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Ultrasound-based strategies for the recovery of microalgal carotenoids: Insights from green extraction methods to UV/MS-based identification

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ABSTRACT

Carotenoids, versatile natural pigments with numerous health benefits, face environmental concerns associated with conventional petrochemical-based extraction methods and limitations of their synthetic equivalents. In this context, this study aims to introduce eco-friendly approaches using ultrasound-based strategies (probe and bath) for the extraction of carotenoids from microalgae, initially focusing on *Microchloropsis gaditana* and subsequently evaluating the versatility of the method by applying it to other microalgae species of interest (*Tisochrysis lutea*, *Porphyridium cruentum*, and *Phaeodactylum tricornutum)* and defatted microalgal residues. Among the approaches evaluated, the 5-min ultrasonic probe system with ethanol showed comparable carotenoid recovery efficiency to the reference method (agitation, 24 h, acetone) (9.4 ± 2.5 and 9.6 ± 3.2 mg g^{-1} carotenoids per dry biomass, for the green and the reference method, respectively). Moreover, the method's sustainability was demonstrated using the AGREEprep™ software (scored 0.62 out of 1), compared to the traditional method (0.22 out of 1). The developed method yielded high carotenoid contents across species with diverse cell wall compositions (3.1 ± 0.2, 2.1 ± 0.3, and 4.1 ± 0.1 mg g^{-1} carotenoid per dry biomass for *T. lutea*, *P. cruentum*, and *P. tricornutum*, respectively). Moreover, the application of the method to defatted biomass showed potential for microalgal valorization with carotenoid recovery rates of 41 %, 60 %, 61 %, and 100 % for *M. gaditana*, *P. tricornutum*, *T. lutea*, and *P. cruentum*, compared to the original biomass, respectively. Furthermore, by using high-performance liquid chromatography with a diode array detector (HPLC-DAD) and high-resolution mass spectrometry (HRMS), we reported the carotenoid and chlorophyll profiles of the different microalgae and evaluated the impact of the eco-friendly methods. The carotenoid and chlorophyll profiles varied depending on the species, biomass, and method used. In summary, this study advances a green extraction method with improved environmental sustainability and shorter extraction time, underscoring the potential of this approach as a valuable alternative for the extraction of microalgal pigments.

1. Introduction

Carotenoids are lipophilic pigments widely distributed in nature, found in photosynthetic bacteria, algae, plants, certain species of archaea and fungi, along with various groups of arthropods (González-Peña et al., 2023; Misawa et al., 2021). Their basic structure comprises eight isoprene units, resulting in a C_{40} backbone. Carotenoids can be divided into two primary groups: carotenes (e.g., α-carotene, β-carotene, γ-carotene, and lycopene) consisting of hydrocarbons, and xanthophylls (e.g., violaxanthin or fucoxanthin) which are oxygenated carotenoids with various functional groups in their molecular structures (**Figure S1**).

The importance of carotenoids is linked to their role in various health-promoting activities. For a long time, carotenoids were primarily known for their contribution to nutrition as precursors of vitamin A and for their antioxidant properties. However, in more recent studies, they have also been associated with a decreased risk of cardiovascular diseases (Hou et al., 2020), the prevention of cataract and age-related macular degeneration (Manayi et al., 2016), inhibition of liver fibrosis (B. Kim et al., 2017), as well as anti-cancer (Kavalappa et al., 2019) and anti-obesity properties (Kurniawan et al., 2023). Moreover, carotenoids have been reported as promising neuroprotective agents for Alzheimer's disease (Batool et al., 2022).

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Fruits and vegetables are commonly recognized as the primary dietary sources of carotenoids since these compounds cannot be synthesized by animals, including humans, and must be obtained through the diet (Santos et al., 2021). Although chemically synthesized carotenoids currently dominate the market due to their faster and more cost-effective production, in some cases, they could lack the health-promoting properties attributed to their natural counterparts (Bogacz-Radomska & Harasym, 2018). These findings, along with the growing global population and the increasing consumer demand for natural products, corroborate the significant need for novel sources. In this context, it is noteworthy that the production of carotenoids in plants is typically limited in quantity. However, microalgae, an emerging alternative source of these compounds, provide a wide range of carotenoids at a much faster growth rate than plants (Bermejo et al., 2021). Moreover, microalgal carotenoids could offer advantages compared to their synthetic analogs. For instance, natural microalgal β-carotene contains a mix of *cis*- and all-*trans* isomers, while the synthetic version consists only of the all-*trans* isomer and exhibits weaker antioxidant capacities (J. Wang et al., 2022). Similarly, natural microalgal astaxanthin is mostly esterified, whereas its synthetic equivalent is unesterified and less effective in combating oxidative stress (Capelli et al., 2013).

Microalgae are unicellular photosynthetic microorganisms present in all aquatic ecosystems. They convert sunlight, water, and carbon dioxide into algal biomass. Microalgae are rich not only in carotenoids and chlorophylls but also in a wide range of high-valuable compounds, including ω-3 fatty acids, proteins, polysaccharides, vitamins, and phenolic compounds, among others, making them an exciting research subject and potential natural sources of these compounds for various industries (Barba, 2017; Castejón & Señoráns, 2019). Regarding pigments, certain species, such as *Dunaliella salina* and *Haematococcus pluvialis*, have gained significant commercial recognition as prominent sources of β-carotene and astaxanthin, respectively (Sousa et al., 2023). However, many other species remain unexplored.

The extraction of carotenoids from microalgae poses challenges due to their intracellular location within chloroplasts, which is a disadvantage because the presence of cell walls and membranes often restricts access to these valuable compounds (Spain & Funk, 2022). Conventional methods to recover carotenoids are based on solvent extraction using acetone or petrochemical solvents such as hexane or petroleum ether. Moreover, these conventional methods, including, for example, distillation, agitation, or centrifugation, have long been known for their energy-intensive nature, which poses significant challenges in aligning with sustainable and environmentally friendly practices (Viñas-Ospino et al., 2023). However, multiple new extraction techniques have been reported in the literature, focusing on less toxic solvents, time- and energy efficiency (Gallego et al., 2021; Sánchez-Camargo et al., 2018). Ultrasound-assisted extraction (UAE) has gained recognition as an efficient technique for disrupting the cell walls of numerous microalgae species. This process facilitates solvent penetration into the cells, enhancing mass transfer and releasing the targeted compounds; however, its effectiveness relies on the choice of solvent (Tiwari, 2015). The importance and potential of green solvents in combination with UAE for microalgal carotenoids have been explored. Examples include the use of 2-methyltetrahydrofuran and ethyl lactate (Morón-Ortiz et al., 2024), ethanol (Vintila et al., 2022, Deenu et al., 2013) or ethanol and water mixtures (Jaeschke et al., 2017). Another critical concern today is the sustainable valorization of microalgal biomass. The simultaneous or sequential extraction of valuable compounds from microalgae minimizes waste and fully utilizes the feedstocks, making the biorefinery concept a more sustainable option (Katiyar et al., 2021). Thus, the search for new integrated approaches that would follow the principles and guidelines of Green Chemistry is a primary objective in the current landscape.

Identification and quantification of carotenoids also present challenges. In high-performance liquid chromatography (HPLC), the typical detectors for carotenoid identification are UV–Vis and diode-array detector (DAD). However, the UV–Vis spectra of many carotenoids exhibit similarities, making their identification difficult (Rivera & Canela-Garayoa, 2012). The adoption of mass spectrometry (MS) since pioneering works in the 1960 s (Schwieter et al., 1969) and with new advances in the early 2000 s marked a substantial advancement compared to the spectrophotometric UV–Vis analysis techniques (Rivera et al., 2014). Different improvements have been made to distinguish between co-eluting carotenoids (Rivera & Canela-Garayoa, 2012), to assess the presence of functional and end groups in carotenoid structures (Lacker et al., 1999), to gain a rapid overview of the carotenoid composition, and to classify samples (Fraser et al., 2007).

Therefore, this study introduces environmentally sustainable extraction approaches for microalgal carotenoids using ultrasound-based strategies (probe and bath). Initially, we explored different green solvents and extraction conditions using *Microchloropsis gaditana* (formerly *Nannochloropsis gaditana*) as a model microalga while assessing the method's sustainability. The developed green method was applied to other microalgae of interest (*Tisochrysis lutea, Porphyridium cruentum*, and *Phaeodactylum tricornutum*) to assess the method's adaptability and limitations across different species. To overcome concerns about single-component approaches, we also explored the potential of biomass valorization by utilizing defatted microalgal biomass to assess the co-production of lipids and carotenoids. Furthermore, this study aimed to comprehensively identify and quantify the carotenoid content within all four microalgae species using HPLC-DAD and MS analysis and assess the impact of the extraction methods on the carotenoid profile. The findings of this study have the potential to establish greener strategies for extracting valuable microalgal pigments with applications in the food and nutraceutical industries.

2. Materials and methods

2.1. Samples and reagents

The spray-dried microalgal biomass of *Microchloropsis gaditana* (batch 02092021Ng), *Tisochrysis lutea* (batch 02092021TL), *Phaeodactylum tricornutum* (batch 02092021Pt), *Porphyridium cruentum* (batch 02092021Pc) was purchased from Cianoalgae SI (Gipuzkoa, Spain).

Acetone, absolute ethanol (both analytical reagent grade), methanol (HPLC grade), and β-carotene (99 %) were purchased from Fisher Scientific GmbH (Vienna, Austria). Acetonitrile (HPLC grade), 2-propanol (≥99.8 %, ACS reagent), butylated hydroxytoluene (BHT), vitamin K_1 (≥99.0 %, HPLC), chlorophyll *a* and *b* (analytical standard grade), violaxanthin (analytical standard grade), and neoxanthin (analytical standard grade) were obtained from Sigma-Aldrich (Munich, Germany). Astaxanthin (≥96 %) and fucoxanthin (≥98 %) were acquired from Carbosynth (now Biosynth AG, Staad, Switzerland).

2.2. Ultrasound-assisted extraction (UAE)

For the UAE, the samples were subjected to two different treatments: (i) an ultrasonic bath and (ii) an ultrasonic probe. Different solvents were investigated: ethanol and acetone, both authorized for food use in the European Union (European Union, 2009), and their mixtures with water (70:30, 50:50, 30:70, *v/v*).

2.2.1. Ultrasonic bath

The extractions were conducted using an ultrasound water bath apparatus (Elmasonic P 30H, Elma Schmidbauer GmbH, Singen, Germany), which featured automated regulation of time and temperature, following the procedure previously described with minor modifications (Ruiz-Domínguez et al., 2023; Zou et al., 2013). Dried microalgal biomass was dispersed in the solvent at a ratio of 1:10 (*w/v*). The mixture was sonicated (37 kHz and 100 W) for 15 min at room temperature and then centrifuged for 10 min at 3148 rcf (4000 rpm in a ROTINA 420R, Hettich Lab, Tuttlingen, Germany) at 20 °C. The

supernatant was collected and evaporated using CentriVap Complete Vacuum Concentrators from Labconco (Kansas City, MO, USA). The extraction yield was determined gravimetrically and calculated as a weight percentage of dry biomass (Equation (1)).

$$Yield(\%) = \frac{weightofdryextract}{weightofdrybiomass} \times 100 \quad (1)$$

For further analysis, the extracts were redissolved in methanol (10–20 mg mL $^{-1}$) and filtered with a sterile polytetrafluoroethylene (PTFE) filter (Carl Roth GmbH + Co., Karlsruhe, Germany) (0.22μ m, 13 mm). Samples were stored in solution at −21 °C.

2.2.2. Ultrasonic probe

Extractions were carried out using an ultrasound probe system (Branson Sonifier 450 with probe (model: 102C (CE), Danbury, CT, USA) following the procedure previously described with minor modifications (da Silva Lima et al., 2020). Dried microalgal biomass was dispersed in the solvent at a ratio of 1:10 (*w/v*). The mixture was sonicated for 5 min with a duty cycle of 50 % and a wave amplitude of 20 %, with the probe immersion depth set to half of the medium's volume, approx. 2 cm. The temperature was maintained using an ice bath to prevent it from increasing. After the treatment, samples were treated as previously described for the ultrasonic bath.

3. Reference method for carotenoid extraction

To select the optimum green method, results were compared using a protocol previously described (reference method), using *M. gaditana* biomass (Castro-Puyana et al., 2013; Gallego et al., 2021). Dried microalgal biomass was dispersed in acetone at a ratio of 1:100 (*w/v*) with 0.1 % (*w/v*) BHT. The mixture was agitated (500 rpm) at room temperature for 24 h and then filtered using a cellulose filter (MN 615 ff 110 mm, Macherey-Nagel GmbH & Co. KG, Dueren, Germany). The filtrate was collected and evaporated using a rotary evaporator (Heidolph Hei-VAP Value HB/G3, Schwabach, Germany) under reduced pressure at room temperature. The extraction yield was determined gravimetrically (Equation (1), and the extracts were treated as previously described for the ultrasonic bath.

3.1. Sustainability assessment of the extraction methods

AGREEprep™ open access software was used to assess the greenness of the proposed extraction methods (Wojnowski et al., 2022). This software was recently proposed by Wojnowski *et al.* as a practical tool to study the environmental impact of the sample preparation step. This evaluation involves the consideration of 10 impact categories, which are subsequently recalculated and converted into sub-scores on a 0–1 scale. These sub-scores are then utilized to calculate the final assessment score. A score of 1 denotes the most environmentally friendly method, while a score of 0 indicates the less sustainable approach, and the results are illustrated with a pictogram. The assessment criteria encompass various factors based on ten principles of green sample preparation (GSP) (López-Lorente et al., 2022).

3.2. Production of defatted microalgal biomass

Lipid extraction was performed according to a previously developed method using ultrasounds (30 min, 30 °C) (Castejón & Marko, 2022). After lipid extraction, the residual microalgal biomass (defatted biomass) was dried overnight at room temperature and stored at −21 °C until further use. Carotenoid extraction from defatted biomass was carried out using the optimized ultrasound method (5 min, ultrasonic probe system with ethanol) following the procedure described in section 2.2.2.

3.3. Chemical characterization of microalgal extracts

The identification of chlorophylls and carotenoids from microalgal extracts was performed using HPLC-DAD and high-resolution mass spectrometry (HRMS), as described below.

3.3.1. High-performance liquid chromatography with diode-array detection (HPLC-DAD)

The applied HPLC method is based on the method described by Li *et al.* with some minor modifications (Li et al., 2021). The equipment used was an Agilent 1200 series HPLC-DAD system (Santa Clara, CA, USA) and ChemStation for LC 3D system software (Rev. B.04.01 SP1 [647]) for the data evaluation. For separation, the HPLC system was equipped with a YMC-C30 reversed-phase column (250 × 4.6 mm, 5 µm particle size; YMC Europe, Schermbeck, Germany) with a pre-column of the same material (10 × 4 mm, 5 µm particle size). The mobile phase (A) comprised 100 % methanol, and the mobile phase (B) was a mixture of isopropanol and acetonitrile (1:1, *v/v*). Carotenoids and chlorophylls were eluted according to the following gradient: a 5-min equilibration time at 5 % B was followed by a linear gradient to 20 % B within the next 5 min. The percentage of B was further increased to 30 % in the next 10 min and to 45 % within another 8 min. Thereafter, the gradient was switched to 95 % within 1 min, and the column was flushed for 10 min with this composition. Thereafter, a fast switch back to the starting conditions was performed, and the column was equilibrated until the end of the total run time of 45 min. A flow rate of 1 mL min^{-1} and capillary diameters of 0.25 mm were used. The DAD recorded signals at 450, and 660 nm.

3.3.2. Total carotenoid content determination

For the semi-quantification of the total carotenoid content of all microalgal extracts, the previously described HPLC-DAD method at 450 nm was used. First, an external calibration curve plotting the peak area versus the concentration of β-carotene (0.0025 – 0.5 mg mL^{-1}) was used to determine the concentrations of the other carotenoids ($R^2 = 0.9971$). To consider the differences in detector response, a molecular-weight-correction factor (determined by dividing the molecular weight of the carotenoid to be quantified by that of the standard) was applied according to Urreta et al. (2014). Results were expressed as mg carotenoids per g of biomass. Relative abundance (%) was calculated by dividing the peak area of a compound by the total area of all compounds identified within the same sample multiplied by 100. The limit of detection (LOD) and the limit of quantification (LOQ) were determined from the calibration curve following the equations previously described (Vial & Jardy, 1999): LOD = 0.036 mg mL^{-1}, LOQ = 0.11 mg mL^{-1}.

3.3.3. Identification of carotenoid fractions using high-resolution mass spectrometry (HRMS)

For MS analysis, fractions of the main pigments were collected according to the retention time of the HPLC-DAD chromatogram at 450 nm. Thereafter, 250 µL of each fraction were injected into a maXis UHR APCI-Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) by direct infusion in the positive ionization mode. The sum formulas of the detected ions were determined using Bruker Compass DataAnalysis 4.1 based on the mass accuracy ($\Delta m/z \leq 5$ ppm) and isotopic pattern matching (SmartFormula algorithm). MS and MS-MS data were evaluated using the SIRIUS + CSI:FingerID GUI (MS2 mass accuracy 10 ppm, candidates scored 10, Mg added as an extra element, $[M + H]^+$, $[M + K]^+$ and $[M + Na]^+$ ionizations) and CLI (Version 5.8.2) and the GNPS database (M. Wang et al., 2016). The levels of identification in MS were defined according to Schymanski *et al.*; an overview is shown in **Table S1** (Schymanski et al., 2014).

3.4. Statistics

All the extraction experiments were conducted in triplicate, and the

results were presented as the mean value ± the corresponding standard deviation (SD). A one-way ANOVA was employed to assess the impact of both the extraction method and the conditions on the carotenoid content, followed by the Tukey post-hoc test for pairwise comparisons (statistical significance was determined at $p < 0.05$). The statistical evaluation was carried out using OriginPro 2021 (version 9.8.0.200) by OriginLab Corporation (Northampton, MA, USA).

4. Results and discussion

4.1. Developing a green extraction method for microalgal carotenoids

To assess the efficacy of ultrasonic bath and probe systems, *M. gaditana* was selected as a model microalga. This choice was made because it possesses one of the most robust cell walls, characterized by a bilayer structure consisting of cellulose in the inner layer and a hydrophobic algaenan layer on the outer surface (Scholz et al., 2014). Thus, the extraction yield and the total carotenoid content were investigated for this microalga species using ultrasonic bath and probe systems with eight different solvents, namely acetone, ethanol, and their mixtures with water (70:30, 50:50, 30:70, *v/v*) (Table 1).

During our investigations, we observed that the use of the probe system yielded better overall recovery of carotenoids than the ultrasound bath. Moreover, single solvents proved to be a better alternative than mixtures with water for both ultrasonic probe and bath systems. Notably, while the introduction of water was chosen to determine the optimal percentage of pure solvents in the mixtures, the nonpolar nature of carotenoids led to significantly lower rates when solvent–water mixtures independent of the percentage water used ($p < 0.05$ compared to pure solvents). Moreover, the solvent–water mixtures increased the extraction yield compared to single solvents, suggesting the potential co-extraction of interfering substances with a higher polar nature when water is in the mixture.

The most favorable results in terms of total carotenoid content were observed for the ultrasound probe system using acetone and ethanol. When compared to the reference method (16.9 % ± 3.4 % extraction yield and 9.6 ± 3.2 mg g^{-1} carotenoids per dry biomass), the use of the probe system with ethanol demonstrated a significantly similar carotenoid recovery ($p > 0.05$), while the use of acetone yielded slightly lower results than the reference method ($p < 0.05$). Importantly, these approaches considerably reduced the extraction time (24 h for the reference method vs. 5 min for the ultrasonic probe system), and also, the need to incorporate BHT as an antioxidant was eliminated due to the shorter duration of the process. Subsequently, these three approaches were evaluated to determine their environmental sustainability using AGREEprep™ software, as discussed in section 3.2.

It is worth highlighting that comparing results with the literature may be challenging in some cases, as the initial amount of carotenoids present in the starting materials can vary even when working with the same microalgae species. It is known that the cultivation conditions of microalgae (such as light intensity, temperature, nutrient availability, and pH) can significantly influence their composition, including lipids, chlorophylls, and carotenoids (Mitra et al., 2015), thus complicating the comparison between different sources. Another point to consider is the expression of the results, which in some cases could vary between total carotenoids per extract, per dry biomass or per volume of culture. Considering these factors, it is essential to undertake the comparison with caution. Overall, our findings were in the range of previous studies conducted on *M. gaditana*. For instance, the total carotenoid content was comparable to that reported by Menegol et al., ranging between 1.0 and 1.2 % of dry weight (Menegol et al., 2019). Di Lena *et al.* reported a lower total carotenoid content (4.46 ± 4.2 mg g^{-1} carotenoids per dry biomass) for the same microalgae species compared to this study, using a similar approach involving ultrasounds but using an acetone-methanol mixture (Di Lena et al., 2019). Other authors reported a similar carotenoid content but using supercritical fluid extraction (SFE) with pure CO_2 and ethanol (Sánchez-Camargo et al., 2018) or high-pressure processes (pressurized liquid extraction (PLE) or ultra-high pressurized extraction UHPE)) for the last one using *Nannochloropsis oceanica*, a microalga from the same family (*Monodopsidaceae*) (Gallego et al., 2021). Our method could offer simplified techniques, distinguishing it from SFE and UHPE, which require substantial quantities of biomass and solvent, along with specialized equipment.

4.2. Assessing the environmental sustainability of the extraction methods

Fig. 1 shows the environmental sustainability using AGREEprep™ software of the most promising extraction methods in terms of total carotenoid content selected in section 3.1: (a) the reference method, (b) 5-min ultrasonic probe system with acetone, and (c) 5-min ultrasonic probe system with ethanol.

The 5-min ultrasonic probe system with ethanol achieved the highest AGREEprep™ score (0.62 out of 1), indicating its superior environmental friendliness, followed by the same system with acetone (0.43 out of 1), while the reference method received the lowest score (0.22 out of 1). Notably, important distinctions between the different extraction approaches are evident across several categories evaluated by AGREEprep™ software. For instance, categories 2 and 10 provide insights into the employment of hazardous materials and associated risks to the operator. In our approach, the choice between solvents, either ethanol or acetone, was guided by the CHEM21 solvent classification guide (Prat et al., 2016). According to this guide, ethanol is classified as recommended, while acetone raises concerns due to its potential to produce volatile organic compounds (VOCs) and flammability. Consequently, in

Table 1
Extraction yield and total carotenoid content of *Microchloropsis gaditana* extracts using ultrasonic bath and probe systems with different solvents.

Solvent	Ultrasonic bath		Ultrasonic probe	
	Yield [%]	Total carotenoid content [mg carotenoids g^{-1} dry biomass]	Yield [%]	Total carotenoid content [mg carotenoids g^{-1} dry biomass]
Acetone	$3.7 \pm 0.2^{c, B}$	$3.8 \pm 0.6^{ab, B}$	$7.6 \pm 0.9^{d, A}$	$7.2 \pm 0.4^{a, A}$
Acetone: H_2O (70:30)	$12.7 \pm 1.5^{bc, A}$	$2.8 \pm 0.4^{c, A}$	$9.1 \pm 3.4^{d, A}$	$2.8 \pm 1.1^{b, A}$
Acetone: H_2O (50:50)	$8.2 \pm 0.2^{c, A}$	$0.7 \pm < 0.1^{e, B}$	$6.8 \pm 0.4^{d, B}$	$0.9 \pm 0.1^{b, A}$
Acetone: H_2O (30:70)	$25.4 \pm 3.9^{b, A}$	$0.9 \pm 0.1^{de, B}$	$14.0 \pm 1.4^{bcd, B}$	$2.3 \pm 0.3^{b, A}$
Ethanol	$7.0 \pm 0.4^{c, B}$	$4.5 \pm 0.2^{a, B}$	$11.6 \pm 2.3^{cd, A}$	$9.4 \pm 2.5^{a, A}$
Ethanol: H_2O (70:30)	$12.8 \pm 0.3^{bc, B}$	$3.1 \pm 0.1^{bc, A}$	$21.9 \pm 0.8^{b, A}$	$1.2 \pm 0.1^{b, B}$
Ethanol: H_2O (50:50)	$42.1 \pm 3.4^{a, A}$	$1.9 \pm 0.1^{d, A}$	$34.6 \pm 3.5^{a, A}$	$1.0 \pm 0.1^{b, B}$
Ethanol: H_2O (30:70)	$41.5 \pm 12.0^{a, A}$	$1.5 \pm 0.4^{de, A}$	$19.8 \pm 9.3^{bc, A}$	$1.1 \pm 0.4^{b, A}$

Data are shown as mean ± standard deviation (n = 3). The extractions for the ultrasonic bath were conducted at room temperature for 15 min, 1:10 biomass to solvent ratio, and for the ultrasonic probe, on ice, for 5 min, 1:10 biomass to solvent ratio. Lowercase letters indicate statistically significant differences in each column (i.e., statistically significant differences in solvents), whereas capital letters indicate statistically significant differences between the ultrasonic bath and probe systems (one-way ANOVA with post-hoc Tukey, $p < 0.05$).

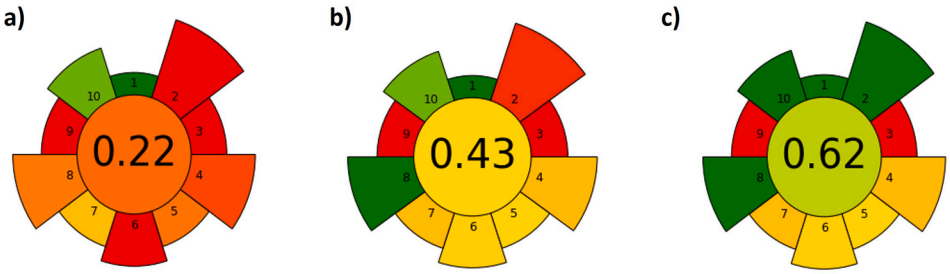

Fig. 1. AGREEprep™ scores of different extraction methods: (a) reference 24-h method (acetone with 0.1 % (*w/v*) butylated hydroxytoluene (BHT), 24 h, 1:100 biomass to solvent ratio), (b) 5-min ultrasonic probe system with acetone (acetone, 5 min, 1:10 biomass to solvent ratio) and (c) 5-min ultrasonic probe system with ethanol (ethanol, 5 min, 1:10 biomass to solvent ratio).

the context of our study, we consider acetone as potentially hazardous. This further supports our preference for ethanol-based extractions, which are both operator- and environmentally friendly. In categories 4 (waste) and 5 (size economy), improvements are evident when transitioning from the reference method, which used a 1:100 biomass to solvent ratio (*w/v*), to the ultrasonic methods with a 1:10 ratio. Category 6, which assesses sample throughput, focuses on the number of samples processed per hour. The introduction of ultrasound-based approaches has notably reduced the extraction times from 24 h in the reference approach to just 5 min, resulting in a remarkable increase in sample throughput. Moreover, in terms of energy consumption (category 8), a comparative evaluation reveals that the reference method consumes 191 Wh, whereas the 5-min ultrasonic probe systems consume considerably lower amounts of energy (7.33 Wh). Thus, considering all the above mentioned, these findings unequivocally position the 5-min ultrasonic probe system using ethanol as the optimal choice within the scope of our investigation.

4.3. Application of the green method to other microalgae species

The efficacy of the developed green method (5-min ultrasonic probe system with ethanol) was evaluated on three different photoautotrophic eukaryotic microalgae species of interest, aiming to explore its versatility (Fig. 2). The microalgae species *T. lutea, P. tricornutum,* and *P. cruentum* were selected due to their different nature and their potential as sources of carotenoids and chlorophylls. Moreover, by selecting these species, we achieved a diverse taxonomic spectrum across different phyla: Haptophyta (*T. lutea*), Heterokontophyta (*P. tricornutum* and *M. gaditana*), Rhodophyta (*P. cruentum*). Overall, while using the green approach, the extraction yield was similar to the reference method for all the microalgae species tested, however, the carotenoid recovery rates were lower than expected (38 %, 50 %, and 54 % for *T. lutea*, *P. cruentum*, and *P. tricornutum*, respectively). Even the ultrasonic probe system with ethanol did not work as well as it did for *M. gaditana*. It is worth noting that a high carotenoid content was extracted in only 5 min, potentially facilitating high-throughput sampling. The distinct cell wall compositions may elucidate the method's efficacy across species. For instance, the composition of monosaccharides in cell wall polysaccharides varies across the different species. In *M. gaditana*, cell wall polysaccharides are mainly composed of glucose (75 %), whereas in *P. tricornutum*, they are composed of mannose (46 %) and xylose (14 %). On the other hand, cell wall polysaccharides in *P. cruentum* and *T. lutea* consist of a combination of monosaccharides, including glucose, galactose, and xylose (Bernaerts et al., 2018). Additionally, the presence of extracellular structures has been described for these microalgae, such as the thick silica-based cell wall in diatoms (*P. tricornutum*) (Pajot et al., 2022), the dense layer of calcified scales covering the cell wall in *T. lutea* (Gonçalves de Oliveira-Júnior et al., 2020), or the presence of extracellular polysaccharides in *P. cruentum* (Bernaerts et al., 2018). Therefore, these structural differences could explain the method's versatility and highlight the challenges of developing eco-friendly extraction methods for microalgae, which may require individual optimization in some cases.

In general, our results agree with the literature, as discussed below

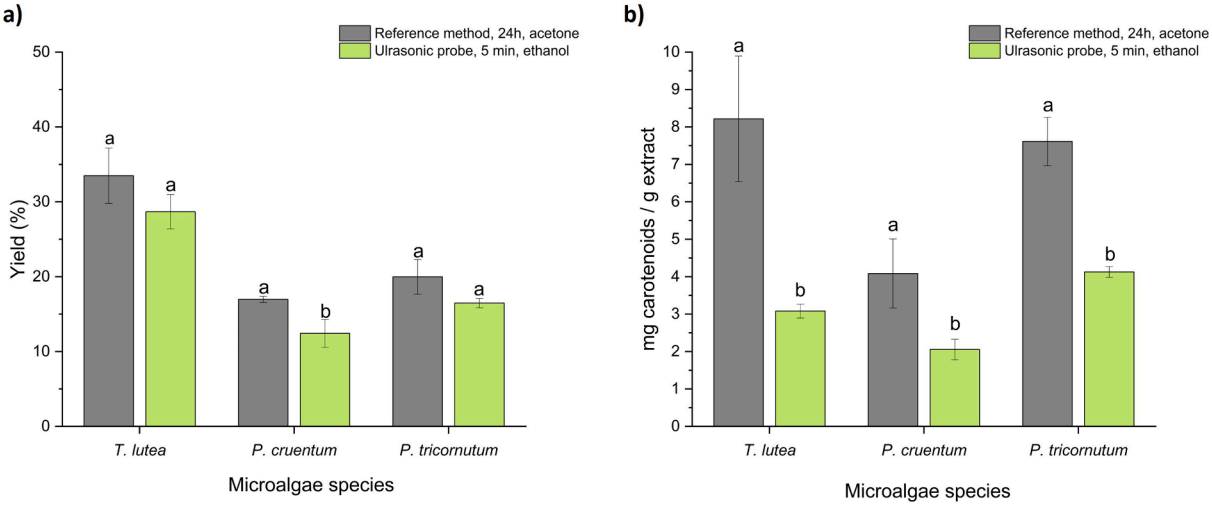

Fig. 2. Impact of the 5-min ultrasonic probe system with ethanol on extraction yield (a) and total carotenoid content (b) of different microalgae species (*Tisochrysis lutea, Porphyridium cruentum,* and *Phaeodactylum tricornutum*) compared to the reference method. Results are expressed as a percentage of dry weight (extraction yield) and mg of carotenoids per gram of dry biomass (total carotenoid content). Error bars denote the standard deviation of three independent extractions (n = 3). Different letters indicate statistically significant differences at $p < 0.05$ (one-way ANOVA with post-hoc Tukey, a–b).

for each microalga individually. For instance, in the case of *T. lutea*, Alkhamis and Qin reported a total carotenoid content of 4.8 to 11.5 mg g^{-1} carotenoids per biomass using a 24-h extraction with 90 % acetone (Alkhamis & Qin, 2016). Pajot *et al.* employed a 30-min ultrasound probe-assisted extraction using ethanol and distillation, achieving an approximate carotenoid recovery of 40 mg g^{-1} carotenoids per extract (Pajot et al., 2023). Moreover, In another study, Gallego *et al.* reported a higher carotenoid recovery (132.81 mg g^{-1} carotenoids per extract) than the present study (24.4 and 12.6 mg g^{-1} carotenoids per extract for the reference and the green method, respectively), using PLE (40 °C with a single static extraction cycle of 20 min) and ethyl acetate, which may be attributed to the optimal microalgal growing conditions used (Gallego et al., 2020). For *P. cruentum*, Gallego *et al.* applied a 20-min PLE process using ethanol at 125 °C, achieving a carotenoid recovery of 43.15 mg g^{-1} carotenoids per extract (Gallego et al., 2019). Our methodology achieves 42 % of the carotenoid content for the same microalgae species in only 5 min without using the special equipment needed for PLE and without external heat. Moreover, as mentioned before, the specific microalgal growing conditions might also explain the high carotenoid content. Furthermore, Di Lena *et al.* showed a total carotenoid content of 1.67 mg g^{-1} carotenoids per biomass using solvent extraction for 30 min (acetone-methanol mixture (70:30 *v/v*) containing BHT) (Di Lena et al., 2019), which is approximately three times lower than the carotenoid content observed in this study. Other authors also reported a lower total carotenoid content (2.0 and 5.2 mg g^{-1} carotenoids per extract for *P. cruentum*) than in the present study using a 2-h process with supercritical CO_2 and *n*-butane, respectively (Feller et al., 2018). Regarding *P. tricornutum*, Di Lena *et al.* documented a similar carotenoid recovery (10.22 mg g^{-1} carotenoids per biomass) to this study using the same methodology described before (Di Lena et al., 2019). Moreover, Wang *et al.* showed a carotenoid recovery of 1.6 mg g^{-1} carotenoids per dry weight using a 15-min PLE extraction at 40 °C with dimethyl sulfoxide (M. Wang et al., 2023).

Therefore, the 5-min ultrasonic probe system with ethanol offers a promising green approach for extracting carotenoids from microalgae. It significantly reduces the extraction time, biomass, and solvent requirements, utilizes a greener solvent than most published studies, and eliminates the need for external heat, thus reducing energy consumption. Specific optimizations are still needed, mainly due to microalgae's different cell wall compositions. Nevertheless, these attributes collectively position our approach as a more energy-efficient and environmentally conscious alternative, which may contribute to developing greener approaches.

4.4. Application of the green method to defatted microalgal biomass

In previous studies conducted by our research group, we focused on the potential of microalgae as a source of omega-3 lipids (Castejón & Marko, 2022; Pühringer et al., 2024), which inspired our idea to explore biomass valorization further. For that, we proposed applying the ultrasonic probe system to microalgal residues obtained after lipid extraction (defatted biomass). This initiative aligns with our goal of progressing toward a circular economy, emphasizing waste reduction. Through this approach, we aimed to assess the feasibility and effectiveness of utilizing microalgal biomass as a valuable resource for multiple sustainable products.

The lipid yield varied among the microalgae species, with *T. lutea* yielding the highest percentage (30.6 %), followed by *P. tricornutum* (23.6 %) and *P. cruentum* (16.9 %) and *M. gaditana* (14.7 %) with no statistically significant differences between the last two microalgae species (**Figure S2**). Regarding the total carotenoid content of the defatted biomass, as expected, it decreased compared to the original biomass for three out of four microalgae species tested ($p < 0.05$) (**Figure S3**) This decrease can be attributed to the coextraction of lipids and carotenoids, as indicated in previous studies (Delbrut et al., 2018; Uquiche et al., 2022). Nevertheless, the carotenoid recoveries were notably high: *M. gaditana*, *T. lutea*, and *P. tricornutum* exhibited recovery rates of 41 %, 61 %, and 60 %, respectively, while *P. cruentum* showed statistically similar results ($p > 0.05$) between the defatted and original biomass. Additionally, beyond the total carotenoid content, significant differences in the relative abundance of the main pigments were observed between the original and defatted biomass, enriching the extracts with specific minor compounds, as discussed in section 3.5. Thus, our proposed method demonstrates the possibility of biomass valorization by utilizing the residues from the extraction of already high-value products, such as microalgal lipids.

4.5. UV- and MS-based identification of carotenoids

A comprehensive chemical analysis of all microalgae extracts was conducted using HPLC-DAD and MS to shed light on the carotenoid profile and other potential minor compounds. Fig. 3 shows an example of HPLC-DAD chromatograms for the four microalgae species investigated in this study.

4.5.1. Microchloropsis gaditana

Table 2 presents the corresponding peak assignments, providing a detailed identification of the compounds detected in the extract obtained from *M. gaditana*. In the HPLC-DAD and MS analysis of *M. gaditana* (Fig. 3**a**), several pigments were identified using commercial standards (identification level 1): violaxanthin (peak 3), chlorophyll *a* (peak 9) as well as neoxanthin (peak 4). In the case of violaxanthin, two isomers with an *m/z* of 601.42 corresponding to the protonated ion species were detected at 9.6 min (peak 3) and 11.7 min (peak 5). They expressed a ratio of 6.7:1 based on the peak height in the UV signal, and both peaks were present in the standard. Resulting MS/HRMS fragments showed the cleavage of the specific fragment *m/z* 221 also in both cases, therefore, peak 5 was assigned as violaxanthin derivative. Regarding chlorophyll *a* (peak 9), the analysis revealed the presence of a precursor ion at *m/z* 893.54, which corresponds to the protonated molecule $[M + H]^+$. Additionally, two distinct fragments were observed: *m/z* 615.24, indicative of the detachment of the phytyl group ($C_{20}H_{39}$), and *m/z* 555.22, illustrating the concurrent loss of both the phytyl group and an acetate group (CH_3COO). This identification corresponds to the recognized MS patterns previously described for chlorophyll *a*, affirming the consistency of our findings with previously reported spectral characteristics (Wei et al., 2013). Furthermore, peaks 1 and 2 were tentatively assigned to lipid derivatives (identification level 3), but their exact structures could not be identified conclusively. Peak 7 was tentatively identified as halocynthiaxanthin acetate (identification level 3). Additionally, using the GNPS database (identification level 2), peak 10 was assigned a probable structure of chlorophyll *a* derivative and peak 11 a probable structure of pheophytin *a*.

In terms of relative abundance, *M. gaditana* extracts exhibited similar major pigments regardless of the method used, with a few exceptions (Fig. 4**a** and **Table S2**). For example, for the green method, violaxanthin and its derivative accounted for 31 % of the total pigments (22 % and 9 %, respectively), while chlorophylls (including chlorophyll *a*, and their derivatives) contributed a combined total of 53 %. Similar results were found for the extracts produced by the reference method, except for violaxanthin and its derivate (23 % and 31 % for the reference and green method with the original biomass, respectively) and neoxanthin (9 % and 1 % for the reference and green methods, respectively). For the defatted biomass, there was a significant increase in chlorophylls, specifically in chlorophyll *a* (49 %), compared to the reference approach and the green method with the original biomass ($p < 0.05$). Moreover, in comparison to the original biomass and using the green method, a decrease in violaxanthin and its derivative was observed for the defatted biomass ($p < 0.05$); meanwhile, the relative abundance of neoxanthin significantly increased (1 % and 8 % for original and defatted biomass using the green method, respectively, $p < 0.05$), suggesting a possible conversion of violaxanthin to neoxanthin (Jian et al., 2021). The

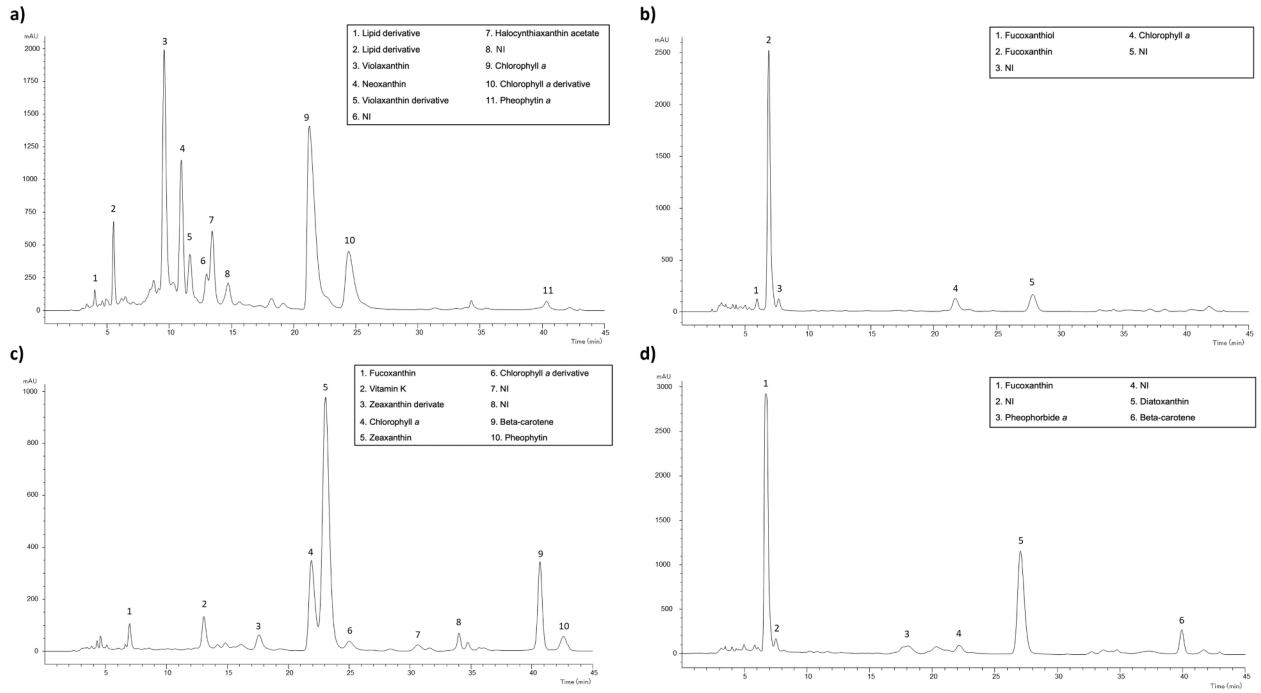

Fig. 3. Examples of HPLC-DAD chromatograms (450 nm) of *Microchloropsis gaditana* (a), *Tisochrysis lutea* (b), *Porphyridium cruentum* (c) and *Phaeodactylum tricornutum* (d) extracts. NI, not identified.

Table 2
Peak assignment, retention times (RT), and MS information of the separated peaks in *Microchloropsis gaditana*.

Peak	Identification	Formula	Identification level	RT (min)	$[M + H]^+$	MS/MS fragments [*m/z*] # and other significant ions [*m/z*] in HRMS
1	Lipid derivative	–	3 (accuracy – 89 %)	4.0	–	HRMS: 659.46; 359.26; 675.46
2	Lipid derivative	–	3 (accuracy – 73 %)	5.5	–	HRMS: 661.48; 840.56
3	Violaxanthin	$C_{40}H_{56}O_4$	1	9.6	601.42	@CE25: 221.15; 583.41: 491.3
4	Neoxanthin	$C_{40}H_{56}O_4$	1	11.0	601.42 623.41 $[M + Na]^+$	@CE30: 149.10; 181.12; 215.14; 563.39
5	Violaxanthin derivative	$C_{40}H_{56}O_4$	1*	11.7	601.42	@CE30: 221.15; 165.09; 203.14
6	NI	–	5	13.0	–	HRMS: 549.49; 401.34
7	Halocynthiaxanthin acetate	$C_{42}H_{56}O_5$	3 (accuracy – 83 %)	13.5	641.42	@CE30: 181.12; 163.11; 149.09; 215.14
8	NI	–	5	14.7	–	HRMS: 518.49; 661.48; 1124.77
9	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	1	21.3	893.54	@CE30: 615.24; 555.22; 583.22; 833.52
10	Chlorophyll *a* derivative	$C_{55}H_{72}MgN_4O_5$	2 (ppm error – 1.0)	24.4	893.54	@CE30: 615.24; 555.22; 583.22; 833.52
11	Pheophytin *a*	$C_{55}H_{74}N_4O_5$	2 (ppm error – 7.7)	40.3	871.57	@CE30: 593.27; 533.25

NI, not identified; *this impurity was detected in the standard as well; #fragments sorted according to intensity at the indicated collision energy; accuracy corresponds to the accuracy of the structure assignment in SIRIUS software; ppm error was determined compared to the theoretical masses. MS and selected MS/MS spectra for each peak are provided in the supplementary materials.

reported carotenoid profile of *M. gaditana* agrees with previous findings. For instance, other authors have already reported neoxanthin (Hita Peña et al., 2015), violaxanthin (Sales et al., 2020), chlorophyll *a* and pheophytin *a* (Sánchez-Camargo et al., 2018). Furthermore, to the extent of our knowledge, the tentatively identified halocynthiaxanthin acetate (level 3) would represent the first documented occurrence of this compound in this microalgae species.

4.5.2. *Tisochrysis lutea*

For *T. lutea* extracts (Fig. 3**b** and Table 3), the highest level of identification (level 1) was achieved for fucoxanthin (peak 2) and chlorophyll *a* (peak 4). In the case of fucoxanthin, *m/z* 659.43 corresponding to the $[M + H]^+$ ion and the respective ion indicating a water loss (*m/z* 641.42 $[M + H - H_2O]^+$) were observed. Regarding chlorophyll *a*, the same fragments as in *M. gaditana* were detected and corresponded to the literature (Wei et al., 2013). Furthermore, peak 1 was tentatively identified as fucoxanthinol (identification level 3) while in the case of peaks 3 and 5, a precise structural assignment was not possible. However, significant ions were observed and are provided in Table 4.

The predominant pigments in *T. lutea* extracts, as indicated by their relative abundance (Fig. 4**b** and **Table S3**), were fucoxanthin (including fucoxanthinol) and chlorophyll *a*, collectively comprising 85 % and 14 % of the total minor compounds in the extracts produced with the green method. Notably, the green approach, independent of the biomass used (original or defatted), positively affects the extraction of these two main pigments. The pigment profile reported herein is consistent with those previously published, which documented the existence of fucoxanthin and its derivates, along with chlorophyll *a*, within this microalgae species (Di Lena et al., 2019; Gonçalves de Oliveira-Júnior et al., 2020; Serive et al., 2017).

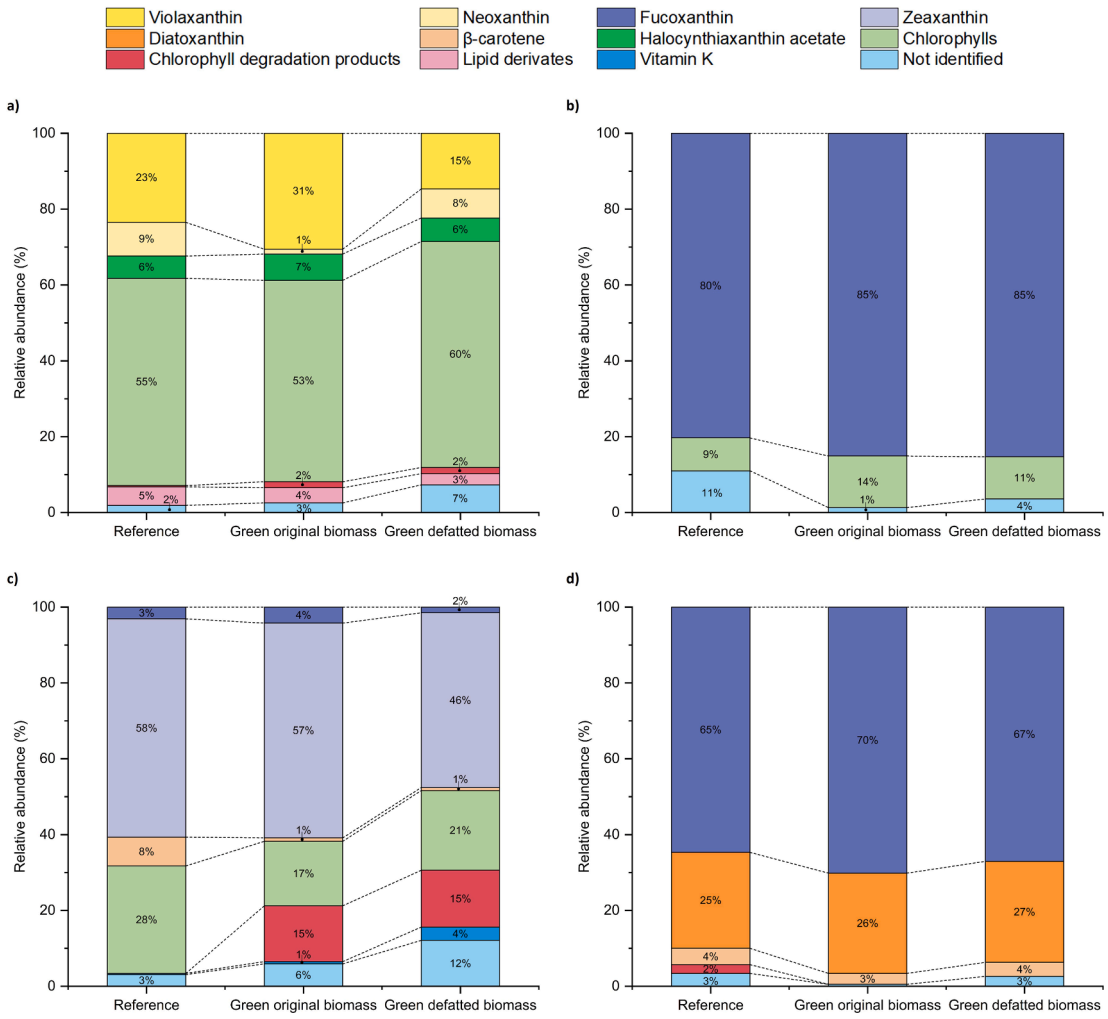

Fig. 4. Relative abundance (%) of carotenoids and chlorophylls in *Microchloropsis gaditana* (a), *Tisochrysis lutea* (b), *Porphyridium cruentum* (c) and *Phaeodactylum tricornutum* (d) extracts using different extraction approaches: reference method (24 h, agitation, acetone, 1:100 biomass to solvent ratio) on original biomass, and the green extraction method (5 min, ultrasonic probe, ethanol, 1:10 biomass to solvent ratio) on original and defatted biomass. Violaxanthin, fucoxanthin, and zeaxanthin include their respective derivatives; chlorophylls include chlorophyll and its derivatives; chlorophyll degradation products include pheophytin *a* and pheophorbide *a*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Peak assignment, retention times (RT), and MS information of the separated peaks in *Tisochrysis lutea*.

Peak	Identification	Formula	Identification level	RT (min)	$[M + H]^+$	MS/MS fragments $[m/z]$ # and other significant ions $[m/z]$ in HRMS
1	Fucoxanthinol	$C_{40}H_{56}O_5$	3 (accuracy – 82 %)	5.9	599.41 $[M-H_2O + H]^+$	@CE30: 147.1; 109.1; 167.11; 233.15
2	Fucoxanthin	$C_{42}H_{58}O_6$	1	6.8	659.43 & 641.42 $[M-H_2O + H]^+$	@CE30: 149.1; 109.1; 263.18; 355.24; 411.27
3	NI	–	5	7.6	–	HRMS: 429.37
4	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	1	21.5	893.54	@CE30: 555.2; 614.24; 481.19
5	NI	–	5	27.5	–	HRMS: 788.6; 889.7

NI: not identified; #fragments sorted according to intensity at the indicated collision energy; accuracy corresponds to the accuracy of the structure assignment in SIRIUS software. MS and selected MS/MS spectra for each peak are provided in the supplementary materials.

4.5.3. *Porphyridium cruentum*

For *P. cruentum* extracts (Fig. 3**c** and Table 4), our analysis unveiled the identification of five minor compounds at identification level 1: fucoxanthin (peak 1), vitamin K1 (peak 2), chlorophyll *a* (peak 4), zeaxanthin (peak 5), and β-carotene (peak 9). Additionally, two compounds were assigned with a probable structure using GNPS database (identification level 2): chlorophyll *a* derivative (peak 6), and pheophytin (peak 10). Furthermore, peak 3 was tentatively identified as a zeaxanthin derivative with *in silico* fragmentation and SIRIUS software (identification level 3). The structure of peaks 7 and 8 remained unidentified and requires further investigation.

The primary pigments identified in *P. cruentum* extracts were zeaxanthin and its derivative, collectively constituting 58 %, 57 %, and 46 % of the total relative abundance for the reference method, original biomass, and defatted biomass with the green approach, respectively ($p < 0.05$ for the defatted biomass) (Fig. 4**c** and **Table S4**). Additionally, chlorophylls showed a relative abundance ranging from 17 % to 28 %, with no significant differences observed between the methods ($p >$

Table 4
Peak assignment, retention times (RT), and MS information of the separated peaks in *Porphyridium cruentum*.

Peak	Identification	Formula	Identification level	RT (min)	$[M + H]^+$	MS/MS fragments $[m/z]$ # and other significant ions $[m/z]$ in HRMS
1	Fucoxanthin	$C_{42}H_{58}O_6$	1	7.0	641.42 $[M-H_2O + H]^+$	@CE20: 149.1; 109.1; 581.40; 411.3
2	Vitamin K1	$C_{31}H_{46}O_2$	1	13.1	451.36	@CE20: 187.08; 227.11
3	Zeaxanthin derivative	–	3 (accuracy – 80 %)	17.6	–	HRMS: 569.43; 647.46
4	Chlorophyll *a*	$C_{55}H_{72}MgN_4O_5$	1	21.9	893.54	@CE30: 615.24; 555.22; 583.22
5	Zeaxanthin	$C_{40}H_{56}O_2$	1	23.7	569.43	@CE30: 175.15; 145.10
6	Chlorophyll *a* derivative	$C_{55}H_{72}MgN_4O_5$	2 (ppm error – 4.0)	25.0	893.54	@CE50: 555.22; 614.24; 481.18
7	NI	–	5	30.6	–	HRMS: 749.62; 369.38; 647.46
8	NI	–	5	34.0	–	HRMS: 730.53; 338.34; 887.57
9	β-carotene	$C_{40}H_{56}$	1	40.7	537.44	@CE20: 177.16; 203.18; 137.13
10	Pheophytin	$C_{55}H_{80}N_4O_5$	2 (ppm error – 1.4)	42.6	899.60*	@CE50: 535.27; 621.30; 813.56

NI: not identified; *corresponds to $[M + Na]^+$; #fragments sorted according to intensity at the indicated collision energy; accuracy corresponds to the accuracy of the structure assignment in SIRIUS software; ppm error was determined compared to the theoretical masses. MS and selected MS/MS spectra for each peak are provided in the supplementary materials.

0.05). While no significant variations were found for chlorophylls regardless of the method used, the presence of pheophytin was predominantly noted in extracts produced with the green method (<1% for the reference method and 15 % for the original and defatted biomass with the green approach). The cause of the formation of pheophytin during the ultrasound process remains unclear. Even though *M.gaditana* extracts exhibited a higher relative abundance of chlorophyll *a*, we did not observe this effect. In general, the pigment composition detailed here aligns with previous studies. For instance, the presence of zeaxanthin, chlorophyll *a*, pheophytin, and β-carotene was also reported (Gallego et al., 2019; Liberti et al., 2023), although other authors have also described fucoxanthin derivatives as a minor compound of this species (Huang & Cheung, 2021). Notably, the occurrence of vitamin K in *P. cruentum* was described several years ago (Antia et al., 1970), but to the extent of our knowledge, this is the first time reported by MS-based identification.

4.5.4. *Phaeodactylum tricornutum*

In the case of *P. tricornutum* extracts, (Fig. 3**d** and Table 5), MS analysis revealed the identification of fucoxanthin (peak 1) and β-carotene (peak 6), both validated by commercially available standards (identification level 1). Moreover, pheophorbide *a* (product of chlorophyll degradation) was assigned as a probable structure using the GNPS database (peak 3, identification level 2), while peak 5 was tentatively identified as diatoxanthin (identification level 3). Peaks 2 and 4 have not been assigned any precise composition, but some *m/z* fragments have been observed and are provided in Table 5.

In terms of relative abundance (Fig. 4**d** and **Table S5**), the main pigments in *P. tricornutum* extracts were fucoxanthin (65–70 %, with the green method the best strategy to extract this pigment, $p < 0.05$) and diatoxanthin (25–26 % with no significant differences between the methods, $p > 0.05$), with a slight contribution from β-carotene and pheophorbide *a* to the overall pigment profile. These results align with the previous findings, where fucoxanthin, diatoxanthin, and β-carotene were reported for this microalga (Celi et al., 2022; Di Lena et al., 2019; S. M. Kim et al., 2012).

5. Conclusion

This study presents significant findings contributing to eco-friendly methodologies that utilize ultrasound strategies for extracting minor compounds, specifically carotenoids and chlorophylls, from microalgae. Among the various solvents and approaches evaluated, the 5-min ultrasonic probe system with ethanol demonstrated a similar efficiency in recovering carotenoids compared to the reference 24-h extraction using acetone and BHT for the microalgae *M. gaditana*. The results from the green metrics assessed by AGREEprep™ software demonstrated the superior environmental friendliness of the ultrasonic probe system, especially due to the absence of organic or toxic solvents, drastic reduction of extraction time, lower energy requirements, and decreased biomass-to-solvent ratio. However, the efficacy of this method across other species was limited, probably due to differences in cell wall compositions. Nevertheless, the potential to process a higher number of samples per hour and the relatively high recovery percentage in comparison to the reference method still demonstrate the versatility of this green approach. Moreover, the application of the method to microalgae residues (defatted biomass) showcases the rich potential for biomass valorization. Additionally, a comprehensive assessment of the carotenoid profile across the four microalgae species was successfully performed, allowing the evaluation of the impact of the extraction methods. Thus, these outcomes provide valuable insights into the carotenoid composition of microalgae, potentially catalyzing the emergence of innovative ways for environmentally conscious production of these high-value compounds with promising applications in the food and nutraceutical industries.

CRediT authorship contribution statement

Mariia Zazirna: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Sonja**

Table 5
Peak assignment, retention times (RT), and MS information of the separated peaks in *Phaeodactylum tricornutum*.

Peak	Identification	Formula	Identification level	RT (min)	$[M + H]^+$	MS/MS fragments $[m/z]$ # and other significant ions $[m/z]$ in HRMS
1	Fucoxanthin	$C_{42}H_{58}O_6$	1	6.7	641.42 $[M-H_2O + H]^+$	@CE25: 149.1; 109.1; 549.39
2	NI	–	5	7.5	–	HRMS: 429.37; 381.35
3	Pheophorbide *a*	$C_{35}H_{36}N_4O_5$	2 (ppm error – 0.1)	18.1	593.28	@CE50: 460.22; 445.20; 506.22
4	NI	–	5	22.1	–	HRMS: 369.4, 583.55
5	Diatoxanthin	$C_{40}H_{54}O_2$	3 (accuracy – 95 %)	27.0	567.42	@CE30: 199.15; 173.13; 145.10
6	β-carotene	$C_{40}H_{56}$	1	39.9	537.44	@CE30: 137.14; 177.16;

NI: not identified; #fragments sorted according to intensity at the indicated collision energy; accuracy corresponds to the accuracy of the structure assignment in SIRIUS software; ppm error was determined compared to the theoretical masses. MS and selected MS/MS spectra for each peak are provided in the supplementary materials.

Tischler: Writing – review & editing, Methodology, Investigation. **Doris Marko:** Visualization, Supervision, Resources, Conceptualization. **Elisabeth Varga:** Writing – review & editing, Visualization, Validation, Supervision, Methodology, Investigation, Conceptualization. **Natalia Castejón:** Writing – review & editing, Visualization, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Natalia Castejón reports financial support was provided by European Commission. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114354.

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