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Green extraction of pigment from astaxanthin-producing algae using natural deep eutectic solvents

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ABSTRACT

The high-value carotenoid astaxanthin is a powerful antioxidant with various purported health benefits. The alga *Haematococcus lacustris* (formerly *pluvialis*) represents the main natural (farmed) source of astaxanthin. Additionally, *Chlamydomonas reinhardtii* has been engineered to produce ketocarotenoids including canthaxanthin, astaxanthin, and intermediates that accumulate with its native carotenoids and chlorophylls. Carotenoid extraction from biomass conventionally employs organic solvents such as acetone and ethanol. Here, the use of natural deep eutectic solvents (NADES), composed of food-grade components, was explored as green alternative for the extraction of total pigments, including astaxanthin, from engineered *C. reinhardtii* and wild-type *H. lacustris*. Hydrophobic menthol-based NADES extracted up to 2.0 mg of astaxanthin g^{-1} of dry algal biomass from engineered *C. reinhardtii* and 13.4 mg g^{-1} of wildtype *H. lacustris*, respectively, in single two-hour extractions, giving an extraction efficiency of 79 % and 204 % compared to organic-solvents, respectively. The extractions were carried out at room temperature without necessitating additional energy inputs like heating or sonication and without any pretreatments. The food-grade nature of NADES suggests the feasibility of utilizing the extracted materials in supplements and health applications, offering a cost-effective and sustainable means of converting waste biomass into valuable products.

1. Introduction

Astaxanthin is a xanthophyll ketocarotenoid responsible for the red-orange color of some microorganisms and marine animals [1]. Its potent antioxidant activity has made it of interest for various potential health benefits [2]. Studies have shown that astaxanthin plays a role in managing oxidative stress, inflammation, lipid metabolism, and cell death [3]. As a result, it can bring various benefits to the skin [4,5], eyes [6,7], cardiovascular system [8,9], neurological function [10,11], exercise performance [12,13], and immune response [14,15]. Astaxanthin can only be obtained from dietary sources and food supplements as it is not produced by humans [2]. It is biosynthesized de novo by a few microorganisms, including the microalga *Haematococcus lacustris*, some yeasts, bacteria and flowers [16]. Alternatively, astaxanthin can be bioaccumulated by other organisms through diet or synthesized from other ingested carotenoids [17,18].

Astaxanthin is reported to represent the largest share of the global market for carotenoids [19]. Organic solvents, such as acetone and ethanol, are conventionally used for the extraction of astaxanthin from biomass [20]. However, the toxicity and flammability of organic solvents represent a downside. Alternatively, supercritical CO_2 and ionic liquids have been employed for astaxanthin recovery [21,22]. These methods are less hazardous and can offer faster and more efficient extractions [23]. Nevertheless, they require special equipment that might not be accessible everywhere or can be costly [24]. Moreover, besides the choice of solvent, recovering extracted materials from the extraction solvent is often complicated and requires additional process steps [25]. Therefore, developing new alternative routes for green and efficient astaxanthin recovery from algal biomass can enable broader use-cases.

Deep eutectic solvents (DES) are emerging green alternatives to

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conventional organic solvents [26,27]. They are non-volatile, non-flammable, non-toxic, and composed of low-cost materials [26,27]. DES combine two or more compounds, forming low-melting-point liquids [28,29]. Due to the variety of possible DES combinations, they can be tailored to dissolve polar, non-polar, and intermediately-polar materials [30]. Their case-by-case tailorable solubilization ability makes them suitable for applications in the fields of extraction [31], solubilization [32], and cleaning [33], among others. DES have been used for the extraction of several water-insoluble compounds, including carotenoids [34]. For example, DES were recently used to extract the carotenoid fucoxanthin from *Fucus vesiculosus*. [35]

DES are being growingly explored as green extraction alternatives to recover by-products, including astaxanthin, from natural substances. Recently, hydrophobic DES have been used to extract astaxanthin from shrimp waste with the aid of ultrasonication [36]. In the case of algae, previous studies mainly used hydrophilic DES as pretreatments to weaken the algal cell walls prior to extraction with other solvents [37]. However, to our knowledge, only a single study reported the extraction of astaxanthin from *H. lacustris* using hydrophobic oleic-acid-based natural DES (NADES) without biomass pretreatment [25]. This study reported 60 % astaxanthin extraction efficiency with reference to organic-solvent extraction [25]. NADES can be composed of food-grade materials, which can enable the direct use of carotenoid-NADES solutions.

The accessibility of astaxanthin from *H. lacustris* is limited, partly due to inefficient extraction from algal biomass caused by the hard cell wall of the alga in the red phase [38,39]. Exploration of alternative sources of astaxanthin or more efficient extraction protocols from *H. lacustris* is generally desirable. It was recently demonstrated that the over-expression of a native β-carotene ketolase (BKT), which is normally silenced in vegetative growth, is a key enzyme in the biosynthetic pathway towards astaxanthin in the microalga *C. reinhardtii* [40]. *Cr*BKT overexpression in alga results in the ketolation of beta carotene and zeaxanthin, which produces canthaxanthin and astaxanthin, respectively. The native activity of the beta carotene hydroxylase (*Cr*CHYB) does not match that of overexpressed *Cr*BKT, which leads to mostly canthaxanthin and partially hydroxylated carotenoid intermediate accumulation in the engineered alga [40]. Further studies have shown that also co-overexpression of *Cr*CHYB and *Cr*BKT can result in the accumulation of mostly astaxanthin in alga [16]. It was also demonstrated that these two enzymes can be overexpressed in other algae to generate astaxanthin from native carotenoids [41]. Modifying the native carotenoid biosynthesis has been found to deregulate the isoprenoid precursor pathway in *C. reinhardtii* contributing to improved rates of engineered volatile isoprene and sesquiterpenoid production from the alga [42,43]. The *Cr*BKT-overexpressing *C. reinhardtii* transformant was shown to accumulate up to ~ 6 mg L^{-1} of ketocarotenoids, or 2.5 mg g^{-1} dry biomass [44]. If *C. reinhardtii* is making engineered products that are outside of the cell, like volatile isoprene [43], extraction of co-produced ketocarotenoids can add value to the algal process.

Here, we investigate the use of NADES as green alternatives for the extraction of cellular hydrophobic pigments from modified *C. reinhardtii* as well as wild-type *H. lacustris* with a focus on astaxanthin yields. NADES of varying hydrophobicity and component acidity were screened, and the most suitable candidates were selected. The selected menthol-based hydrophobic NADES were explored for the first time here for the extraction of carotenoids from algal biomass, and the recovery of both free and esterified astaxanthin was evaluated. Hydrophobic menthol-based NADES show promising astaxanthin extraction capacities that can exceed the efficiency of organic solvent extraction, representing a greener extraction alternative.

2. Materials and methods

2.1. Engineering of C. reinhardtii for astaxanthin accumulation

A cell-wall deficient strain of *C. reinhardtii,* UPN22 was used for all experiments [45]. This strain was derived from UVM4 [46], which had its nitrate metabolism restored by the transformation of native nuclear genes *nit1/nit2* [47,48] and plastid genome engineered to express phosphite oxidoreductase (*ptxD*) from *Pseudomonas stutzeri* WM88 [49]. This enables high-density contamination-free cultivation of this strain in phosphite- and nitrate-containing media. The alga was maintained on solidified agar TAPhi-NO_3 medium at ~150 μ mol photons m^{-2} s^{-1} [45]. To alter the astaxanthin content of UPN22, a plasmid was kindly developed by Dr. Thomas Baier (Bielefeld University, Germany) to enable the over-expression of *C. reinhardtii β-carotene hydroxylase* (*Cr*CHYb, Cre04.g215050) and *β-carotene ketolase* (*Cr*BKT, UniProt: Q4VKB4.1), based on the results reported by Amendola et al., 2022 [16]. Synthetic coding sequences of *Cr*CHYB 5-terminally tagged with fluorescent protein mScarlet (Uniprot: 5LK4) (*Cr*CHYb-mScarlet) and *Cr*BKT 5-terminally tagged to aminoglycoside (3″) (9) adenylyltransferase (*aadA,* Uniprot: P0AG05) as previously reported by Pivato et al., 2021 [44] were combined into a single plasmid (pOpt3_PsaD_CrChyB-mScarlet_CrBKT-aadA). The PsaD promotor (*PsaD*) and chloroplast-targeting peptide (PsaD-CTP) enable the nuclear expression and plastid localization of both *Cr*CHYB-mScarlet *Cr*BKT-aadA, while *aadA* confers resistance to the antibiotic spectinomycin to enable selection of transformants.

Nuclear transformation of UPN22 was carried out by the glass bead protocol as previously reported [47]. In brief, 10 μg of plasmid DNA was linearized by restriction enzymes (*Xba*I + *Kpn*I, ThermoFisher FD) in 100 μL reactions. 50 mL of mid-log phase algal cells were pelleted by centrifugation at 1000 ×*g* for 4 min, mixed with plasmid DNA, and agitated by vortexing in a 20 % PEG-4000 solution with 1 g of 0.6 mm glass beads for 15 s. Cells were transferred into 45 mL of TAPhi-NO_3 liquid medium for 6 h before plating on TAPhi-NO_3 agar selective medium, containing 200 mg L^{-1} spectinomycin and left under constant illumination at ~150 μmol photons m^{-2} s^{-1} for ~7 d prior to colony picking. 96 transformants were picked using a PIXL (Singer, US) colony-picking robot and transferred to TAPhi-NO_3 agar, containing 200 mg L^{-1} spectinomycin. After a week, colonies were screened for mScarlet fluorescence (excitation filter: 560/10, emission filter: 600/10) by UVP ChemStudio PLUS (Analytik Jena, USA) [50] to detect the expression of *Cr*CHYB-mScarlet. 24 colonies with high mScarlet fluorescence and red-brown phenotype indicative of altered carotenoid content were transferred into 1 mL TAPhi-NO_3 liquid media and grown for 3 days with 190 rpm shaking at 150 μmol photons m^{-2} s^{-1} illumination. A single transformant with vigorous growth and strong red-brown phenotype was selected for further scale-up [50].

2.2. Mid-scale cultivation of C. reinhardtii for generating biomass

A single transformant of UPN22_*Cr*CHYb-mScarlet_*Cr*BKT-aadA was scaled up to 45 mL in TAPhi-NO_3 and used to inoculate 1 L of 6xPhi nitrate media in a CellDEG HD1000 high-cultivation reactors (CellDEG, Germany) and cultivated with the program outlined in Table 1 until reaching stationary phase (~1×10^8 cells/mL). Cells were harvested by centrifugation at 1000 x*g* for 10 min and the supernatant was discarded before snap-freezing in liquid N_2. Samples were then lyophilized for 24 h in Sentry 2.0 Lyophilizator (VirTIS, USA) and then stored at −80 °C, protected from light before carotenoid extraction.

2.3. Source of H. lacustris biomass

Lyophilized *H. lacustris* biomass, reported by manufacturer to contain 5 wt% astaxanthin (Lot No. AKEY_0047_1), was obtained from Algikey S.A. (Póvoa de Santa Iria, Portugal).

Table 1
CellDeg photobioreactor program used for high-cell-density cultivation of astaxanthin-producing *Chlamydomonas reinhardtii*. Initial and final CO_2 concentration and light intensity. Progression indicates the model used for the increase over the specified duration.

CO_2				Light			
Initial	Progression	Duration	Final	Initial	Progression	Duration	Final
3 %	Linear	12 h	5 %	100 μE	Exponential	48 h	1000 μE
Maintained at 5 % CO_2 for the remainder of the experiment				Maintained at 1000 μE for the remainder of the experiment			

2.4. NADES preparation

DL-menthol, 98 + % (Alfa Aesar); acetic acid glacial (Fisher Scientific); lactic acid, 85 % (Sigma Aldrich); D-glucose (Sigma Aldrich), glycerol, ACS, 99.5 + % (Alfa Aesar); octanoic acid, 99 % (Thermo Scientific); betaine, anhydrous, 98 % (Alfa Aesar); urea (Fisher Chemical); and ultrapure water (Sigma Aldrich) were used without further purification. NADES were prepared in the molar ratios specified in Table 2 using the heating method reported by Dai et al., 2013 [30]. The NADES were selected based on the inclusion of hydrophobic or acidic components.

2.5. NADES extraction of algal biomasses

The extraction was performed according to the protocol reported by Pitacco et al., 2022 [25]. The algal culture was collected and freeze-dried to enable the accurate quantification of the initial algal biomass weight and the final %wt of the extracted compounds. The freeze-dried biomass was suspended in the extraction solvents in a 2.5 %w/v ratio. The samples were briefly vortexed and then incubated at room temperature for 2 h in the dark. Following the incubation, the samples were centrifuged at 2600 $\times g$ for 10 min then the supernatant was recovered. Aliquots from the supernatants were diluted in methanol in a 1:72 volume ratio for ultraviolet-visible (UV–vis) spectroscopy and in a 1:4:20 extract-DMSO-methanol volume ratio for high-performance liquid chromatography (HPLC) analyses.

2.6. Analyses of NADES extracts

Diluted algal extracts were analyzed by HPLC-UV–vis system (Agilent 1290 Infinity, Agilent Technologies, Milan, Italy) coupled with a UV–vis diode array detector using an Eclipse Plus C8 column with the dimensions: 95 Å, 5 μm, 4.6 mm × 100 mm (Agilent Technologies, Milan, Italy), and an injected volume of 10 μL. The mobile phase was constituted of deionized water as solvent A and methanol as solvent B. A gradient elution of 80–100 % B from 0 to 10 min, followed by 100 % B from 10 to 16 min was performed under a flow rate of 0.7 mL min^{-1} from 0 to 16 min and of 1.7 mL min^{-1} from 16 to 30 min. The astaxanthin was quantified using a calibration curve ($R^2 = 0.9964$, adjusted $R^2 = 0.9946$) obtained from standard astaxanthin (Sigma Aldrich) dissolved in DMSO. For all extracts, the values are expressed as mean ± the standard deviation (SD) for $n = 3$ and analyzed using two-tailed, unpaired Student's *t*-test. For statistical tests, the 0.05 level of confidence was accepted for statistical significance. The mean, SD, and relative SD (%RSD) values are reported in Table S1. The estimated limit of detection (LOD) and limit of quantification (LOQ) are 0.88 and 2.66 mg g^{-1}, respectively. LOD and LOQ were calculated based on the formulas: $3.3\sigma/S$ and $10\sigma/S$, respectively, where σ is the standard error of the regression and S is the slope of the standard curve. The astaxanthin extraction efficiency was determined by dividing the amount of astaxanthin extracted with NADES by the astaxanthin extracted with acetone. The extracts were diluted in methanol and analyzed by a double-beam UV–vis spectrophotometer (UV-1900i, SHIMADZU, Kyoto, Japan) in the spectral range of 90–900 nm, with methanol in the reference cell.

Table 2
NADES investigated for total pigment extraction from *Chlamydomonas reinhardtii*.

NADES	A	B	C	D	Molar ratio
N1	Menthol	Acetic acid	–	–	1:1
N2	Menthol	Lactic acid	Water	–	3:3:1
N3	Glucose	Glycerol	Water	–	1:1:4
N4	Glycerol	Citric Acid	Water	–	1:1:4
N5	Glucose	Citric acid	Water	–	1:1:5
N6	Menthol	Caprylic acid	–	–	1:1
N7	Betaine	Urea	Water	–	1:1:3
N8	Glucose	Glycerol	Citric acid	Water	1:1:1:3

3. Results and discussion

3.1. NADES selection for total pigment extraction from algal biomass

Carotenoids contain a polyene chain of multiple conjugated double bonds and an end group at both ends of the chain [51]. The conjugated chain functions as a chromophore that absorbs light in the wavelength region between 400 and 500 nm, producing the characteristic color of carotenoid pigments, ranging from light yellow to dark red [52]. Some carotenoids, including astaxanthin, contain polar ionone rings, which can scavenge reactive oxygen species, giving rise to the strong antioxidant properties. The long carbon chain of carotenoids contributes to their poor solubility in aqueous media, making organic solvents the conventional choice for extraction. NADES are explored here as green alternatives for the extraction of cellular hydrophobic pigments, including accumulated astaxanthin, from modified *C. reinhardtii* and wild-type *H. lacustris*.

The effectiveness of the NADES is influenced by several factors, including the type of hydrogen bond donors and acceptors, which can impact the solvation by altering the solvent polarity and hydrogen bonding capacity [53]. Solvent viscosity and acid-base interactions with the solute (i.e. protonation and deprotonation) are other factors that can affect the extraction efficiency [54]. In order to select the most efficient solvent for extraction, eight NADES were tested for their pigment extraction capacity from algal biomasses (Table 2). Menthol- and thymol-based NADES are among the commonly explored hydrophobic NADES [55]. Typically, menthol-based NADES combine menthol with carboxylic acids [56]. Here, hydrophobic menthol-based NADES (N1, N2, and N6) were used as they should be suitable for the recovery of hydrophobic carotenoids. These NADES have not been used previously for the extraction of carotenoids and were investigated here for the first time. NADES combining sugars and sugar alcohols (N3) and sugars or sugar alcohols with acid (N4, N5, and N8), were also tested. A single NADES composed of basic components (N7) was also tested for comparison with those containing acids.

These NADES are formed from food-grade chemicals. Menthol occurs naturally in mint plants, such as peppermint and spearmint [57], and is commonly used as a flavoring additive in beverages, gum, and candies [58]. The United States Food and Drug Administration (FDA) generally considers menthol a safe substance. According to the World Health Organization (WHO), when taken orally, the acceptable daily intake (ADI) of menthol must not exceed 4 mg kg^{-1} of body weight [59]. Acetic (ethanoic) acid is produced by fermenting fruit such as apples and pineapples and is used up to 8 % volume of common vinegar [60]. Lactic

acid is naturally present in soy and dairy products [61,62], and caprylic (octanoic) acid is naturally found in palm oil, coconut oil, and milk [63–65]. These components facilitate the direct use of carotenoid-containing NADES in food-related applications.

The pigment extraction performance of these eight NADES was evaluated first on *C. reinhardtii* engineered to accumulate astaxanthin through co-expression of *Cr*BKT and *Cr*CHYB as recently described [16]. Algal biomass often requires pretreatment methods, such as sonication, to disrupt the cells and enable the extraction of biomaterials. However, the engineered *C. reinhardtii* strain UPN22 used here is cell-wall deficient, eliminating the need for pretreatments. 2.5 %*w/v* of freeze-dried algal biomass was directly suspended in acetone or NADES and incubated in the dark to prevent light-induced astaxanthin degradation. The supernatants were collected after 6 h to provide a sufficient period for the extraction. When the supernatants were isolated, acetone extraction yielded a green color, mainly attributed to chlorophylls (Fig. 1b). Except for N3 and N7, NADES extracts had colors ranging from light yellow to reddish brown, associated with carotenoids. N1, N2, and N6 extracts had the most intense pigment coloration (Fig. 1b).

UV–vis spectroscopy indicated a typical carotenoid absorbance spectrum for the acetone extract (Fig. 1c). The wavelength at which the maximum absorption occurs (λ_{max}) for astaxanthin is 470 nm. The λ_{max} values of chlorophyll *a* are 430 and 662 nm, and those of chlorophyll *b* are 453 and 642 nm. These absorption peaks were observed in acetone extracts, indicating the extraction of chlorophylls alongside the carotenoids. N1, N2, and N6 exhibited the highest absorbances and the most similar profiles to acetone, with a slight blue shift (Fig. S1). Based on these results, N1, N2, and N6 were selected for further investigation and quantification. Although the extracts by menthol-based NADES were not visibly green, chlorophyll absorption peaks were observed in comparable intensities to acetone extracts. The hydrophobicity of the selected NADES resulted in extraction of all hydrophobic pigments, including carotenoids, from biomass. All three menthol-based NADES contain an acidic component. Acidification or thermal processing results in pheophytinization [66], which is the conversion of chlorophylls to pheophytins through the substitution of the magnesium in the porphyrin ring of chlorophyll with two hydrogen ions (Fig. 1d). Pheophytinization is preceded or followed by dephytylation [67], the hydrolytic removal of the phytol tail of chlorophyll. These events change the color from bright green to olive brown or olive yellow [66–68], which is observed in the samples extracted by acid-containing NADES. This phenomenon explains the different colors observed in N3 and N7 extracts, both NADES lack an acidic component (Fig. 1b).

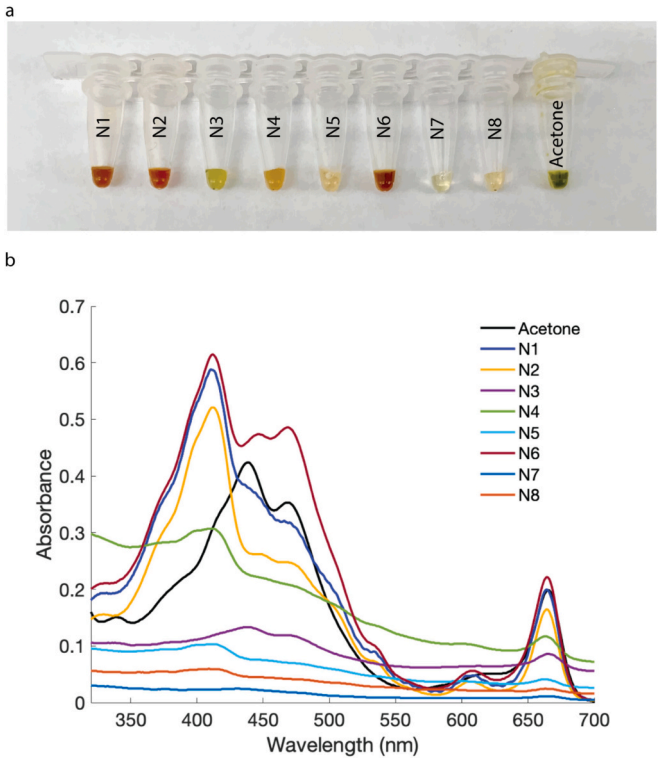

Fig. 1. Total pigment extraction from modified *Chlamydomonas reinhardtii* using different NADES. (a) Aliquots from *C. reinhardtii* extracts using N1-N8 and acetone. (b) UV–vis spectra of *C. reinhardtii* extracts using N1-N8 and acetone.

3.2. Optimizing total pigment extraction from modified *C. reinhardtii* with NADES

A previous study has shown that the yield of astaxanthin extraction from *H. lacustris* was enhanced by increasing the incubation time, reaching a plateau after 2 h [25]. Here, to determine the impact of incubation time on the extraction efficiency, *C. reinhardtii* dry biomass was suspended in N1 and an aliquot of the supernatant was collected after 1, 2, 4, and 6 h. Similar to *H. lacustris,* UV–vis spectroscopy of N1 extracts collected at different time points shows that the yield increased with 2 h of incubation but was not notably affected beyond the 2 h (Fig. 2a and Fig. S2). Therefore, 2 h was selected as the optimum incubation time. Moreover, we evaluated the effect of temperature in improving the extraction. The extraction by N1 was performed at room temperature (21 °C) and 40 °C and the UV–vis spectra were compared (Fig. 2b and Fig. S3). The increase in temperature did not increase the absorption intensity of the evaluated extracts. Additionally, heating the samples seems to have caused some degradation in the extracts, suggested by the disappearance of the shoulder at ~480 nm. Therefore, subsequent extractions were performed at room temperature.

The extracts were examined by HPLC-UV–vis to quantify the extraction yield. Only astaxanthin was quantified as a target molecule in the total NADES hydrophobic extracts. The extracts were analyzed for the presence of the astaxanthin peak at 470 nm using a pure astaxanthin standard as a reference. HPLC chromatograms show that acetone and NADES extracts have similar peaks except for small variations (Fig. 3a). The peak retained at ~7 min is associated with free astaxanthin, as confirmed by the chromatogram of the astaxanthin standard. Other peaks are associated with other carotenoids such as canthaxanthin and β-carotene. The most notable variation is the absence of the peak at ~10.8 min in N2, likely associated with β-carotene, which is present in all other chromatograms. Additionally, some peaks at ~7.3 and 7.6 min are present only in NADES extracts and are absent in the acetone chromatogram. The quantification of the area under the curve of the peak at ~7 min indicated that acetone extracted 2.5 mg g^{-1} astaxanthin per dry biomass (0.25 wt%). N1, N2, and N6 NADES were able to extract 50, 36, and 79 % astaxanthin compared to acetone, respectively (Fig. 3b). Thus, N6, comprising 1:1 menthol-caprylic acid, had the best extraction capacity among the tested NADES and was not significantly different than that of acetone.

Since N6 showed the highest extraction efficiency, we examined the possibility of improving the extraction yield by decreasing the biomass-solvent ratio. The extraction was performed with a 1.25 %*w/v* biomass-solvent ratio instead of an initial 2.5 %w/v. Altering the biomass-solvent ratio did not improve the extraction efficiency (Fig. 3b and Fig. 3c). Additionally, the biomass was double extracted to ensure the collection of the remaining material in the biomass after the first extraction. Double extracting the biomass slightly increased the yield by 9.18 %, indicating the presence of some leftover pigments in residuals after the first extraction; however, the effect was not significant (Fig. 3c).

These results show that the tested NADES could successfully extract up to 79 % of the astaxanthin content from modified *C. reinhardtii*. It is worth noting that this high extraction efficiency was achieved in a single, unassisted, two-hour extraction at room temperature without any pretreatment. Therefore, NADES offer a green low-cost solution for a waste-to-product conversion of the accumulated astaxanthin in

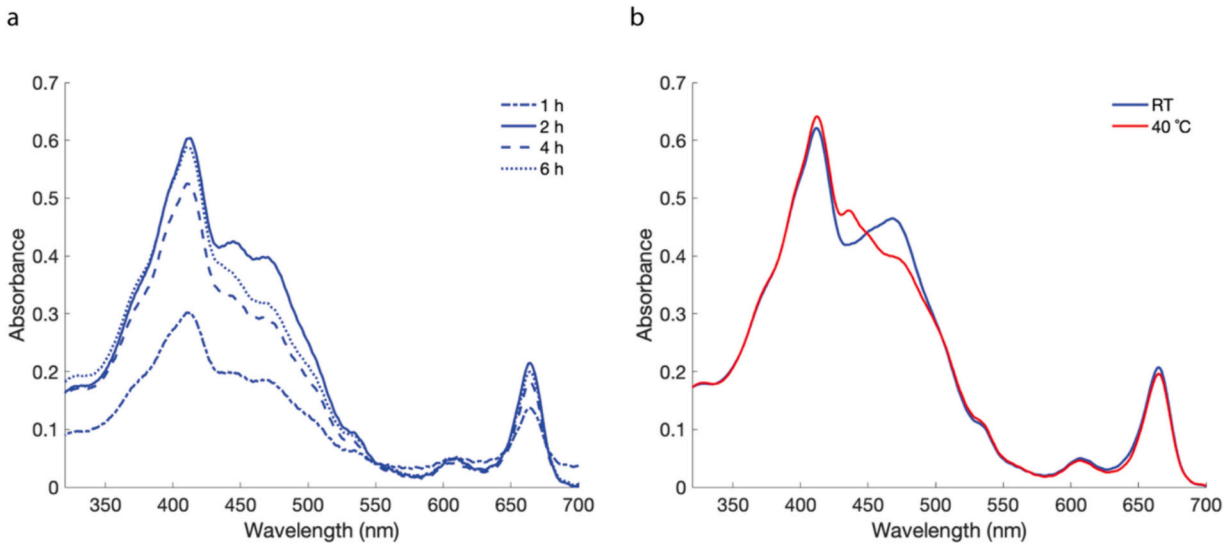

Fig. 2. Evaluation of NADES extraction conditions on engineered *Chlamydomonas reinhardtii* biomass. (a) UV–vis spectra of *C. reinhardtii* extracts by N1 collected after 1 h (dash-dotted line), 2 h (solid line), 4 h (dashed line), and 6 h (dotted line). (b) UV–vis spectra of *C. reinhardtii* extracted by N1 at room temperature (RT, blue) or at 40 °C (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

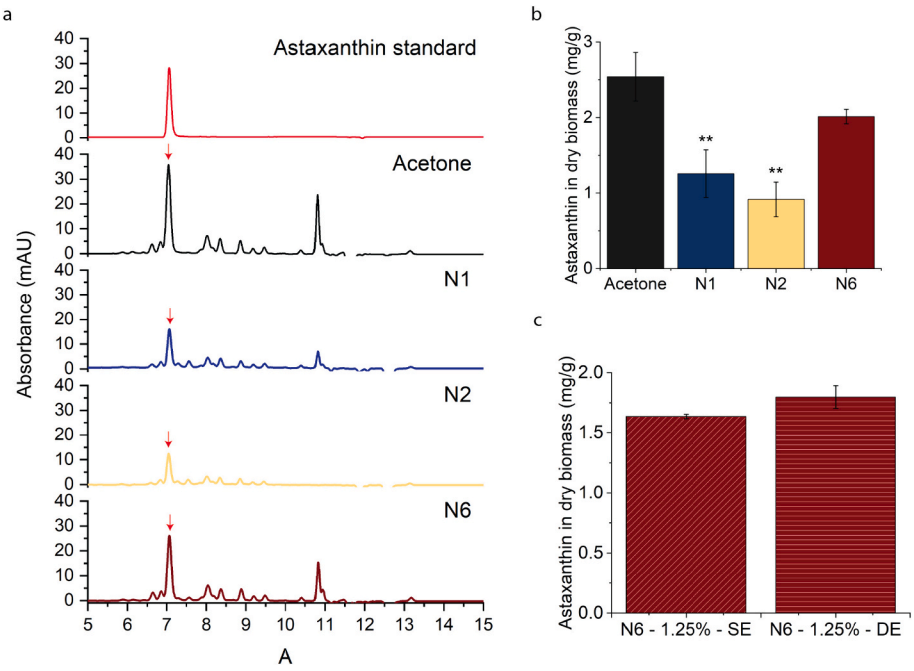

Fig. 3. Total pigment extraction from modified *Chlamydomonas reinhardtii* by menthol-based NADES. (a) HPLC-UV–vis chromatograms at 470 nm for astaxanthin standard, acetone, N1, N2, and N6 extracts (top to bottom). The peaks considered for the following quantification are indicated by arrows. (b) Concentrations of extracted astaxanthin from the areas under the curves retained at ~7 min and 470 nm, indicated by arrows in (a). (C) Recovered astaxanthin by N6 starting with a 1.25 % biomass-solvent weight-volume ratio obtained from a single extraction (SE) and a double extraction (DE). For (b) and (c), the values are presented as the mean ± SD for $n = 3$. The statistical significance is presented as $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ with respect to the leftmost bar. The absence of the asterisks indicates a nonsignificant difference.

modified *C. reinhardtii* that does not require additional biomass processing steps. Being composed of food-grade materials, pigment-containing NADES have the potential to be used as food or nutraceutical additives. In the future, statistical optimization of the NADES composition can be explored to further enhance the extraction efficiency.

3.3. Total pigment extraction from H. lacustris with NADES

The green microalga, *H. lacustris,* is the main natural (farmed) source of astaxanthin and is the most widely used on a commercial scale. Astaxanthin content in the *H. lacustris* varies from 1.5 to 3 % in commercially grown biomass [69]. Other reports have shown that the content could be further enhanced up to 5.34 % by weight [70]. Astaxanthin in *H. lacustris* occurs in a free form, or in forms where the hydroxyl group in one or both ionone rings are esterified. In *H. lacustris*,

approximately 70 % of astaxanthin is present as monoesters, 25 % as diesters, and only 5 % is unesterified [71].

Research has shown that the esterification degree of astaxanthin is positively correlated with its stability [72]. However, oral absorption analyses in mice revealed that the degree of astaxanthin esterification is negatively correlated to its bioavailability [72]. Moreover, free astaxanthin reached its maximum concentrations in the serum at an earlier time than esterified forms [72]. The lower bioavailability and slower uptake of astaxanthin esters could be attributed to their need to be hydrolyzed to free astaxanthin prior to their uptake by the gastrointestinal epithelium [73].

The cultivation of *H. lacustris* consists of two main stages [74]. At the green vegetative stage, the motile algal cells are under favorable conditions for growth and reproduction and do not accumulate high levels of astaxanthin. When cells are placed under stress conditions, they transition into a non-motile red phase during which they accumulate astaxanthin and reduce contents of other pigments. During the red phase, *H. lacustris* develops a rigid three-layered cell wall, mainly composed of algaenan biopolymers and cellulose-like polysaccharides [75].

The cell wall in *H. lacustris* presents a barrier to solvent extraction of its pigments, which typically necessitates pretreatments to improve the extraction yield. Mechanical pretreatment methods including grinding, milling, microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE) have been used [76]. The main downside of these methods is the generation of heat and energy or infrastructure requirements. Alternatively, chemical pretreatments such as acid hydrolysis have been shown [76], however, these involve corrosive acids like hydrochloric, sulfuric, and nitric acids that also require high working temperatures.

For sake of simplicity, total pigment extraction from *H. lacustris* will be described as astaxanthin extraction below, as this represents the major pigment in the cells harvested at this stage. Recently, oleic acid-based NADES were shown to extract ~60 % of the astaxanthin from *H. lacustris* in 6 h without any pretreatment [25]. Since N1, N2, and N6 showed high efficiency in astaxanthin extraction from *C. reinhardtii*, these hydrophobic NADES were tested for their ability to extract astaxanthin from red-phase *H. lacustris* dry biomass. The extraction yield was previously shown to reach its maximum after incubating the biomass in NADES for 2 h [25], therefore, all extractions were performed here for 2 h.

In agreement with previous reports [77], the extracts mostly contained astaxanthin monoesters and some diesters, demonstrated by the peaks between ~11.2–12.6 min in the HPLC measurements (Fig. 4a). The quantification of the areas under the three major astaxanthin monoester peaks observed at ~11.4, 11.6, and 11.9 min clearly demonstrates pigment extraction capabilities of N1, 2, and 6 on *H. lacustris* biomass (Fig. 4b). N1, 2, and 6 were able to extract significantly higher amounts of astaxanthin from the dry biomass compared to acetone, which were 10.4, 12.8, and 13.4 mg g^{-1}, compared to 6.6 mg g^{-1}, respectively (Fig. 4b). Estimating the absolute astaxanthin content in the *H. lacustris* dry biomass to be 50 mg g^{-1} (5 wt%) as reported by the manufacturer, these values correspond to ~20.8, 25.6, 26.8, and 13.2 % of the total astaxanthin content for N1, 2, 6, and acetone extracts, respectively. In agreement with these results, previous reports indicated that unassisted acetone could recover ~14 % of the total astaxanthin from *H. lacustris*.

As observed for *C. reinhardtii*, N6 exhibited the highest astaxanthin extraction yield for *H. lacustris* among the three tested NADES. The efficiency of N6 extraction was ~204 % that of acetone, compared to a maximum extraction efficiency of 60 % reported by Pitacco et al. with reference to organic solvents [25]. Assuming 70 % of the astaxanthin content is present as monoesters, N6 was able to extract ~38 % of the astaxanthin monoesters in a single extraction. These results were estimated by calculating the amounts of the major astaxanthin monoesters in the extracts; thus, the total extracted amounts are slightly higher. Yet,

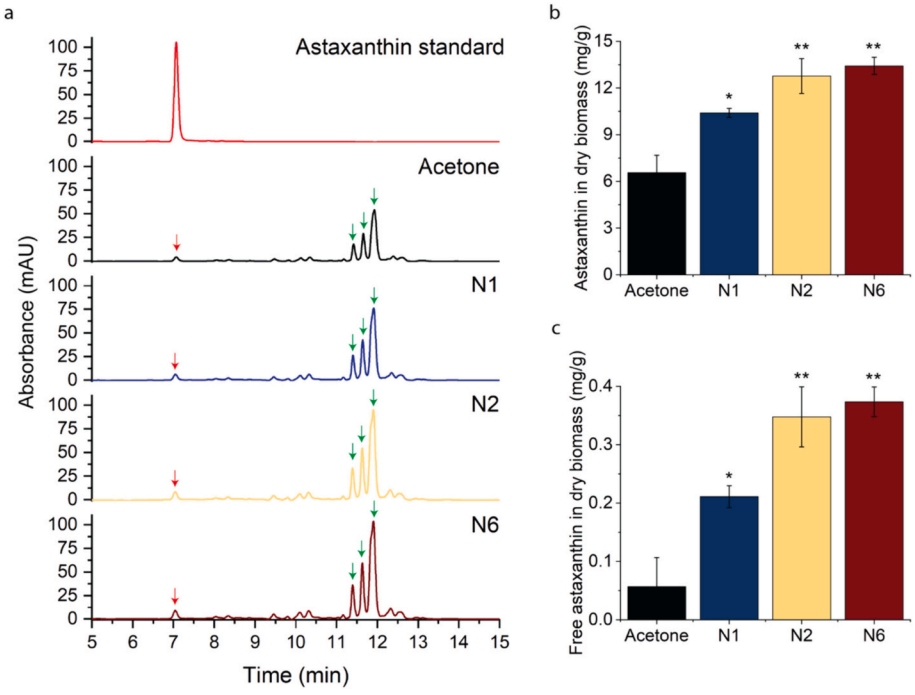

Fig. 4. Total pigment extraction from *Haematococcus lacustris* using menthol-based NADES. (a) HPLC-UV–vis chromatograms at 470 nm for acetone, N1, 2, and 6 extracts (top to bottom). (b) Concentrations of extracted astaxanthin from the areas under the three largest monoester peaks retained at ~11.4, 11.6, and 11.9 min and 470 nm, which are indicated by green arrows in (a). (c) Concentrations of free astaxanthin, calculated from the peak areas at ~7 min and 470 nm, indicated by red arrows in (a). For (b) and (c), the values are presented as the mean ± SD for $n = 3$. The statistical significance is presented as $^{*}P \leq 0.05$ $^{**}P \leq 0.01$ with respect to the leftmost bar. The absence of the asterisks indicates a nonsignificant difference. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

to our knowledge, this is the highest reported NADES extraction yield of astaxanthin from *H. lacustris*. These results indicate that menthol-based NADES can efficiently extract both free and esterified astaxanthin.

NADES were able to outperform acetone extraction in *H. lacustris* but were slightly less efficient than acetone at the extraction from *C. reinhardtii*. The disparity in the results could suggest that, compared to acetone, NADES are more efficient at extracting esterified astaxanthin and are slightly less efficient at extracting free astaxanthin. However, NADES were also superior in extracting free astaxanthin from *H. lacustris* compared to acetone (Fig. 4c). These results suggest that the NADES tested may be able to disrupt the *H. lacustris* cell wall more effectively than acetone. Previous studies have demonstrated that NADES are able to solubilize extracellular polymeric substances from membrane biofilm, and a variety of polysaccharides, such as starch, chitin, and chitosan [33,78,79]. Moreover, since acid treatment has been shown to induce algal cell lysis [76], the acidic components in the three NADES could have contributed to the disruption of the algal cell walls, consequently improving the extraction yield. Further studies are needed to elucidate the interactions between the NADES and *H. lacustris* cell wall components.

Taken together, these results present NADES as superior solvents for astaxanthin extraction. The employed extraction protocol presented a distinct advantage as it did not require any biomass pretreatment, heating, or sonication. These factors not only simplified the extraction procedure but also substantially contribute to reducing the environmental and economic impact. The food-grade components of these NADES suggest the feasibility of directly using the carotenoid extracts as food ingredients or in antioxidant applications. The recommended daily dosage of astaxanthin typically ranges between 4 and 12 mg [80]. Thus, the current maximum astaxanthin-NADES ratio in the extracts (~0.4 mg g^{-1}) is lower than necessary to allow their direct use as stand-alone oral supplements. The ratio could be improved by increasing the initial extraction %*w/v* (i.e., lowering the volume of NADES) or reusing the liquid for multiple rounds of extraction. Alternatively, the extracts could be used as topical antioxidants [81]. Furthermore, carotenoids have a plethora of other applications. They are used as food coloring agents and to provide vibrant shades in cosmetics. They also have cleansing, moisturizing, and anti-aging effects and can protect against UV-induced skin damage [82].

4. Conclusion

This work presents an unassisted green extraction of total carotenoids from engineered ketocarotenoid-producing *Chlamydomonas reinhardtii* and wild-type *Haematococcus lacustris* (formerly *Haematococcus pluvialis*). Explored for the first time for carotenoid extraction, the presented menthol-based NADES exhibited the highest extraction yields and efficiently extracted different astaxanthin forms. Although *H. lacustris* contains more than double the total astaxanthin content of the modified *C. reinhardtii*, the latter is a better source of free astaxanthin. The NADES also extracted other carotenoids and chlorophylls alongside astaxanthin, showcasing their suitability for extracting other structurally related compounds. The explored protocol efficiently extracted carotenoids from both cell-wall-deficient and three-layered-cell-wall algal strains. Therefore, it has the potential to extract valuable compounds from other microalgal species and other organisms.

The food-grade components of these NADES suggest the feasibility of directly using the carotenoid extracts in food and pharmaceutical applications. When considering these pigments for such applications, the chemicals used for extraction must be of the highest quality, which is usually more expensive than other chemical grades. The applications of carotenoids extend beyond food and health. Their molecular structure makes them ideal candidates for photo-sensitization and controlled electrical conduction [83]. Thus, they can be potentially used for solar and photoelectrochemical cells, semiconductor surface modification, and organic electronics in general. Lower commercial-grade quality chemicals can be used for such applications, further reducing costs without significantly impacting the extraction efficiency.

CRediT authorship contribution statement

Lamya Al Fuhaid: Writing – original draft, Methodology, Investigation, Data curation. **Gordon B. Wellman:** Writing – review & editing, Methodology, Investigation, Formal analysis, Conceptualization. **Najeh Kharbatia:** Methodology, Formal analysis, Data curation. **Andreia S.F. Farinha:** Writing – review & editing, Investigation, Formal analysis. **Johannes S. Vrouwenvelder:** Resources, Investigation, Funding acquisition. **Rob Verpoorte:** Methodology, Formal analysis. **Young Hae Choi:** Methodology, Formal analysis. **Geert-Jan Witkamp:** Writing – review & editing, Resources, Investigation, Funding acquisition. **Kyle J. Lauersen:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Formal analysis. **Luca Fortunato:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

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

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.algal.2024.103668.

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