of cycles (1 or 3), considering a total of 20 min of static extraction time.

A sequential platform composed of two steps was designed for the recovery of bioactives from P. cruentum: a first step using water as the extraction solvent to extract B-phycoerythrin, and a second step for the recovery of carotenoids and PUFAs using different GRAS solvents. Other extraction parameters are detailed as follows:
Step 1: This step targeted the recovery of B-phycoerythrin using water at 25 °C for 20 min. Different ultra-high pressures (100, 300, 600 MPa) were evaluated.

Step 2: The residual biomass from step 1 was used to extract bioactives at 70 °C for 20 min. In this step, different GRAS solvents (ethanol, ethyl acetate and a mixture of ethanol:D-limonene 1:1 (v/v)) at different pressures (100, 300 and 600 MPa) were evaluated with the goal to extract carotenoids and PUFAs.

2.3. Conventional extraction (CE) methods

2.3.1. Conventional B-phycoerythrin extraction

*P. cruentum* biomass (0.5 g of dry algae) was resuspended in 50 mL of Milli-Q water and shaken for 1 h under agitation at 20°C, away from direct light exposure. After centrifugation (2,057 x g at 4°C for 15 min), the supernatant was collected. The extraction was carried out in duplicate. Water extracts were lyophilized in a freeze-drier (Lyobeta, Telstar, Terrassa, Spain) and stored in the dark at −20 °C to prevent any degradation until further analysis.

2.3.2. Conventional carotenoid extraction

Carotenoids were extracted in duplicate using the method described elsewhere (Reyes, Mendiola, Ibáñez, & del Valle, 2014). Briefly, 200 mg of lyophilized biomass was mixed with 20 mL of acetone containing 0.1% (w/v) BHT, and the mixture was shaken for 24 h in a thermostatic shaker (Thermomixer comfort (Eppendorf AG, Germany)) at 500 rpm and 20 °C. Then, the sample was centrifuged at 12,857 x g and 4 °C for 10 min. The supernatant was collected, and the solvent was removed using a nitrogen stream. The extracts were weighed to calculate the extraction yield and then stored protected from light at −20 °C for further analysis.

2.3.3. Conventional lipid extraction
Conventional lipid extraction was performed in duplicate according to the method reported by Axelsson & Gentili (2014) to compare the fatty acids (FAs) obtained during step 2 of *P. cruentum* sequential process approach. Briefly, 25 mg of freeze dried *P. cruentum* biomass were mixed with 8 mL of chloroform/methanol 2:1 (v/v) under agitation. Then, 2 mL of 0.73% NaCl (w/v) was added and the mixture was strongly agitated. Samples were centrifuged at 350 \( \times \) g for 5 min at 25°C, allowing the separation of the two phases. The lower layer (lipidic fraction) was collected with a glass pipette and transferred to a pre-weighed glass vial, while the upper layer was discarded. The solvent was evaporated under a nitrogen stream and the total lipids were calculated by a gravimetrical determination. The extracts were stored in the dark at −20 °C.

**2.4. Compressed fluid extraction processes**

**2.4.1. Ultra-high pressure extraction**

A multivessel system (Apparatus U111 Unipress, Warszawa, Poland) was used for the extractions at ultra-high pressures up to 600 MPa. Figure 2 shows a scheme of the UHPE system. Briefly, each one of the four vessels has a capacity of 8 mL. The vessels were heated with a circulator thermostat (Lauda Proline RP 855 Low Temperature, Lauda-Konigshofen, Germany) using propylene glycol as the pressure transmission fluid. Polypropylene tubes (Cryogenic vial, Fisher Scientific, Pittsburgh, PA) of 3 mL were filled with 0.135 g of microalgae biomass and then, the tubes were completed with the GRAS solvent of interest. *H. pluvialis* samples in ethanol at 50 °C were pressurized to 100, 300, and 600 MPa at a rate of 10 MPa/s. Similar pressures were studied previously for extraction of bioactives from *Nannochloropsis oceanica* microalgae by our research group (Gallego, et al., 2021) and also used in high pressure homogenization (HPH) in other studies (Carullo, et al., 2018; Zhang, 235 Grimi, Marchal, Lebovka, & Vorobiev, 2019). In addition, the effect of one static extraction cycle (20 min) or three static extraction cycles (7 + 6 + 7 min) was evaluated. At the end of each extraction, the vessels were depressurized, and the samples were removed immediately from
the high-pressure vessels, cooled down with ice and centrifuged at 4629 x g for 10 min. Supernatants from three polypropylene tubes, corresponding to the same experimental conditions, were combined together to form a replicate. Experimental data were obtained in duplicates. Combined ethanolic extracts were evaporated under nitrogen stream, protected from light and frozen at −18 °C to prevent degradation until further analysis.

In the first step of P. cruentum sequential approach, microalgae biomass was pressurized to 100, 300, and 600 MPa using water at 25 °C with a holding time of 20 min. Samples were pressurized at a rate of 10 MPa/s. At the end of the holding time, supernatants were collected and combined as described above, lyophilized and stored at −18 °C for further analysis. The remaining residues were recovered, lyophilized and used as raw material for the next extraction step. The second step was carried out adding to the polypropylene tubes, ethanol, ethyl acetate or a mixture of ethanol:D-limonene 1:1 (v/v). Then, samples were extracted at 70 °C for 20 min, using the same pressures as in the first step. Subsequently, the samples were collected, cooled in ice and centrifuged. Supernatants were combined and kept in the dark at −18 °C. Experiments were performed in duplicate.

2.4.2. Pressurized liquid extraction (PLE)

Previously optimized PLE condition at 10 MPa was employed for comparative purposes (Gallego, et al., 2020; Gallego, et al., 2019b). Extractions of the different microalgae biomass were performed in an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller unit. For the extractions, 1.0 g of dried algal biomass was loaded into an 11-mL stainless steel extraction cell sandwiched between two layers of 2.0 g of sea sand, maintaining the same solvent to feed ratio than in the UHPE experiments. Extraction time was set at 20 min for all the extractions. The first step of P. cruentum sequential extraction was carried out using water at 25 °C, and the second step was performed at 75 °C and 125 °C. H. pluvialis extracts were obtained...
using ethanol at 50 °C, as this temperature was the optimum condition (Gallego, et al., 2020).

Water extracts were lyophilized, while other solvents extracts were evaporated under nitrogen stream. Samples were protected from light and stored at –20 °C until further analysis. All experiments were done at least in duplicate.

2.5. Characterization of extracts

2.5.1. B-phycoerythrin content determination

The amount of B-phycoerythrin in the aqueous extracts from P. cruentum was calculated using the spectrophotometric method and equations (1-3) proposed by Bermejo-Roman, Alvarez-Pez, Acien-Fernandez, & Molina-Grima (2002). Extracts were diluted in Milli-Q water at 1 mg/mL and absorbances were measured at 545, 565, 620, and 650 nm \( (A_{545}, A_{565}, A_{620}, A_{650}, \text{respectively}) \) using 1 cm UV fused quartz cuvettes. Each extract was measured in triplicate using a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). B-phycoerythrin content was determined in mg/mL and was then converted in mg/g extract, applying the following formulae:

\[
\begin{align*}
\text{R-phyocyanin: } [\text{R-PC}] (\text{mg/mL}) & = \frac{(A_{620} - 0.7 \times A_{650})}{7.38} \\
\text{Allophycocyanin: } [\text{APC}] (\text{mg/mL}) & = \frac{(A_{650} - 0.19 \times A_{620})}{5.65} \\
\text{B-Phycoerythrin: } [\text{B-PE}] (\text{mg/mL}) & = \frac{(A_{565} - 2.8 \times [\text{R-PC}] - 1.34 \times [\text{APC}])}{12.27}
\end{align*}
\]

The purity index (PI) was calculated using equation (4):

\[
\text{Purity index} = \frac{A_{545}}{A_{280}}
\]

2.5.2. Total carotenoid content determination

The total carotenoid content in the extracts was determined spectrophotometrically based on their characteristic absorbance as described by Gilbert-Lopez, et al. (2015). UHPE and PLE extracts from H. pluvialis were dissolved in ethanol at a concentration of 0.1 mg/mL, whereas the extracts from step 2 of P. cruentum sequential process were dissolved in methanol at a concentration of 0.3 mg/mL. The absorbance of samples was recorded at 470 nm. A standard
calibration curve of astaxanthin (0.16–10 μg/mL) or zeaxanthin (0.2–20 μg/mL) was used to calculate the concentration of total carotenoids from *H. pluvialis* and *P. cruentum*, respectively, as they are the major carotenoids present in each microalga. The carotenoid content was expressed as mg of carotenoids extracted per g of extract. Analyses was carried out in triplicate.

### 2.5.3. Carotenoid characterization by HPLC–DAD–MS

Carotenoids were characterized by high-performance liquid chromatography coupled to a diode array detector and mass spectrometry detector (HPLC–DAD–MS), according to a previously described method (Castro-Puyana, et al., 2013), with some modifications. Analyses were performed using an Agilent 1100 series liquid chromatograph (Santa Clara, CA, USA) equipped with a DAD and using a YMC-C30 reversed-phase column (250 mm × 4.6 mm inner diameter, 5 μm particle size; YMC Europe, Schermbeck, Germany) and a YMC-C30 precolumn (10 mm × 4 mm i.d., 5 μm). The mobile phase was a mixture of methanol–MTBE–water (90:7:3, v/v/v) (solvent A) and methanol–MTBE (10:90, v/v) (solvent B). Compounds from *H. pluvialis* extracts were eluted according to the following gradient: 0 min, 0% B; 20 min, 30% B; 35 min, 40% B; 45 min, 80% B; 50 min, 100% B; 52 min, 0% B. The linear gradient employed for the elution of pigments from *P. cruentum* was as follows: 0 min, 0% B; 20 min, 30% B; 35 min, 40% B; 38 min, 80% B; 43 min, 100% B; 45 min, 0% B. The flow rate was 0.8 mL/min while the injection volume was 10 μL in both cases. The detection was performed at 280, 450, and 660 nm, although the spectra from 240 to 770 nm were recorded using the DAD (peak width >0.1 min (2 s) and slit 4 nm). The instrument was controlled by LC ChemStation 3D Software Rev. B.04.03 from Agilent.

Extracts were dissolved in pure ethanol at an appropriate concentration (1–10 mg/mL for *H. pluvialis* and 0.3-3 mg/mL for *P. cruentum*) and filtered using 0.45 μm nylon filters before analysis. Each dilution was injected in triplicate. For calibration plots, at least five different concentrations of astaxanthin (from 3.1 to 50.0 μg/mL), lutein (from 3.1 to 50.0 μg/mL),
zeaxanthin (from 3.9 to 31.3 μg/mL), cantaxanthin (from 31.3 to 250 μg/mL) and β-carotene (from 0.78 to 6.25 μg/mL) dissolved in ethanol were analyzed in triplicate. The same instrument was directly coupled at the exit of the DAD to an Agilent ion trap 6320 mass spectrometer (Agilent Technologies) via an atmospheric pressure chemical ionization interface. Analyses were conducted under the positive ionization mode (further details can be found in section S1 of the Supporting Information). Automatic tandem mass spectrometry (MS/MS) analyses were also performed fragmenting the two highest precursor ions. External standards were also reinjected for the identification of some compounds. Furthermore, a high number of compounds were tentatively identified as derivatives of main H. pluvialis carotenoids (astaxanthin monoesters and diesters) since they had similar UV–Vis spectra and compatible mass spectrometry fragmentation patterns.

2.5.4. Fatty acid derivatization and analysis by GC-MS

Fatty acid methyl esters (FAMEs) of the lipidic extracts (from conventional lipid extraction and step 2 of the UHPE of P. cruentum) were derivatized using a variation of the method described earlier (Golmakani, Mendiola, Rezaei, & Ibáñez, 2012). Briefly, 0.6 mg of internal standard (heptadecanoic acid) were added to 5 mg of the lipid extract and the mixture was treated with methanol/acetyl chloride (95:5 v/v) solution. The PTFE capped glass vial was sealed under nitrogen atmosphere and incubated at 85 °C for 1 h. Subsequently, the vial was cooled down to room temperature and 1 mL of water was added and mixed with a vortex. Then, FAMEs were extracted by adding 3 mL of hexane containing 0.01% BHT and 1 mL of the extract (upper layer) was transferred into a clean vial for gas chromatography-mass spectrometry (GC-MS) analysis. Two microliters of each extract were injected in a Shimadzu QP-2010 gas chromatograph (Kyoto, Japan) equipped with a Shimadzu AOC-20i autosampler coupled to a QP-2010 Plus single quadrupole mass spectrometer. The standard split/splitless injector was operated in split mode (split ratio, 1:2) and the temperature of the injector was kept at 220 °C. The carrier gas was He.
at a constant linear velocity of 38.6 cm/s (=1 mL/min flow rate). The column was a Zebron™ ZB-
WAX capillary column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) from Phenomenex
(Torrance, CA, USA). The oven programmed temperature started at 120 °C, and raised to 240 °C
at 4 °C/min. The final temperature was held for 10 min. The ion source was operated in
electronic impact (EI) mode. The temperature of the ion source was 230 °C, while the transfer
line was kept at 260 °C. The mass analyzer operated in SCAN mode using a mass interval ranging
from m/z 40 to 550. The solvent cut window selected was 2.5 min. Shimadzu GC Solution
software was used to process all the data. The identity of FAMEs was determined through the
mass spectra using the NIST Mass Spectral database and their linear retention indexes, which
was confirmed by the injection of a marine source standard (PUFA No. 1, Sigma-Aldrich). Each
sample was analyzed in duplicate.

2.6. Scanning electron microscopy (SEM)
The integrity of the cell walls before and after the different UHPE processes applied to *H.
pluvialis* and *P. cruentum* biomass was observed using a Zeiss Sigma Field Emission scanning
electron microscopy (SEM) (Carl Zeiss AG, Oberkochen, Germany). Samples were dried and
covered with carbon using a Leica EM SC005 evaporative carbon coater.

2.7. Statistical analysis
Extraction yield of all the experiments was calculated as the percentage of extract dry weight
per initial biomass dry weight. Experimental data are shown as mean value ± standard deviation.
Results were analyzed by analysis of variance (ANOVA), whereas mean values were compared
by Tukey’s test (SPSS Statistics v.15 IBM, Armonk, NY, USA). The value of *p* ≤ 0.05 was considered
statistically significant, and alphabetical letters were used along means in the tables.

3. RESULTS AND DISCUSSION
We investigated the use of UHPE as a viable green alternative for the valorization of two microalgae in the search for bioactive compounds that could potentially be used in the food or cosmetic industries. We hypothesized that the use of ultra-high pressures could weaken the integrity of the cell wall and therefore, facilitate the extraction of carotenoids, PUFAs and proteins from microalgae. This possibility would avoid the need for cell disruption using any additional pre-treatment. As the bioactives of interest were carotenoids (H. pluvialis and P. cruentum microalgae) and proteins and PUFAs (P. cruentum microalgae), we proposed two different extraction approaches (Fig. 1) and the results obtained were discussed separately for each microalgae.

3.1. UHPE of carotenoids from H. pluvialis

Ethanol at 50 °C was selected as the extraction solvent based on our previous study of H. pluvialis chemical composition in terms of the number and relative polarity of its carotenoids (Gallego, et al., 2020). Furthermore, the use of low temperature avoids the occurrence of potential degradation reactions, influencing the antioxidant activity of this type of extract (Jaime, et al., 2010). For these reasons, in this study, the extraction behavior of ethanol at ultra-high pressures from 100 to 600 MPa was studied. Likewise, the number of static extraction cycles (from 1 to 3) was evaluated as more than 3 UHPE cycles did not increase neither the extraction yield nor carotenoids content (data not shown).

To evaluate the UHPE process, the content on total carotenoids in the extract was considered. Experimental conditions, extraction yield as well as total carotenoid content of the different extracts are shown in Table 1. All pressurized conditions produced extracts significantly richer in carotenoids than the conventional extraction. The solid/liquid ratio was the same (ratio solid/liquid = 0.05) for the UHPE and PLE processes. However, for the conventional extraction, a solid/liquid ratio of 0.01 g/mL was used. Even with more solvent and time for the conventional extraction than for the PLE or UHPE, the total carotenoids were less compared to those obtained
by the UHPE. Furthermore, ultra-high pressure extracts obtained in 1 cycle of 20 min had significantly higher amounts of carotenoids than the PLE, but not showing statistically significant differences among pressures (100-600 MPa). This fact is in agreement with recent literature in which *H. pluvialis* cell microalgae dispersed in distilled water was treated using high pressure homogenization up to 207 MPa, and the cells were significantly broken or fully ruptured at 69-207 MPa (Praveenkumar, et al., 2020).

On the other hand, with regards to the number of static extraction cycles, the total carotenoid content obtained using 3 cycles was not significantly different from that using the PLE. This observation, connected with the fact that UHPE yield was positively affected by the number of cycles, indicated that the addition of fresh solvent favored the co-extraction of other compounds with similar polarity/solubility properties from the algae. However, most of the extracted compounds (around 70%) were obtained in the first extraction cycle, which implied that in a single 7 min static extraction, more than 90% of the total yield was obtained (data not shown) using a static extraction cycle of 20 min. We also performed some preliminary PLE experiments to evaluate the relevance of number of cycles and the increase of the extraction yield was not significantly enough to consider a second cycle (i.e. the extraction yield increased from 5.44% to 6.17% when two extraction cycles were used, with the same amount of total carotenoids obtained, data not shown). Consequently, considering a global view of the results, only one cycle of pressurization-depressurization was enough for extracting carotenoids. Moreover, the duration of this extraction cycle could be an important factor and imply a significant advantage over PLE and conventional extraction.

In addition, SEM images of *H. pluvialis* cells before (Figure 3A) and after (Figure 3B, 3C and 3D) 1 cycle of pressurized ethanol extraction at ultra-high pressures were examined to visualize possible cell structure damage. At the lowest studied pressure of 100 MPa, cell walls were affected, while some cell walls seemed completely broken at 600 MPa. However, cell wall integrity could be independent of membrane integrity, suggesting that 100 MPa was probably
good enough for damaging the cell membrane without a complete rupture of the cell wall, which was in agreement with the results obtained for the content of total carotenoids (Table 1).

Furthermore, extracts obtained using UHPE were analyzed by HPLC-DAD-MS to collect more information about the specific quantities recovered of each carotenoid. The identification of carotenoids was accomplished by combining the information provided by UV–Vis spectra, MS spectra, and MS/MS fragmentation patterns, and bibliographic published information. An example of the chromatographic profiles achieved is shown in Figure S1 of the Supporting Information. As expected, no evidence of new compounds was found regarding the identification of carotenoids with respect to the PLE extracts. The main identified pigments were astaxanthin (free form) and its derivatives (more stable pigments, mono- and di-esterified with different fatty acids), lutein, zeaxanthin, canthaxanthin and β-carotene. Even in extracts with less total carotenoid content (3 cycles), the amounts of astaxanthin and derivatives obtained at 100 and 600 MPa were satisfactory (Figure 4). No significant differences were found between the extraction carried out at 100 MPa and optimum PLE, whereas values obtained at 600 MPa were significantly higher than the optimum PLE (Figure 4). Moreover, the use of UHPE process resulted in a significant increase (p<0.05) on the total amount of zeaxanthin (from $0.17 \pm 0.04$ to $0.25 \pm 0.02$ mg/g extract) and β-carotene (from $0.46 \pm 0.07$ to $0.59 \pm 0.01$ mg/g extract) regardless of the pressure exerted, meaning 47% and 28% increase, respectively. For canthaxanthin, a statistically significant 105% increase was found (p<0.001), reaching a concentration of $10.12 \pm 0.46$ mg/g extract (UHPE), independently of the pressure used. Lutein with a mean value of $4.99 \pm 0.40$ mg/g extract was the only carotenoid not significantly affected, comparing extracts obtained by UHPE with that of PLE.

### 3.2. UHPE as a green alternative for the extraction of bioactive compounds from *P. cruentum*

The sequential strategy was selected considering the nature of the bioactive compounds present in *Porphyridium cruentum* microalgae (proteins, mainly B-phycoerythrin (B-PE), carotenoids and
Moreover, the decreasing polarity order of the different extraction steps has been selected considering previous PLE results obtained using water and ethanol in our laboratory (Gallego, et al., 2019b). According to those results, B-phycoerythrin was better extracted at 25 °C and its extraction should be carried out before the lipid extraction, since proteins are very sensitive to thermal degradation. If the extraction of bioactives was carried out in reverse order, the optimal conditions for the extraction of lipids would be detrimental for the subsequent recovery of the protein. Furthermore, the re-extraction of the residual biomass from the previous step, the yield of the extraction process and the use of green solvents at ultra-high pressure condition were also considered to improve the sustainability, efficiency and the green character of the sequential process, respectively. The proposed UHPE process may open a wide range of possibilities for extracting compounds of different polarities from microalgae.

3.2.1. First step: B-phycoerythrin extraction

In the first step of the P. cruentum sequential extraction approach, B-PE was easily recovered from the supernatant of a single UHPE extract. It is worth to remark that this study is not focused on the total extraction and purification of this pigment, but rather on obtaining selective B-PE-enriched extracts. Therefore, B-PE results shown in Table 2 are expressed in mg/g extract instead of mg/g dry biomass. UHPE yields and B-PE content in the first step were above 7% and 34 mg/g extract, respectively, significantly higher than the values obtained using the PLE. The amount of B-PE recovered increased with pressure up to 300 MPa (144 mg/g extract), obtaining a richer B-PE extract than with the conventional extraction (93 mg/g extract). Nevertheless, results indicated that at the highest studied pressure (600 MPa), not only cell wall disruption was observed but also protein denaturation could occur. These results are in agreement with those obtained by Tran, et al. (2019b), who observed by micro-differential scanning calorimetry and electrophoresis, aggregation of B-PE protein at 500 MPa, while B-PE structure was not affected.
when treated for 5 min with pressures up to 300 MPa in protein extracts of *P. cruentum* previously obtained.

Similarly, Li, et al. (2020) studied the effect of different processes on the extraction of phyococyanin, which is another pigment-protein complex from the light-harvesting phycobiliprotein family. They extracted phyococyanin from *Arthrospira platensis* using high-pressure processing (HPP) among other techniques (pulsed electric fields and ultrasound). In this case, pressures up to 200 MPa did not seem to improve the release of the protein into the extraction buffer. Moreover, pressures above 400 MPa seemed to denature the conformation of this phycobiliprotein, as it occurred with phycoerythrin at 600 MPa.

Purity is another key response to consider, the protein purity values obtained at 100-300 MPa are higher than 2, which were also significantly higher than the obtained using PLE or conventional extraction. Therefore, the use of UHPE could be justified as B-PE has a high value in the market (approx. 637 € per mg, Sigma Aldrich). Generally, B-PE extraction required total cell disruption methods, such as sonication or grinding, which main drawback was the mixing of all intracellular components (Jubeau, et al., 2013). As a consequence, long and complex procedures, involving a combination of several techniques such as centrifugation or microfiltration, ammonium sulfate precipitation and different chromatographic techniques (ion-exchange chromatography or size exclusion) must be applied to purify B-PE (Bermejo-Roman, et al., 2002; Gaignard, et al., 2019). The B-PE purity index (PI) obtained with these methods achieved values up to 5 (Tang, et al., 2016) and extracts were considered to have a high purity level (for pharmaceutical or fluorescent uses) when values were above 4 (Camara-Artigas, et al., 2012). To decrease the number of unit operations below three, different authors proposed two-step processes for the B-PE extraction and purification. The purity index obtained in this study at 300 MPa is lower than the obtained after cell lysis by sonication and B-PE recovery by aqueous two-phase partition using poly(ethylene glycol) (PI = 3.2) (Benavides & Rito-Palomares, 2006), but it is similar to the one obtained after a two-step membrane filtration process (PI = 2.3).
Highlighting that the UHPE methodology only consists of a single and simple step of simultaneous disruption and extraction. In addition, it was observed how the purity decreased at 600 MPa, as well as the amount of extracted B-PE, which indicated protein denaturation. According to the SEM images (Figure 5), high pressure produced intense disruption of P. cruentum cell walls in the first sequential step. As observed in Figure 5B2 and B3, the cells were surrounded by shapeless rests, which may be the result of exopolysaccharides or cellular debris at pressures greater than 300 MPa. This fact partially disagreed with the observations done after confocal fluorescence microscopy pictures of P. cruentum resuspensions (Tran, et al., 2019a). In that study, no effects on cell morphology were observed after 5 min treatment up to 500 MPa. Therefore, the influence of the extraction time at ultra-high pressures should be further investigated to gain further insights on this issue.

### 3.2.2. Second step: carotenoids and fatty acids extraction

Starting from the residual biomass after B-PE extraction, the extraction of carotenoids was studied using ethanol, ethyl acetate and a mixture of ethanol and D-limonene 1:1 (v/v). These GRAS solvents were selected as other PLE studies at lower pressures demonstrated their potential application in the extraction of carotenoids from microalgae (Castro-Puyana, et al., 2013; Gallego, et al., 2019a; Sanchez-Camargo, et al., 2017). In order to compare the carotenoid extraction after UHPE at different pressures, a conventional acetone extraction at 20 °C and PLE using ethanol at 75 and 125 °C were performed in parallel starting from dried raw biomass. This step targeting carotenoids extraction was performed at 70 °C due to limitations of the UHPE equipment. Since ethanol and ethyl acetate have similar boiling points, the same temperature was used with ethanol and D-limonene 1:1 (v/v) mixture for comparative purposes. The results of the extractions are reported in Table 3. Regarding the extraction yield, the best results were
achieved using PLE at 125 °C, as expected. High temperature increases compounds solubility, favoring the mass transfer rate and decreasing solvent viscosity, which leads to higher yields. In terms of total carotenoids, all pressurized extractions produced significantly richer extracts than the conventional extraction, and the composition of the extracting solvent had a relevant influence on this factor as observed in Table 3. The most selective extraction of carotenoids was achieved using UHPE at 300 MPa with ethyl acetate. These compounds were characterized by HPLC-DAD-MS as described in section 3.1. Figure S2 in the Supporting Information shows an example of the chromatographic profiles of P. cruentum extracts. The number of pigments obtained by UHPE with all the solvents tested were the same, and also comparable to the PLE profile. Another common element among the carotenoids profiles was the fact that peaks 5 and 10 stood out as the most relevant ones, and they were confirmed as zeaxanthin and β-carotene, respectively. Their quantification showed significant differences among solvents (Table 3). Also, the use of ethyl acetate at 100-300 MPa was better for carotenoids extraction than the PLE optimum (125 °C), whereas the content of carotenoids using this solvent at 600 MPa had similar results as the PLE at 75 °C. Again, ethyl acetate at 300 MPa was the most suitable extraction condition for zeaxanthin and β-carotene recovery, improving the values obtained by PLE. Regarding the other two tested solvents, there were no significant differences between them in terms of total carotenoid extraction; however, better results were obtained using ethanol than using the mixture ethanol/limonene for zeaxanthin and β-carotene selective extraction. Moreover, we explored the potential of the UHPE process for the simultaneous extraction of carotenoids and fatty acids. Conventional extraction methods for fatty acids employed organic solvents, mainly hexane or chloroform:methanol 50:50 v/v (Goncalves, Pires, & Simoes, 2013), which are classified as hazardous for health and the environment (Boutekedjiret, Vian, & Chemat, 2014). Recent methods that assess the theoretical solubility parameters of solvents predicted D-limonene as the more environmentally respectful option for hexane substitution for the green selective extraction of low polarity bioactive compounds from natural sources.
For example, pure D-limonene was employed for the extraction of algae fatty acids from wet *Nannochloropsis oculata* and *Dunaliella salina* biomasses (Dejoye Tanzi, Vian, & Chemat, 2013). However, considering that in this case the objective was the simultaneous extraction of carotenoids and fatty acids, the use of pure D-limonene might not be the best option. As previously discussed, zeaxanthin, a xanthophyll that belongs to the group of the most polar carotenoids, is one of the main carotenoids present in *P. cruentum*. For this reason, the mixture ethanol:D-limonene 1:1 (v/v) was selected for this step. Also, earlier this mixture was used for the PLE of lipids from marine microorganisms (Golmakani, Mendiola, Rezaei, & Ibanez, 2014).

The GC-MS analysis of ultra-high pressure extracts revealed the same lipid profile as the conventional extracts carried out directly on the dry biomass. As an example, Figure S3 in the Supporting Information shows the fatty acid profile of a *P. cruentum* lipid extract obtained from the second step of the sequential process after UHPE. Major FAs in *P. cruentum* extracts were palmitic acid (C16:0), linoleic acid (C18:2ω6), arachidonic acid (C20:4ω6) and eicosapentanoic acid (EPA, C20:5ω3), in agreement with the observations of Rebolloso-Fuentes, et al. (2000), who used conventional extraction from the same matrix, *P. cruentum*. In addition, other FAs were identified in lower abundance, such as myristic acid (C14:0) and stearic acid (C18:0), among others.

Figure 6 shows the sum of the relative area per mg of extract for saturated FAs (SFAs), monounsaturated FAs (MUFAs) and polyunsaturated FAs (PUFAs). The specific case of EPA was also shown as an example of the behavior of PUFAs. Also, significant decreases of SFAs and MUFAs were achieved in the second UHPE step in comparison to the amounts of FAs in the extract obtained by conventional extraction (CE). Nevertheless, better results were obtained for PUFAs, including EPA and linoleic acid (data not shown), which are the most interesting compounds in terms of bioactivity. For these compounds, no significant differences were found between the extractions carried out at 100 and 300 MPa than the ones following the CE.
Therefore, these results were satisfactory since they demonstrated that an ethanol/limonene mixture at 100-300 MPa produced an effective disruption of cell walls and provided the same or higher recovery of PUFAs and carotenoids than the CE (Figure 6 and Table 3, respectively) after B-phycoerythrin extraction, avoiding the use of organic solvents. Furthermore, bioactive compounds such as B-phycoerythrin, carotenoids, and PUFAs were obtained from *P. cruentum* using this new sequential UHPE process with GRAS solvents (water, ethanol, ethyl acetate and D-limonene), showing its potential use for extraction from other microalgae.

4. CONCLUSIONS

The potential of ultra-high pressure extraction (UHPE) process was demonstrated as a fast and viable eco-friendly alternative for the extraction of bioactives of two microalgae biomasses, *Haematococcus pluvialis* and *Porphyridium cruentum*. UHPE improved the selectivity of carotenoid extraction from *H. pluvialis* in comparison with the conventional extraction, indicating that ultra-high pressure might affect the integrity of cell membrane and help the recovery of bioactive compounds. Moreover, when one extraction cycle was used in the UHPE, a more carotenoid-enriched extract was obtained compared to the pressurized liquid extraction (PLE), reaching values up to 119 mg of total carotenoids per g of extract. For *Porphyridium cruentum*, high-value compounds such as B-phycoerythrin, carotenoids and PUFAs were effectively and separately extracted using a two step-UHPE process. In particular, a first extraction step using water at 300 MPa provided extracts significantly enriched in B-phycoerythrin with a satisfactory purity index of 2.37. Subsequently, the extraction of the residual biomass in the second step, provided extracts enriched in carotenoids when ethyl acetate was used, and enriched in PUFAs when a mixture of ethanol and limonene (1:1 v/v) was used. In summary, results obtained demonstrated the efficiency and green character of the UHPE process when more environmentally friendly solvent alternatives are employed for the valorization of microalgae biomass.
ACKNOWLEDGMENTS

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Supplementary material

HPLC-DAD-MS/MS parameters. Example of carotenoid profile from *H. pluvialis* obtained by the UHPE. Example of HPLC-DAD profile of the *P. cruentum* carotenoids obtained in the second step of the UHPE process. Example of GC-MS profile of the *P. cruentum* fatty acids obtained in the second step of the UHPE process.

Conflicts of Interest

The authors declare no conflicts of interest in the publication of this research.

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**Figure caption**

**Figure 1.** Scheme of the UHPE processes proposed for the valorization of microalgae biomass.

**Figure 2.** Scheme of the UHPE multivessel system apparatus U111 Unipress. Reprinted from Ref. (Martinez-Monteagudo, et al., 2015) with permission from Elsevier, Copyright 2015.

**Figure 3.** SEM images of *H. pluvialis* initial biomass (A) and after 1 cycle of 20 min using ethanol ultra-high pressure extraction at 100 MPa (B), 300 MPa (C), and 600 MPa (D).

**Figure 4.** Ultra-high pressure extraction of carotenoids from *H. pluvialis* using 3 cycles of 7+7+6 min with ethanol at 50°C. Different letters show significant differences (*p*<0.05).

**Figure 5.** SEM images of *P. cruentum* before the extraction process (A), and residues after the first step of UHPE using water (25 °C) at 100 MPa (B1), 300 MPa (B2) and 600 MPa (B3).

**Figure 6.** UHPE of fatty acids using ethanol:D-limonene (1:1, v/v) mixture at different pressures. Different letters within the same group of fatty acids indicate significant differences (*p*<0.05). SFAs: saturated fatty acids; MUFAs: monounsaturated fatty acids; PUFAs: polyunsaturated fatty acids; EPA: eicosapentanoic acid.
**Figure 1**

- **H. pluvialis** biomass → Ethanol, 50°C, 20 or 7+7+6 min → Residual biomass
- **P. cruentum** biomass → Water, 25°C, 20 min → GRAS solvents, 70°C, 20 min → Residual biomass

- Proteins
- Carotenoids or carotenoids + PUFAs
Figure 2

Hydraulic system

Vessel 4  Vessel 3  Vessel 2  Vessel 1

Thermostat Circulator
Figure 3
Figure 4
Figure 5
Table 1. Extraction yield and total carotenoids content obtained by conventional extraction, PLE and UHPE from *H. pluvialis* with ethanol at 50 °C.

<table>
<thead>
<tr>
<th>Extraction process</th>
<th>Time (min)</th>
<th>Extraction cycles</th>
<th>Pressure (MPa)</th>
<th>Extraction yield (%)</th>
<th>Total carotenoids (mg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1440</td>
<td>1</td>
<td>0.1</td>
<td>8.52 ± 0.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>59.06 ± 8.99&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLE</td>
<td>20</td>
<td>1</td>
<td>10</td>
<td>5.44 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>91.57 ± 6.83&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>UHPE</td>
<td>20</td>
<td>1</td>
<td>100</td>
<td>4.20 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>119.34 ± 7.34&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>4.06 ± 0.08&lt;sup&gt;c&lt;/sup&gt;</td>
<td>113.78 ± 0.95&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>600</td>
<td>4.09 ± 0.59&lt;sup&gt;c&lt;/sup&gt;</td>
<td>109.74 ± 3.60&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>7+7+6</td>
<td>3</td>
<td>100</td>
<td>5.24 ± 0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>98.69 ± 6.43&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>300</td>
<td>5.02 ± 0.61&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>95.08 ± 5.72&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>600</td>
<td>5.77 ± 0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>102.32 ± 11.61&lt;sup&gt;abcd&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Conventional extraction used acetone at 25 °C; PLE: pressurized liquid extraction; UHPE: ultra-high pressure extraction. Different letters in the same column show significant differences (p<0.05).
Table 2. Extraction yield, total B-phycoerythrin and its purity obtained by UHPE of *P. cruentum* biomass. Other extraction conditions: water, 25 °C, 20 min (or 60 min for conventional).

<table>
<thead>
<tr>
<th>Extraction process</th>
<th>Pressure (MPa)</th>
<th>Extraction yield (%)</th>
<th>B-Phycoerythrin (mg/g extract)</th>
<th>Purity (A545/A280)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>0.1</td>
<td>36.14 ± 0.18</td>
<td>93.07 ± 2.68</td>
<td>1.71 ± 0.05</td>
</tr>
<tr>
<td>PLE</td>
<td>10</td>
<td>3.03 ± 0.19</td>
<td>13.21 ± 0.77</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>12.19 ± 1.71</td>
<td>86.97 ± 7.88</td>
<td>2.16 ± 0.11</td>
</tr>
<tr>
<td>UHPE</td>
<td>300</td>
<td>7.43 ± 0.46</td>
<td>144.43 ± 5.00</td>
<td>2.37 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>16.06 ± 3.42</td>
<td>33.51 ± 3.76</td>
<td>0.96 ± 0.05</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate significant differences (p<0.05). PLE: pressurized liquid extraction; UHPE: ultra-high pressure extraction.
<table>
<thead>
<tr>
<th>Extraction process</th>
<th>T (°C)</th>
<th>Solvent</th>
<th>Pressure (MPa)</th>
<th>Extraction yield (%)</th>
<th>Total carotenoids (mg/g extract)</th>
<th>Zeaxanthin (mg/g extract)</th>
<th>β-carotene (mg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional</td>
<td>25</td>
<td>Acetone</td>
<td>0.1</td>
<td>7.67 ± 0.49&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.25 ± 1.86&lt;sup&gt;f&lt;/sup&gt;</td>
<td>7.49 ± 0.07&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>n.d.</td>
</tr>
<tr>
<td>PLE</td>
<td>75</td>
<td>EtOH</td>
<td>10</td>
<td>4.13 ± 0.16&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36.09 ± 3.95&lt;sup&gt;c&lt;/sup&gt;</td>
<td>13.85 ± 0.76&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>5.78 ± 0.45&lt;sup&gt;cd&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>125</td>
<td></td>
<td></td>
<td>9.00 ± 0.67&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43.15 ± 0.84&lt;sup&gt;b&lt;/sup&gt;</td>
<td>19.11 ± 4.33&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>6.48 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.00 ± 0.17&lt;sup&gt;c&lt;/sup&gt;</td>
<td>24.46 ± 1.79&lt;sup&gt;d&lt;/sup&gt;e</td>
<td>6.27 ± 1.05&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>4.52 ± 0.65&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
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<td>3.44 ± 0.16&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>22.40 ± 1.53&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4.29 ± 0.71&lt;sup&gt;d&lt;/sup&gt;e</td>
<td>4.07 ± 0.07&lt;sup&gt;e&lt;/sup&gt;</td>
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<tr>
<td></td>
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<td></td>
<td>3.73 ± 0.68&lt;sup&gt;c&lt;/sup&gt;</td>
<td>21.31 ± 2.03&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.08 ± 0.08&lt;sup&gt;cde&lt;/sup&gt;</td>
<td>3.62 ± 0.47&lt;sup&gt;ef&lt;/sup&gt;</td>
</tr>
<tr>
<td>UHPE</td>
<td>70</td>
<td>EtOH/D-</td>
<td>100</td>
<td>0.99 ± 0.18&lt;sup&gt;e&lt;/sup&gt;</td>
<td>65.05 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.75 ± 5.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.89 ± 0.34&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td></td>
<td></td>
<td>limonene</td>
<td>300</td>
<td>1.37 ± 0.19&lt;sup&gt;e&lt;/sup&gt;</td>
<td>30.24 ± 0.09&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>10.97 ± 0.01&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>3.92 ± 0.34&lt;sup&gt;e&lt;/sup&gt;</td>
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<td>3.64 ± 0.14&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>26.57 ± 0.15&lt;sup&gt;de&lt;/sup&gt;</td>
<td>4.42 ± 0.07&lt;sup&gt;d&lt;/sup&gt;e</td>
<td>2.40 ± 0.24&lt;sup&gt;fg&lt;/sup&gt;</td>
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<td></td>
<td></td>
<td></td>
<td>3.49 ± 0.05&lt;sup&gt;cd&lt;/sup&gt;</td>
<td>24.81 ± 0.10&lt;sup&gt;de&lt;/sup&gt;</td>
<td>2.94 ± 0.36&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.07 ± 0.21&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.86 ± 0.10&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.03 ± 0.37&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.01 ± 0.01&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.63 ± 0.01&lt;sup&gt;g&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different letters in the same column indicate significant differences (p<0.05); T: temperature; EtOH: ethanol; EtOAC: ethyl acetate; PLE: pressurized liquid extraction; UHPE: ultra-high pressure extraction, n.d: not determined.