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Extraction of astaxanthin from *Haematococcus pluvialis* with hydrophobic deep eutectic solvents based on oleic acid

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KEYWORDS. Astaxanthin; *Haematococcus pluvialis*; algal culture; deep eutectic solvents; terpenes; antioxidant potential.

ABSTRACT. Three novel hydrophobic deep eutectic solvents (DESs) based on oleic acid and terpenes (thymol, DL-menthol, and geraniol) were prepared, characterized, and used to extract astaxanthin from the microalga *Haematococcus pluvialis* without any pre-treatment of the cells. The three DES were composed of Generally Recognized As Safe (GRAS) and edible ingredients. All the tested DESs gave astaxanthin recovery values of about 60 and 30% in 6 hours if applied

on freeze-dried biomass or directly on algae culture, respectively. The carotenoid profile was qualitatively identical to what was obtained by using traditional organic solvents, regardless of the DES used; the monoesters of astaxanthin with C18-fatty acids were the main compounds found in all the carotenoid extracts. The thymol:oleic acid DES (TAO) could preserve astaxanthin content after prolonged oxidative stress (40% of the astaxanthin initially extracted was still present after 13.5 h of light exposure), thanks to the superior antioxidant properties of thymol. The capacity of improving astaxanthin stability combined with the intrinsic safety and edibility of the DES components makes the formulation astaxanthin-TAO appealing for the food ingredients/additives industry.

1. INTRODUCTION

Astaxanthin (3,3'-dihydroxy-β,β-carotene-4,4'-dione) is a secondary carotenoid belonging to the class of xanthophylls and biosynthesized (e.g. by the microalga *Haematococcus pluvialis* or the yeast *Phaffia rhodozyma*) or accumulated (e.g. by marine invertebrates or birds) by a variety of living organisms. The chemical structure of astaxanthin is directly correlated to the organism in which it is produced or found: astaxanthin in the free form is usually found in shrimps, crabs, flamingos or fishes, organisms that cannot synthesize astaxanthin *de novo* but are capable of accumulating such pigment when it is assumed with food (Ambati et al., 2014), while astaxanthin bounded with long-chain fatty acids (monoesters of astaxanthin) is the typical form biosynthesized by *H. pluvialis* (Miao et al., 2006). Moreover, *H. pluvialis* accumulates the astaxanthin monoesters in specific hydrophobic deposits in the cytoplasm composed of (neutral) lipid droplets (e.g. triglycerides) (Shah et al., 2016; Solovchenko, 2015).

The extraction and purification of astaxanthin from *H. pluvialis* is not a trivial process: i) the known chemical instability of astaxanthin (oxidation) over long periods or when exposed to high temperatures, oxygen, light, and extreme pH environments can affect and compromise its practical use (Liu et al., 2016); ii) the presence of lipidic droplets all around astaxanthin molecules with similar solubility behavior hampers their effective separation; iii) the rigid cellular structures of *H. pluvialis* cysts in which astaxanthin is accumulated creates a physical "barrier" that can decrease the efficiency of the extraction; iv) the need of harvesting and dewatering algal cells before the extraction makes the entire recovery challenging and energy-intensive.

The recovery of natural astaxanthin has been accomplished with a variety of solvents, from hazardous traditional organic compounds to safer solvents (e.g. ethyl acetate, acetone, ethanol, Vechio et al., 2021), to unconventional (but sometimes highly-costly) alternatives (de Souza Mesquita et al., 2021) like supercritical CO_2, vegetable oils, ionic liquids, or deep eutectic solvents (Chandra Roy et al., 2021; Desai et al., 2016; Gao, You, et al., 2020; Krichnavaruk et al., 2008; Machmudah et al., 2006; Rodrigues et al., 2020; Samorì, Pezzolesi, et al., 2019). Regardless of the kind of solvent, the dewatering of the algal biomass, its pre-treatment to weaken the cell walls (i.e. cell disruption), the solvent removal to recover astaxanthin, and the color fading and loss of biological activity are unavoidable bottlenecks.

Ionic liquids have been widely used in the recovery of astaxanthin from natural matrices including *H. pluvialis* (Khoo et al., 2019), as "weakening" agents to increase the cell membrane permeability (Bi et al., 2010; Choi et al., 2019; Desai et al., 2016; Liu, Yue, et al., 2018; Liu, Zeng, et al., 2018) and as real lipophilic solvents (Fan et al., 2019; Gao, Fang, et al., 2020; Gao, You, et al., 2020; Khoo et al., 2021; Praveenkumar et al., 2015). In all these cases, dried *H.*

67 *pluvialis* biomass or an "algal" paste at 20 wt% of biomass (Praveenkumar et al., 2015) has been
68 chosen as the matrix for the ILs-assisted extraction, thus no protocol has never been applied
69 directly to algal cultures. Moreover, since ILs are not volatile (apart of the distillable CO_2-based
70 alkyl carbamate ILs developed by Khoo et al., 2021), the solvent removal/separation to recover
71 astaxanthin is a critical issue, performed by the addition of an anti-solvent that was then distilled
72 to regenerate the IL itself (Gao, Fang, et al., 2020; Gao, You, et al., 2020) or by a further
73 liquid/liquid extraction (Praveenkumar et al., 2015). It is worth mentioning that the inherent
74 toxicity of most IL components (especially the first-generation ones) prevents their use without
75 any separation from the extracted astaxanthin.
76 Deep eutectic solvents (DESs) are a new generation of solvents composed of a hydrogen bond
77 acceptor (HBA) like choline chloride or betaine and a hydrogen-bond donor (HBD) (such as
78 amides, amines, alcohols, and carboxylic acids) that self-organize through hydrogen bonds to
79 form a mixture characterized by a shift of the eutectic point from the theoretical one, in terms of
80 both molar ratio of the components and melting point (Pontes et al., 2017). DESs have become
81 quite popular in the scenario of "green extractions", especially if composed of non-toxic and
82 biocompatible hydrogen bond donors and acceptors (HBD and HBA, respectively) (Dai et al.,
83 2013). DESs have been initially considered as "improved ILs" in terms of sustainability, but the
84 characteristics that these two families of neoteric solvents have in common are important but a
85 few, like the non-volatility and the tunability of the properties as a function of different
86 combinations of the components.
87 In analogy with ILs, both hydrophilic (Chandra Roy et al., 2021; Padilha et al., 2021; Zhang et
88 al., 2014) and hydrophobic (Lee & Row, 2016; Rodrigues et al., 2020) DESs have been used so
89 far for the recovery of astaxanthin from natural matrices, mainly from crustacean waste. If

hydrophilic and water-soluble eutectic mixtures of choline chloride and diols/carboxylic acids act as “adjuvants” for weakening algal cell wall and enhancing a subsequent astaxanthin extraction, hydrophobic DESs can work as real solvents for astaxanthin itself (Florindo et al., 2019). The only class of hydrophobic DESs exploited so far as solvents for extracting astaxanthin are terpene-based mixtures, characterized by a low viscosity in comparison to hydrophilic DESs and other phosphonium-based hydrophobic DESs (Lee & Row, 2016; Rodrigues et al., 2020): perillyl alcohol, camphor, eucalyptol, and menthol were used mixed with myristic acid, and the mixture menthol-myristic acid was the most efficient one in the extraction of astaxanthin from crab waste (Rodrigues et al., 2020).

A common feature to both hydrophilic and hydrophobic DESs is their low or absent volatility: if this property confers an intrinsic safety for the operators and the, it is also true that this strictly influences their application since DESs are inseparable from the compounds they dissolve (Samorì, Mazzei, et al., 2019). For this reason, the use of eutectic mixtures composed of inherently safe components is mandatory for developing “bioactive compounds-DES” formulations exploitable in applications for humans. Menthol-fatty acids hydrophobic DESs meet this criterion (Silva et al., 2019) with the additional benefit of being Therapeutic DESs (THeDES, Silva et al., 2018) since
both menthol and fatty acids are anti-inflammatory and antimicrobial compounds (Silva et al., 2019; Van Osch et al., 2019).

The present paper aims to apply terpene-based hydrophobic DESs to the recovery of natural astaxanthin from *H. pluvialis* by exploiting the unique features of three novel mixtures based on oleic acid mixed with DL-menthol (named MAO), thymol (TAO), and geraniol (GAO), here used for the first time:

- being water-immiscible, the three DESs were applied directly to *H. pluvialis* cultures, developing a novel protocol in which the harvesting, dewatering and pre-treatment of the algal cells were avoided, thus decreasing the overall energy consumption and economics of the extraction process (Samorì, Pezzolesi, et al., 2019);
- being composed of non-volatile components, the three DESs were not separated from the extracted astaxanthin but directly incorporated into bioactive formulations, overcoming the difficulties and energy consumption of astaxanthin recovery from non-volatile solvents using an anti-solvent (Rodrigues et al., 2020);
- being composed of edible and Generally Recognized As Safe (GRAS) components, already in use as food additives, the three DESs were used to prepare formulations that could be exploited in the food industry as carriers of natural astaxanthin, approved by both the United States Food and Drug Administration (USFDA) and the European Commission as food colorant/dye;
- being composed of oily components (oleic acid), known to improve the stability and the bioavailability in humans of natural astaxanthin (Ambati et al., 2014), the three DESs could act as stabilizing agents for natural astaxanthin

Therefore, in the present paper, the possibility of extracting, conveying, and stabilizing astaxanthin through oleic acid-based edible DES has been explored and demonstrated, trying to meet the concept of "Green Food Processing" by reducing the use of hazardous solvents and chemicals, minimizing the production of waste, and using renewable compounds (Khoo et al., 2020).

2. MATERIALS AND METHODS

2.1 Chemicals

All solvents and chemicals used in this study were purchased from Sigma-Aldrich (Germany) and were used without purification (purities ≥ 98%). Free astaxanthin, canthaxanthin, and lutein standards (purities ≥ 95%) were purchased from Sigma-Aldrich (Germany).

2.2 Solid-Liquid phase determination and Deep Eutectic Solvents (DESs) preparation

All the hydrophobic DESs (DL-menthol:oleic acid, thymol:oleic acid, geraniol:oleic acid) characterization was based on the comparison between the experimental and the theoretical solid-liquid phase diagrams. The experimental solid-liquid phase curves were obtained by measuring the melting points of the different samples at the different molar ratios with a thermometer via immersion of the samples in an ice/NaCl mixture or solid CO_2/acetone mixture in a Dewar. The melting temperatures were evaluated in triplicate to avoid any kinetic effect on the melting of the mixtures.

The solid-liquid theoretical curves were determined by using equation (1) that represents the solid-liquid equilibrium curve in a eutectic mixture (Rowlinson, 1970):

$$ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T}\right) + \frac{\Delta_m Cp_i}{R} \cdot \left(\frac{T_{m,i}}{T} - ln\frac{T_{m,i}}{T} - 1\right) \quad (1)$$

where χ_i is the mole fraction of component i, γ_i is its activity coefficient in the liquid phase, $\Delta_m h_i$ and $T_{m,i}$ are its melting enthalpy and temperature, respectively, $\Delta_m Cp_i$ is its heat capacity change upon melting, R is the ideal gas constant, and T is the absolute temperature of the system. This equation can be simplified by considering the heat capacity change upon the melting of a substance as negligible, therefore equation (2) was used:

$$ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T}\right) \quad (2)$$

158 The theoretical melting temperatures were determined from the theoretical curves by considering
159 the activity coefficients γ_i = 1. The eutectic points were determined as the minimum in the
160 experimental curves and they were compared to the theoretical ones.
161 The experimental γ_i values were determined via equation (3) by using the experimentally
162 observed melting temperatures:

163 $$\gamma_i = \frac{\exp\left[\frac{\Delta_m h_i}{R}\left(\frac{1}{T_{m,i}} - \frac{1}{T}\right)\right]}{\chi_i} \quad (3)$$

164 The three DESs were then prepared by mixing appropriate molar ratios of oleic acid and
165 the three terpenes to give MAO (DL-menthol:oleic acid, 2:1), TAO (thymol:oleic acid,
166 3:1), and GAO (geraniol:oleic acid, 13:1). The mixtures were heated at 60°C and
167 magnetically stirred until homogeneous liquids were obtained. Particular attention was
168 given to ensure homogeneous heating and prevent terpenes sublimation by limiting the
169 headspace.
170 The water content of the three DESs was measured via Karl-Fisher titration (684 KF
171 Coulometer, Metrohm, US).
172 Three other mixtures were also prepared in the same way and then tested, to compare the
173 extraction efficiency of eutectic and non-eutectic mixtures of oleic acid: i) thymol:oleic
174 acid in a molar ratio 1:1, ii) geraniol:oleic acid in a molar ratio 2:1, and iii) L-α-
175 phosphatidylcoline:oleic acid in a weight ratio 95:5.
176
177 *2.3 Haematococcus pluvialis cultivation*
178 *H. pluvialis* (strain HP5, isolated in July 2014 in a freshwater sample collected in Ravenna, Italy)
179 was cultivated in triplicate in a 1 L air-insufflated bottle using a modified BBM medium at a
180 temperature of 21±1°C, a light intensity of 90-100 μmol of photons per m^2 s^{-1} and a 16 h light:8

h dark cycle. Under these conditions, the cells were kept in a vegetative phase until a dry weight of 0.7 g L^{-1} was reached. Then, the cultures were stressed under high light intensity (450-500 µmol of photons per m^2 s^{-1}) and nutrient starvation by 3-times dilution of the algal culture (Samorì, Pezzolesi, et al., 2019). When mature aplanospores (red cysts) were obtained, astaxanthin was extracted through two different procedures: extraction from freeze-dried algal biomass (see Section 2.4) and from algal culture (see Section 2.5).

2.4 Extraction of astaxanthin from freeze-dried H. pluvialis biomass

Algal culture (100 mL) with an astaxanthin content of 1.6 wt% was collected and centrifuged at 2550 x g for 10 min at 4°C. The supernatant was removed, the algal pellet was freeze-dried and then extracted at rt for 6 h with DESs, oleic acid, and geraniol (50 mg of biomass with 2 mL of solvent, i.e. 2.5 wt%). At the end of such time, the extracts were centrifuged at 2550 x g for 10 min to separate the extracted biomass from the liquid phases, then recovered by pipetting. Aliquots of the recovered liquid phases (0.02 mL) were withdrawn at specific time frames (1, 2, 4, and 6 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV vis at 470 nm, as described below to determine the astaxanthin content. The same extraction procedure was also applied varying specific conditions to evaluate their effect on the kinetics and overall extraction performances:

i) at rt with three non-eutectic mixtures of oleic acid: i) thymol:oleic acid in a molar ratio 1:1, ii) geraniol:oleic acid in a molar ratio 2:1, and iii) L-α-phosphatidylcoline:oleic acid in a weight ratio 95:5;

ii) at 60°C with TAO;

iii) at 60°C with two other biomass/TAO ratios: 50 mg biomass/1 mL TAO (i.e. 5 wt%), and 50 mg biomass/0.5 mL TAO (i.e. 10%).

2.5 Extraction of astaxanthin from H. pluvialis culture

Algal culture (3 mL) with a cell density of 1.3 g L^{-1} and an astaxanthin content of 2.7 wt% was put in contact with DESs, oleic acid, and geraniol (1 mL) and gently stirred with a magnetic bar at 50-100 rpm for 6 h. At the end of such time, the biphasic mixtures were centrifuged at 2550 x g for 10 min to separate the algal cultures from the liquid solvents, lastly recovered by pipetting. Aliquots of the recovered solvent phases (0.02 mL) were withdrawn at specific time frames (1, 2, 4, and 6 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV vis at 470 nm, as described below to determine the astaxanthin content. *H. pluvialis* vitality before and after the extraction experiments was evaluated through pulse-amplitude modulated (PAM) fluorometry measurements in terms of kinetics and parameters of Photosystem II (PSII) (Samorì, Pezzolesi, et al., 2019). The model used was 101-PAM (H. Walz, Effeltrich, Germany) connected to a PDA-100 data acquisition system, high power LED Lamp Control unit HPL-C and LED-Array-Cone HPL-L470 to supply saturated pulses, US-L655 and 102-FR to provide far-red light and light measurement, respectively. Before and after the extraction experiments, aliquots of algal cultures were placed in cuvettes (10 × 10 mm) mounted on an optical unit ED-101US/M. Measurement of the photosynthetic efficiency was derived from the maximum quantum yield of PSII (ΦPSII), calculated from the following equation (4):

$$\Phi\text{PSII} = \frac{F_m - F_0}{F_m} \quad (4)$$

The minimal fluorescence (F_0) was measured on dark-adapted cultures for 20 min, by using modulated light of low intensity (2 μmol m^{-2} s^{-1}). Then, a short saturating pulse of 3000 μmol

226 m^{-2} s^{-1} for 0.8 s induced the maximal fluorescence yield (F_m). Photosynthetic activity (%) was
227 calculated by dividing the maximum quantum yield of PSII (ΦPSII) after the extraction by the
228 maximum quantum yield of PSII (ΦPSII) of the culture before the extraction.
229
230 *2.6 Astaxanthin analysis*
231 To determine the astaxanthin content in the algal cells subjected to the extraction experiments, a
232 freeze-dried algal pellet (50 mg) was extracted twice with a mixture of
233 cyclohexane/ethanol/acetone (2/1/1, 5 mL) for 48 h at rt. An aliquot of solvent (0.02 mL) was
234 withdrawn, diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV-vis
235 at 470 nm. Liquid chromatography analysis was performed using an HPLC system (Agilent 1200
236 series, Agilent Technologies Italia S.p.A, Milan, Italy) coupled with a UV-vis diode array
237 detector. The separation was performed using an XBridge C8 column 137 Å, 3.5 μm, 4.6 mm x
238 150 mm (Waters, Milford, MA, US) maintained at 30°C, with an injected volume of 5 μL. The
239 mobile phase was constituted as follows: H_2O (solvent A) and methanol (solvent B).
240 Chromatographic separation was achieved at a 0.7 mL min^{-1} flow rate under gradient elution
241 conditions: 80–100% B from 0 to 10 min, 100% B from 10 to 18 min, 100-80% B from 18 to 20
242 min; all the changes in the mobile phase composition were linear. The astaxanthin content in the
243 cells was determined using a calibration curve prepared with standard astaxanthin in the free
244 form (2.5-20 μg mL^{-1}). The astaxanthin recovery (%) was determined by dividing the astaxanthin
245 amount extracted with DESs, oleic acid, and geraniol, by the astaxanthin content in the algal
246 cells (determined as described before with the mixture of cyclohexane/acetone/ethanol). The
247 qualitative identification of the astaxanthin monoesters in the extracts was carried out through
248 HPLC-MS analyses performed on an Agilent 1260 Infinity II system coupled to an electrospray
249 ionization mass spectrometer (positive-ion mode, m/z = 100–3000 amu, fragmentor 30 V). The

column was the same used for HPLC/UV-Vis analysis, the mobile phase was modified by adding trifluoroacetic acid 0.1% v/v to both solvents. Chromatographic separation was achieved at a 0.4 mL min^{-1} flow rate under gradient elution conditions: 80–100% B from 0 to 10 min, 100% B from 10 to 30 min, 100-80% B from 30 to 32 min. Chemstation software was used for data processing.

2.7 Light-stability test and DPPH assay

Oxidation tests were performed on the extracts obtained from freeze-dried *H. pluvialis* biomass to evaluate the potential of the different hydrophobic solvents here used to stabilize and preserve astaxanthin. Samples were exposed to light radiation under controlled conditions employing sun simulating OSRAM ULTRA-VITALUX 300W UV-A lamp (220-230 μE m^{-2} s^{-1}, OSRAM spa, Milan, Italy). Aliquots of solvent (0.02 mL) were withdrawn at specific time frames (0.5, 1.5, 3.5, 7.5 and 13.5 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV vis at 470 nm, as described above to determine the astaxanthin content. The astaxanthin stability was expressed as the percentage of astaxanthin amount at specific ageing time with respect to the astaxanthin content in the corresponding unaged sample.

The antioxidant activity of the obtained extracts was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Blois, 1958). An aliquot (25 μL) of the sample was dissolved in ethanol to obtain 2 mL solutions, then 500 μL of these solutions were mixed with 500 μL of 0.06 mM DPPH solution (in ethanol). After 30 min of incubation at rt in the dark, the absorbance at 517 nm was measured with JASCO V-650 UV/Vis spectrophotometer (Jasco, Tokyo, Japan). The DPPH free radical scavenging activity was calculated in terms of the percentage of inhibition of the free radicals by using the following equation (5):

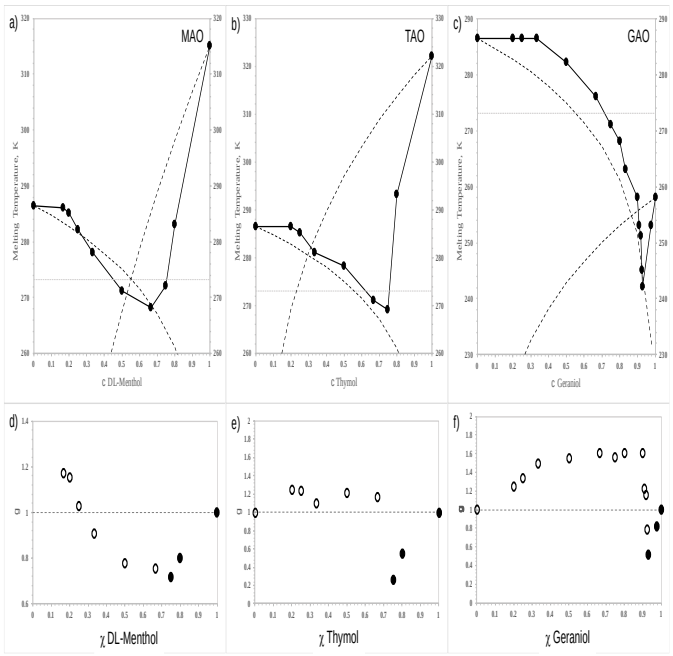
$$\text{DPPH scavenging activity } \% = \frac{A_C - (A_S - A_B)}{A_C} \quad (5)$$

Where: A_S indicates the absorbance of the sample, A_C is the absorbance of the control (prepared by diluting 500 µL of DPPH solution in 500 µL of ethanol) and A_B is the absorbance of blank (prepared by mixing 500 µL of sample solution with 500 µL of ethanol).

3. RESULTS AND DISCUSSION

3.1 Hydrophobic DESs preparation and solid-liquid phase diagrams

Three oleic acid-based mixtures composed of natural and edible components approved by the Flavor and Extract Manufacturers Association (FEMA) as GRAS flavor ingredients (oleic acid: FEMA N° 2815; DL-menthol: FEMA N° 2665; thymol: FEMA N° 3066; geraniol: FEMA N° 2507) were here prepared with the aim of providing an improvement towards the use of hydrophobic DESs (Martins et al., 2018). The solid-liquid phase diagrams of the three mixtures were initially defined and compared with the theoretical melting curves (Figure 1) (Kollau et al., 2019). This approach was necessary to define the identity of the liquid mixtures; in this case, this was particularly important since oleic acid itself is a liquid (m.p. = 16°C), so the resulting mixtures could be solutions rather than eutectic systems. Moreover, a shift of the eutectic point from the theoretical one, in terms of both molar ratio of the components and melting point, is necessary to define the mixtures as "deep eutectic solvents" (Pontes et al., 2017). This shift indicates that the interactions occurring between the different molecules have intensities like the ones occurring between the same species; therefore, the mixtures have a non-ideal behavior (Ashworth et al., 2016).



295

296 **Fig. 1.** Eutectic profiles, experimental melting points (dots) and theoretical curves (dashed lines)
297 *vs* molar ratio of the DESs (a-c); experimental activity coefficients γ of the DESs (d-f).

298

299 All the three mixtures can be considered as DESs as the eutectic points observed differed from
300 the theoretical curves both in terms of eutectic ratio and melting points (Abdallah et al., 2021).
301 MAO had a eutectic point at 2:1 (DL-menthol:oleic acid) molar ratio with a melting point of -
302 5°C (oleic acid m.p. = 16°C; DL-menthol m.p. = 42°C) while the ideal curve showed a minimum
303 at about 0°C and approximately 1:1 molar ratio of the components. TAO had a eutectic point at
304 3:1 molar ratio (thymol/oleic acid) with a melting point of -4°C (thymol m.p. = 49°C); in this
305 case, a higher shift from the theoretical curve was observed (about 7°C and 1/2 molar ratio).
306 GAO had a peculiar and uncommon solid-liquid diagram with a eutectic point at 13:1 molar ratio
307 (geraniol:oleic acid) with a melting point of -31°C (geraniol m.p. = -15°C). However, even the
308 theoretical curve showed a minimum at a high value of the molar ratio (0.85 molar fraction of
309 geraniol) with a temperature of about -18°C. All the DESs showed shifts from ideal values of

activity ($\gamma = 1$) in correspondence to the eutectic points. If MAO showed the fewest difference from the ideal curve in the melting point profile, TAO and GAO showed high differences from the ideal behavior. All these liquid systems can be considered as Type V DESs because they are composed of non-ionic molecules. Moreover, they are hydrophobic because both the components are scarcely soluble in water (<0.01 mM oleic acid; <3 mM DL-menthol; 6 mM thymol; 5 mM geraniol) and their water content (i.e. 2.9 wt% for TAO, 0.94 wt% for MAO, and 2.6 wt% for GAO) was in agreement with literature data on hydrophobic DESs (Tiecco et al., 2019). Mixtures of terpenes (such as geraniol, thymol, and menthol) with carboxylic acids have been already reported and discussed in the literature (Martins et al., 2018); however, in those mixtures, only small shifts of the experimental melting points from the theoretical ones are reported. Differently, in all the oleic acid mixtures here reported, larger differences were observed; this suggests that the hydrogen-bonding networks established in oleic acid-based mixtures are significantly different in intensity to the ones reported for other carboxylic acids. Three other non-eutectic but liquid mixtures of oleic acid were also prepared to understand whether the "eutecticity" could give superior extraction performances: i) thymol:oleic acid in a molar ratio 1:1, ii) geraniol:oleic acid in a molar ratio 2:1, and iii) L-α-phosphatidylcoline:oleic acid in a weight ratio 95:5.

3.2 Extraction of astaxanthin from freeze-dried H. pluvialis biomass

The three hydrophobic DESs here studied were applied to the extraction of astaxanthin from *H. pluvialis* and compared with the single (liquid) components (oleic acid and geraniol) in terms of astaxanthin recovery. From a qualitative point of view, all the hydrophobic phases here tested gave an identical carotenoid profile to what achieved through traditional organic solvents (cyclohexane:acetone:ethanol mixture) (Figure 2).

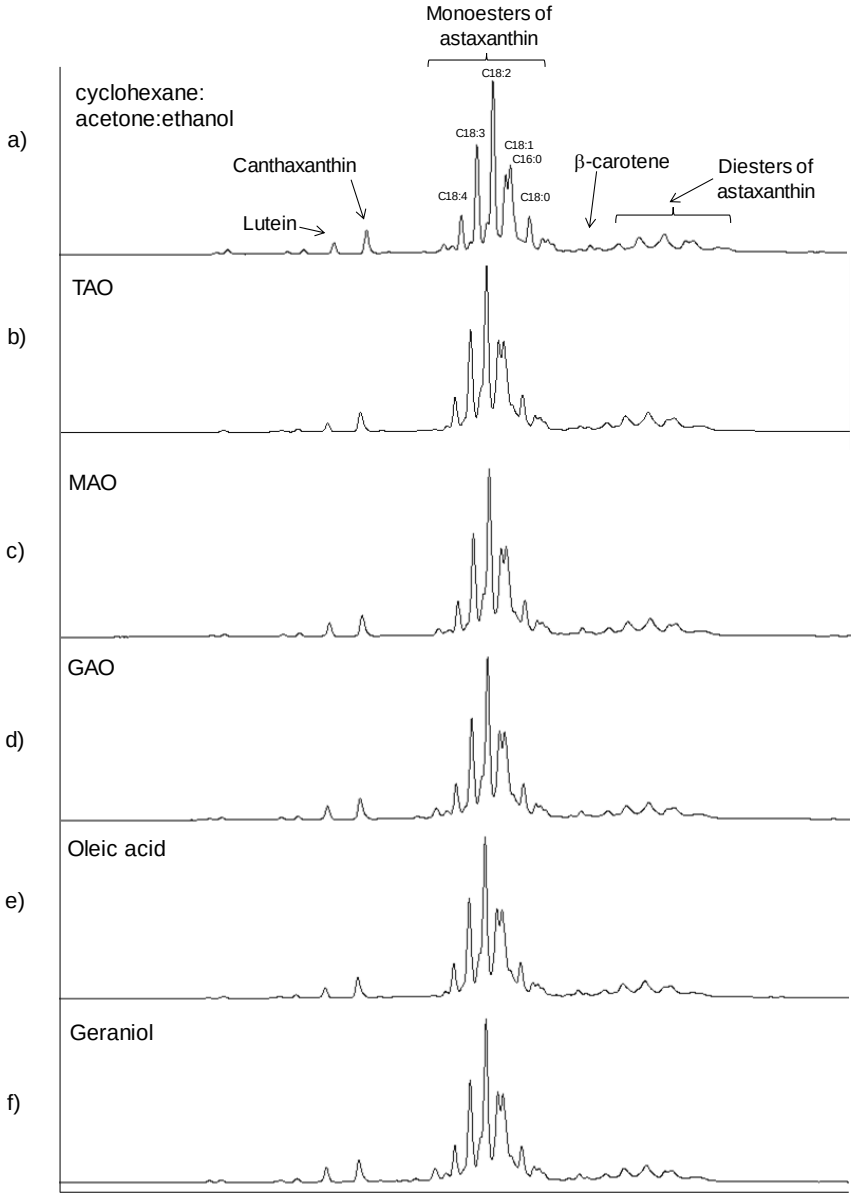


Fig. 2. Carotenoid profile obtained with a) cyclohexane:acetone:ethanol mixture 2:1:1 v/v/v; b) thymol:oleic acid 3:1, TAO; c) DL-menthol:oleic acid 2:1, MAO; d) geraniol:oleic acid 13:1, GAO; e) oleic acid, f) and geraniol.

All the chromatograms obtained by HPLC UV-Vis at 470 nm were predominantly characterized by the peaks of astaxanthin monoesters, identified by LC-MS on the basis of the molecular

342 weight: monoesters of linoleic (C18:2) and linolenic acid (C18:3) were the most abundant peaks,
343 followed by oleic (C18:1) and stearidonic acid (C18:4) monoesters (Figure 2). The monoester
344 with palmitic acid (C16:0) was the only C16-ester detected, in line with previous findings
345 (Samorì, Pezzolesi, et al., 2019). Minor signals ascribable to astaxanthin diesters, lutein,
346 canthaxanthin, and β-carotene were found in all the extracts. The ratio between astaxanthin
347 monoesters and diesters was 4.8±0.3, in line with the literature (Grewe and Griehl, 2008) and
348 independent from the solvent used and the kinetics, highlighting that no specific selectivity
349 occurred in the extraction of the two forms of astaxanthin biosynthesized by *H. pluvialis*. The
350 NMR spectra of the extract obtained with the cyclohexane:acetone:ethanol mixture (see Figure
351 1S in ESI) revealed also the presence of unsaturated triglycerides that constitute the lipidic
352 droplets known to create hydrophobic deposits in the hydrophilic environment of the cytoplasm
353 (Shah et al., 2016; Solovchenko, 2015), and from which astaxanthin is hardly separable even
354 after flash chromatography (see Figure 2S in ESI). Therefore, independently from the solvent
355 used for the extraction, the extracts resulted composed of a mixture of carotenoids, in which the
356 monoesters of astaxanthin dominate, and polyunsaturated fatty acids: all of these components
357 can play a synergic role and confer superior properties to the extract than isolated astaxanthin
358 (Tan et al., 2021).
359 From a quantitative point of view, the performance of the three hydrophobic DESs was similar,
360 giving a recovery of astaxanthin of about 60% in 6 h (Figure 3). MAO was the only one that
361 showed slower kinetics of extraction since in 1 h the recovery of astaxanthin was almost half of
362 that obtained with TAO and GAO. After 24 h, TAO gave the best extraction performances
363 (83±13%), followed by MAO (74±4%), and GAO (66±6%). Geraniol tested alone behaved
364 similarly to GAO, while oleic acid was the worst hydrophobic phase among the tested ones

(41±7% of recovery after 6 h), even after prolonged extraction times (24 h, 64±10%). This suggests that the combination of oleic acid in a DES mixture with all the three terpenes here used effectively improves its extraction ability, probably due to a reduction of oleic acid viscosity or to an increase in "affinity" for astaxanthin, thanks to π–π stacking interactions between the conjugated systems of terpenes (but not DL-menthol) and that of astaxanthin. On the other hand, the extraction performances of the non-eutectic mixtures of oleic acid here prepared (see Table 1S in ESI with the data for thymol:oleic acid in a molar ratio 1:1 and geraniol:oleic acid in a molar ratio 2:1) were worse than the corresponding DESs (TAO and GAO) over a long period; in particular, the recovery of astaxanthin with TAO was 1.4 times higher than a non-eutectic mixture of oleic acid and thymol. The mixture L-α-phosphatidylcoline and oleic acid (95:5 ratio by weight), known as OSMOS^{TM} solvent, was the worst solvent among the ones tested (52% after 24 h), presumably because of higher viscosity than the terpenes mixtures.

The effect of the temperature on the extraction performances was evaluated on TAO, the best solvent among the tested ones. Increasing the extraction temperature from rt to 60°C improved the kinetics and the overall performances, giving an astaxanthin recovery of 75±0.7% after 6 h, 1.2 times higher than what was obtained at rt and higher than all the solvents here tested. Therefore, this temperature was chosen to investigate two other biomass/TAO ratios (i.e. 5 and 10 wt%, see Figure 3S in ESI). The recovery after 6 h did not substantially change, regardless of the used ratio (70.9±2.8% at 10 wt% and 84.9±3.7% at 5 wt%), underlying that it is possible to minimize the amount of solvent used without changing the extraction performances (higher ratios were not tested since the viscosity of the "biomass-TAO" solution hampered an efficient separation of the extracted biomass by centrifugation).

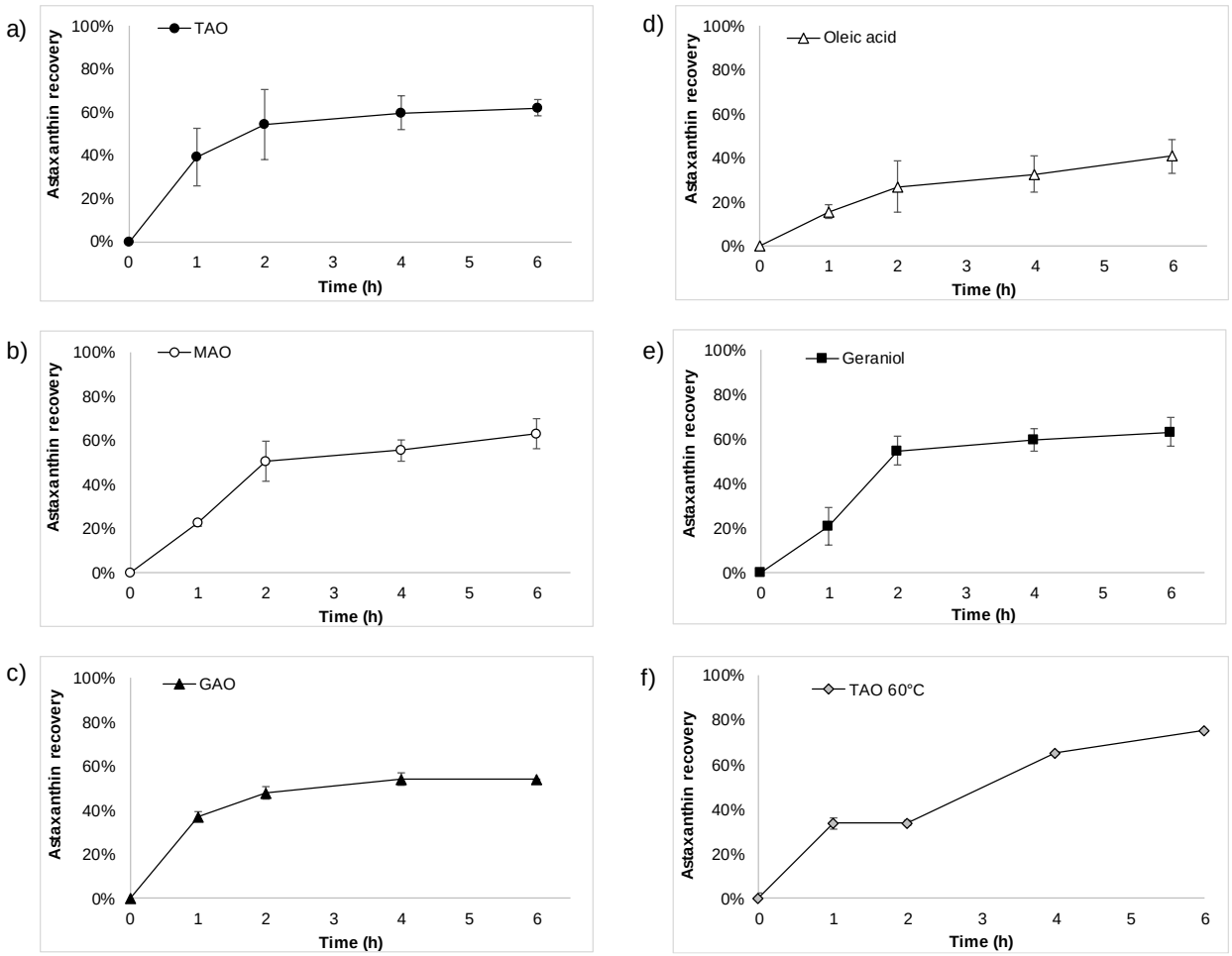


Fig. 3. Astaxanthin recovery from *H. pluvialis* freeze-dried biomass with a) thymol:oleic acid 3:1, TAO; b) DL-menthol:oleic acid 2:1, MAO; c) geraniol:oleic acid 13:1, GAO; d) oleic acid, e) geraniol, and f) thymol:oleic acid 3:1, TAO at 60°C. Data are expressed on the basis of the percentage of astaxanthin content in *H. pluvialis* cells, as mean ± standard deviation of two independent experiments on different freeze-dried algal biomass.

3.3 Extraction of astaxanthin from H. pluvialis cultures

All the three hydrophobic DESs here tested formed a biphasic system with water and were therefore applicable in a direct extraction of astaxanthin from *H. pluvialis* culture. The possibility of by-passing algae harvesting and dewatering is for sure economically appealing even if

extracting astaxanthin from algal cultures is more challenging than extracting astaxanthin from freeze-dried biomass because astaxanthin is accumulated inside algal cells surrounded by a strong and multilamellar cell wall and by a large volume of water. The kinetics of the liquid-liquid extraction with the three hydrophobic DESs and their single (liquid) components was here tested (Figure 4). In parallel, the algal vitality was analyzed by measuring the residual photosynthetic efficiency after the extraction at specific time frames (Figure 5 and Figure 4S in ESI). This evaluation was done to verify the "algal-compatibility" of such hydrophobic solvents in keeping *H. pluvialis* cells alive and reusable for continuous production of astaxanthin (Samorì, Pezzolesi, et al., 2019).

All the three DESs (Figure 4 a-c) followed the same kinetics of extraction: the recovery of astaxanthin increased from values of about 10% achieved in 1 h, up to 30% after 6 h; the "hampering" effect created by water to the contact between solvent and algal cells was evident since the recovery, in this case, was half of what achieved from *H. pluvialis* pellet in the same time frame (Figure 3 a-c). After 48 h, the recovery of astaxanthin reached values of 56, 58 and 68% with MAO, TAO, and GAO, respectively. Diversely from what occurred in the extraction of astaxanthin from algal pellets, oleic acid showed the same extraction pattern of DESs, while geraniol gave the best performance under these conditions (three-times higher astaxanthin recovery than DESs in 1 h, and 44% of recovery in 6 h). Qualitatively, the extracts recovered from algal cultures showed some differences with the extracts obtained from algal pellets (see Figure 5S in ESI): the chromatograms were dominated by the signal of astaxanthin monoesters, but lutein and β-carotene were almost undetectable. The ratio between astaxanthin monoesters and diesters (6.0±0.2), was higher than what was observed in the extracts from freeze-dried biomass (4.8±0.3), but it is known that several biological factors related to algal growth and

physiology (like the cultivation period, cysts age, growth medium composition, Grewe and Griehl, 2008) influence this number and in the present case *H. pluvialis* cultures used for obtaining the pellet came from a different batch than the ones used for the liquid-liquid extraction.

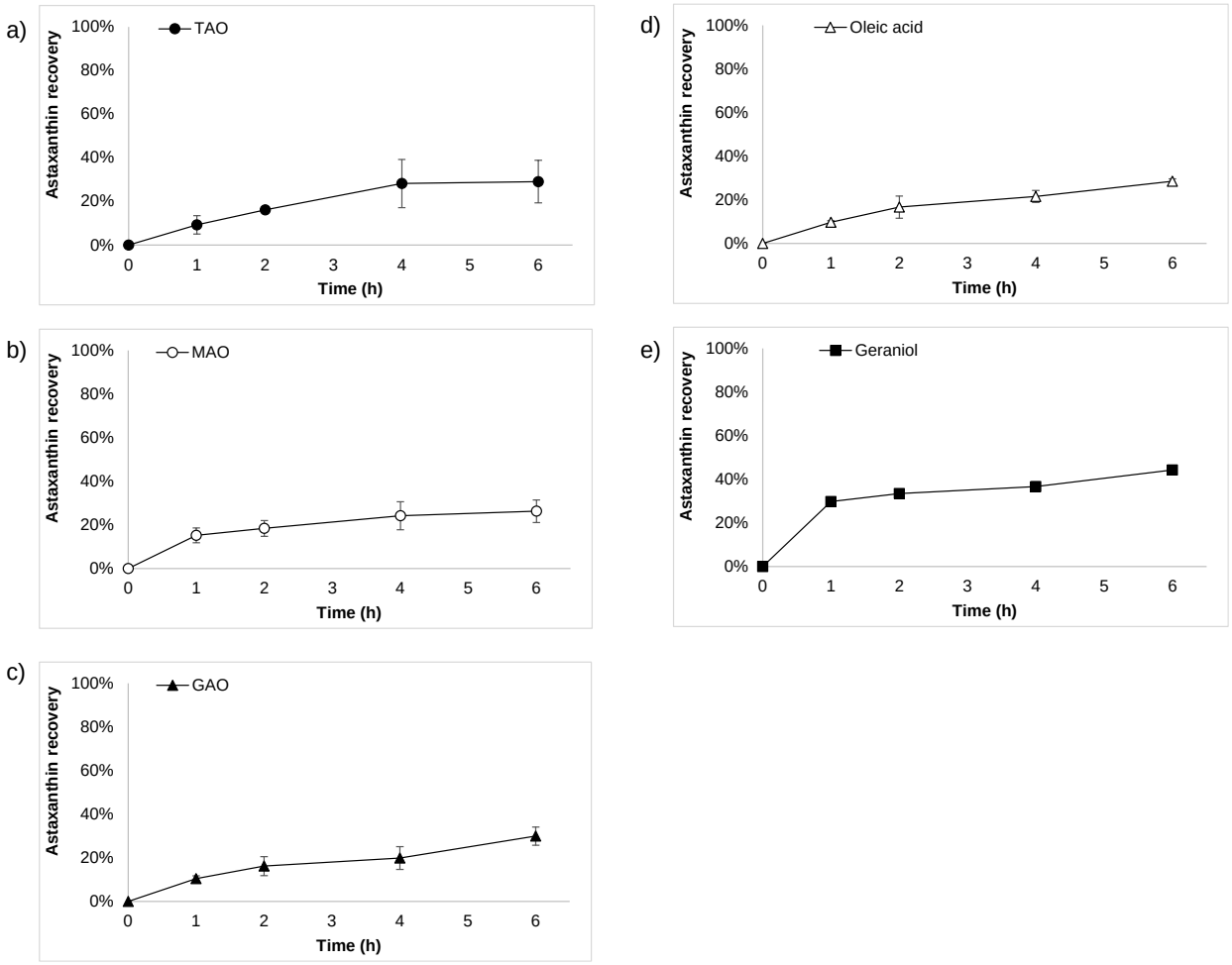


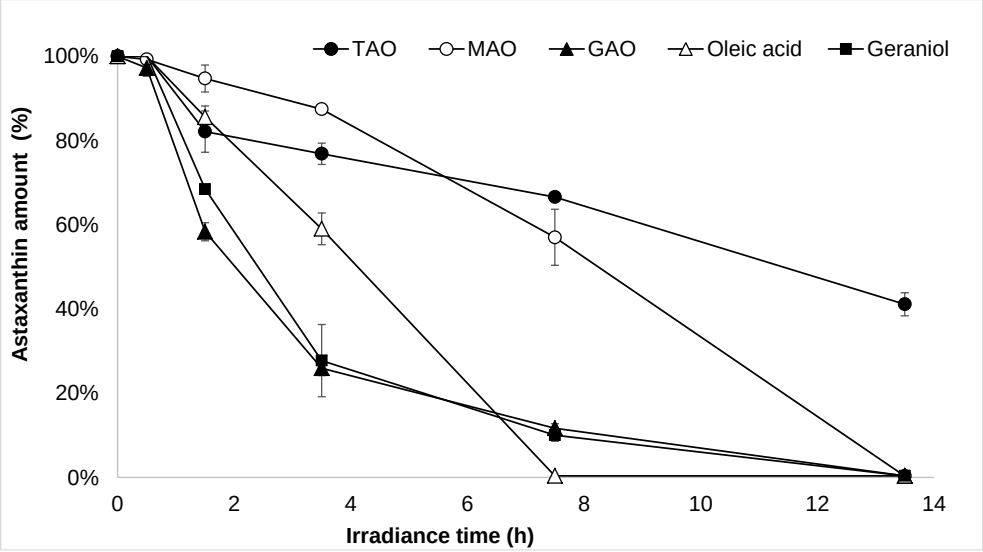
Fig. 4. Astaxanthin recovery from *H. pluvialis* cultures with a) thymol:oleic acid 3:1, TAO; b) DL-menthol:oleic acid 2:1, MAO; c) geraniol:oleic acid 13:1, GAO; d) oleic acid, and e) geraniol. Data are expressed based on the percentage of the astaxanthin content in *H. pluvialis* cells, as mean ± standard deviation of two independent experiments on different algal biomass.

Even if after 1 and 4 h of contact algal cells seemed intact and still rich in astaxanthin or empty under a light microscope (see Figure 10S in ESI), after 1 h no photosynthetic activity was observed for the culture put in contact with TAO, GAO, and geraniol, while the viability of cells extracted with MAO was 50% for the first hour of extraction, before dropping down to 0% after 4 h (see Figure 4S in ESI). In analogy to what was already observed for vegetable oils (Samorì, Pezzolesi, et al., 2019), oleic acid was the most algae-compatible compound, maintaining 80% of algal viability within the first hour of extraction and about 30% even after 6 h of extraction (see Figure 4S in ESI). NMR spectroscopy analysis of the algal cultures after 6 h of contact with the various hydrophobic solvents here tested helped in explaining such behavior: in the case of the three DESs, the presence of the terpenic component of the eutectic mixtures was detected (geraniol >> thymol ~ DL-menthol, Figures 6S for TAO, 7S for MAO and 8S for GAO in ESI), while oleic acid (tested alone or as a component of the DESs) was almost undetectable and largely below the molar ratio of the mixtures used in the extraction step; this suggested that the hydrophobic DESs here used were not completely water-stable (Florindo et al., 2017). Therefore, the algal cell mortality could be related to the toxicity towards algae of each terpene (the growth inhibition of DL-menthol, thymol, and geraniol on freshwater algae after 72 h of exposition is reported to be in the range of 0.1 mM). These data demonstrated that preserving the viability of algal cells after contact with solvents is even more challenging than extracting algal metabolites directly from algal culture.

3.4 Light-stability test and antioxidant activity

The instability of astaxanthin to light, oxygen, and self-oxidation is a serious issue that can affect its practical use, especially for what concerns the Z-isomers, less thermodynamically stable than

455 the all-E-isomers and more prone to isomerize in response to heat and light; different solvent
456 media (e.g. vegetable oils enriched in oleic acid like sunflower, soybean, sesame, and rice bran)
457 and additives (e.g. the antioxidants α-tocopherol and ascorbic acid) have shown their positive
458 effect in improving astaxanthin stability and preventing its degradation during 6-week storage in
459 the dark (Anarjan et al., 2013; Honda et al., 2021). Since the hydrophobic DESs here used
460 contain both oleic acid and terpenes that are known to have antioxidant properties that could
461 have a synergic effect, the stability of extracted astaxanthin contained in DESs, oleic acid, and
462 geraniol was tested under the effect of light, one of the main oxidative factor together with
463 temperature and oxygen (Figure 5) (Armenta & Isabbl, 2009).
464



465

466 **Fig. 5**. Effect of light as oxidative factor on astaxanthin contained in DESs, oleic acid, and
467 geraniol.
468

469 All samples except for TAO extract showed a complete astaxanthin degradation at the end of the
470 13.5 h irradiance. GAO and geraniol extracts followed the same kinetics of degradation, with an

astaxanthin content that rapidly decreased (after 3.5 h, more than 70% of the initial astaxanthin content was degraded). In oleic acid a decrease with a constant rate was observed, reaching a complete degradation after 7.5 h of exposure to light. MAO and TAO extracts performed the best, maintaining the astaxanthin amount above 50% after 7.5 h. After 13.5 h in TAO 40% of the initial astaxanthin content was maintained (Figure 6b), demonstrating TAO superior potential to stabilize astaxanthin due to the antioxidant properties of thymol, higher than those of geraniol and menthol (Ruberto & Baratta, 2000). The antioxidant activity of TAO alone was 30-times higher than that of MAO (Figure 6c, black bars), while oleic acid did not have any antioxidant activity at all. This finding can be attributed to the unique antioxidant properties of thymol, a well-known $^1O_2$ quencher and anti-lipid peroxidation agent, suggested as a valid natural replacement for synthetic antioxidant food additives (Aeschbach et al., 1994; Alam et al., 1999; Kruk et al., 2000). Moreover, it is known that a whole carotenoid extract that contains astaxanthin is more antioxidant than astaxanthin alone, thanks to the synergism that occurs in the extract between astaxanthin and the polyunsaturated lipidic droplets strictly associated with astaxanthin itself; moreover, astaxanthin monoester has a stronger total antioxidant capacity than astaxanthin in the free form (Tan et al., 2021). This could explain the large increase of the antioxidant potential of all the tested solvents (black bars) observed after the extraction process (white bars). However, after the exposition to light, only TAO was capable to maintain such property (grey bars), suggesting TAO as the most promising extractant, carrier and stabilizer of natural astaxanthin among the tested DESs, useful for the development of food additives.

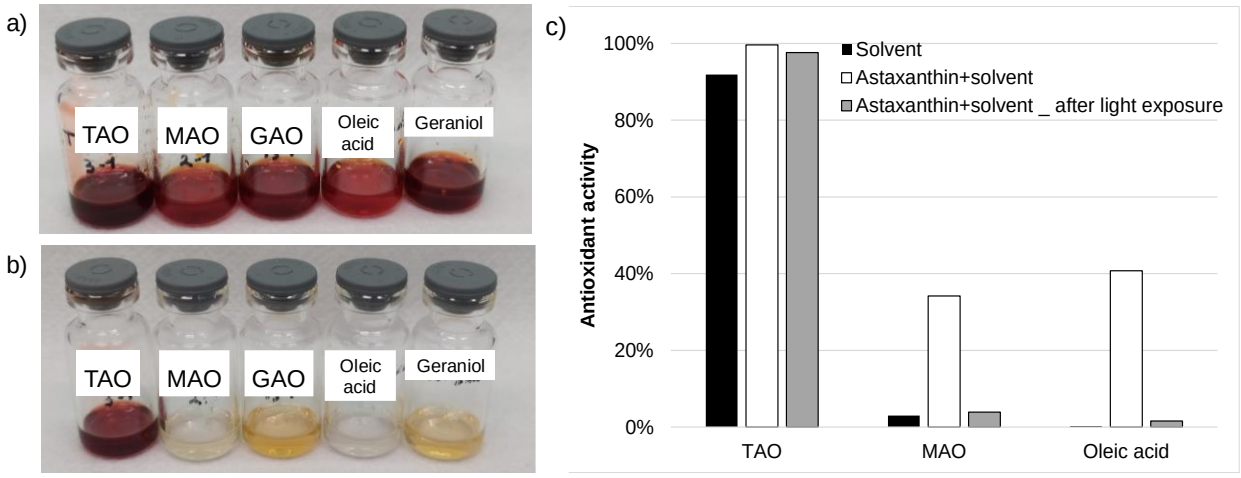


Fig. 6. Extracts of astaxanthin in TAO, MAO, GAO, oleic acid, and geraniol after a) the extraction from *H. pluvialis* freeze-dried cells, and b) 13.5 h of light irradiance. Antioxidant activity of TAO, MAO, and oleic acid tested alone or as extracts of astaxanthin (c).

4. CONCLUSIONS

Three novel DESs based on oleic acid and thymol (TAO), DL-menthol (MAO) and geraniol (GAO) have been here prepared for the first time and applied to the extraction of astaxanthin from *H. pluvialis*. All of them gave good recovery percentages without any thermal, mechanical or chemical pre-treatment; the extraction of dried biomass gave an astaxanthin recovery of about 60% in 6 h, independently from the DES used and significantly higher than the recovery of 40% achieved with oleic acid alone. Increasing the extraction temperature increased the recovery up to 75% under the same time frame, while the performances did not vary with the biomass/solvent ratio used.

A liquid-liquid extraction directly from algal cultures, by-passing dewatering and harvesting steps, known to be energy-intensive and largely impacting on the overall economics of algal-based process/productions, has been here demonstrated. In this case, the three DESs behaved similarly, giving a recovery of about 30% in 6 h and 60-70% in 48 h. Although the three DESs

behaved similarly in terms of extraction efficiency, they had completely different antioxidant potential and stabilizing power of astaxanthin: the eutectic mixture composed of thymol and oleic acid was the best in this sense, maintaining the astaxanthin amount above 40% after 13.5 h of light exposure thanks to the 30-times higher antioxidant potential of thymol in comparison to DL-menthol and geraniol. This finding suggests the possibility to exploit astaxanthin extracts in TAO as improved antioxidant formulations that could be used for human-related applications thanks to the biocompatibility of all the GRAS ingredients of such formulations.

ASSOCIATED CONTENT

Supporting Information. Astaxanthin recovery from *H. pluvialis* freeze-dried biomass with DESs, non-eutectic mixtures of oleic acid, oleic acid and geraniol. ^{1}H-NMR spectra of the crude and purified extracts obtained after extraction of *H. pluvialis* freeze-dried biomass with cyclohexane:acetone:ethanol mixture. Astaxanthin recovery from *H. pluvialis* freeze-dried biomass after 6 h with TAO, varying the biomass/TAO ratio. Comparison of the carotenoid profile obtained with TAO from a) freeze-dried *H. pluvialis* biomass and b) *H. pluvialis* culture. Residual photosynthetic activity of *H. pluvialis* cells after the contact with MAO and oleic acid ^{1}H NMR spectra of the oleic acid-based DESs after contact with water. Optical microscope pictures of algal cells after liquid-liquid extraction with TAO, MAO and GAO. ^{1}H NMR and ^{13}C NMR spectra of TAO before and after the extraction of freeze-dried biomass.

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ABBREVIATIONS

TAO, thymol:oleic acid mixture 3:1; MAO, DL-menthol:oleic acid mixture 2:1; geraniol:oleic acid mixture 13/1.

REFERENCES

Abdallah, M.M., Müller, S., González de Castilla, A., Gurikov, P., Matias, A.A., do Rosário Bronze, M., & Fernandez, N. (2021). Physicochemical characterization and simulation of the eutectic solvent systems. *Molecules*, *26*, 1801. https://doi.org/10.3390/ molecules26061801

Aeschbach, R., Löliger, J., Scott, B. C., Murcia, A., Butler, J., Halliwell, B., & Aruoma, O. I. (1994). Antioxidant actions of thymol, carvacrol, 6-gingerol, zingerone and hydroxytyrosol. *Food Chemistry Toxicology*, *32*(1), 31-36. https://doi.org/10.1016/0278-6915(84)90033-4.

Alam, K., Nagi, M. N., Badary, O.A., Al-Shabanah, O.A., Al-Rikabi, A.C., & Al-Bekairi, A.M. (1999). The protective action of thymol against carbon tetrachloride hepatotoxicity in mice. *Pharmacology Research*, *40*(2), 159-163. https://doi.org/10.1006/phrs.1999.0472.

Ambati, R. R., Moi, P. S., Ravi, S., & Aswathanarayana, R. G. (2014). Astaxanthin: Sources, extraction, stability, biological activities and its commercial applications - A review. *Marine*

Drugs, *12*(1), 128–152. https://doi.org/10.3390/md12010128

Anarjan, N., Nehdi, I. A., & Tan, C. P. (2013). Protection of astaxanthin in astaxanthin nanodispersions using additional antioxidants. *Molecules*, *18*(7), 7699–7710. https://doi.org/10.3390/molecules18077699

Armenta, R. E., & Isabbl, G. L. (2009). Stability studies on astaxanthin extracted from fermented shrimp byproducts. *Journal of Agricultural and Food Chemistry*, *57*(14), 6095–6100. https://doi.org/10.1021/jf901083d

Ashworth, C. R., Matthews, R. P., Welton, T., & Hunt, P. A. (2016). Doubly ionic hydrogen bond interactions within the choline chloride-urea deep eutectic solvent. *Physical Chemistry Chemical Physics*, *18*(27), 18145–18160. https://doi.org/10.1039/c6cp02815b

Bi, W., Tian, M., Zhou, J., & Row, K. H. (2010). Task-specific ionic liquid-assisted extraction and separation of astaxanthin from shrimp waste. *Journal of Chromatography B*, *878*(24), 2243-2248. https://doi.org/10.1016/j.jchromb.2010.06.034

Blois, M. S. (1958). Antioxidant determinations by the use of a stable free radical. *Nature*, *181* (4617), 1199–1200. https://doi.org/10.1038/1811199a0

Chandra Roy, V., Ho, T. C., Lee, H. J., Park, J. S., Nam, S. Y., Lee, H., Getachew, A. T., & Chun, B. S. (2021). Extraction of astaxanthin using ultrasound-assisted natural deep eutectic solvents from shrimp wastes and its application in bioactive films. *Journal of Cleaner Production*, *284*, 125417. https://doi.org/10.1016/j.jclepro.2020.125417

Choi, S. A., Oh, Y. K., Lee, J., Sim, S. J., Hong, M. E., Park, J. Y., Kim, M. S., Kim, S. W., & Lee, J. S. (2019). High-efficiency cell disruption and astaxanthin recovery from *Haematococcus pluvialis* cyst cells using room-temperature imidazolium-based ionic liquid/water mixtures. *Bioresource Technology*, *274*, 120-126.

https://doi.org/10.1016/j.biortech.2018.11.082

Dai, Y., van Spronsen, J., Witkamp, G. J., Verpoorte, R., & Choi, Y. H. (2013). Natural deep eutectic solvents as new potential media for green technology. *Analytica Chimica Acta*, *766*, 61–68. https://doi.org/10.1016/j.aca.2012.12.019

de Souza Mesquita, L. M., Martins, M., Pisani, L. P., Ventura, S. P. M., & de Rosso, V. V. (2021). Insights on the use of alternative solvents and technologies to recover bio-based food pigments. *Comprehensive Reviews in Food Science and Food Safety*, *20*(1), 787–818. https://doi.org/10.1111/1541-4337.12685

Desai, R. K., Streefland, M., Wijffels, R. H., & Eppink, M. H. M. (2016). Novel astaxanthin extraction from *Haematococcus pluvialis* using cell permeabilising ionic liquids. *Green Chemistry*, *18*(5), 1261–1267. https://doi.org/10.1039/c5gc01301a

Fan, Y., Niu, Z., Xu, C., Yang, L., Chen, F., & Zhang, H. (2019). Biocompatible protic ionic liquids-based microwave-assisted liquid-solid extraction of astaxanthin from *Haematococcus pluvialis*. *Industrial Crops and Products*, *141*, 111809. https://doi.org/10.1016/j.indcrop.2019.111809

Florindo, C., Branco, L., & Marrucho, I. (2019). Quest for green-solvent design : from hydrophilic to hydrophobic (deep) eutectic solvents. *ChemSusChem*, *12*, 1549–1559. https://doi.org/10.1002/cssc.201900147

Florindo, C., Branco, L. C., & Marrucho, I. M. (2017). Development of hydrophobic deep eutectic solvents for extraction of pesticides from aqueous environments. *Fluid Phase Equilibria*, *448*, 135-142. https://doi.org/10.1016/j.fluid.2017.04.002

Gao, J., Fang, C., Lin, Y., Nie, F., Ji, H., & Liu, S. (2020). Enhanced extraction of astaxanthin using aqueous biphasic systems composed of ionic liquids and potassium phosphate. *Food*

Chemistry, *309*, 125672. https://doi.org/10.1016/j.foodchem.2019.125672

Gao, J., You, J., Kang, J., Nie, F., Ji, H., & Liu, S. (2020). Recovery of astaxanthin from shrimp (*Penaeus vannamei*) waste by ultrasonic-assisted extraction using ionic liquid-in-water microemulsions. *Food Chemistry*, *325* (December 2019), 126850. https://doi.org/10.1016/j.foodchem.2020.126850

Grewe, C., & Griehl, C. (2008). Time- and media-dependent secondary carotenoid accumulation in *Haematococcus pluvialis*. *Biotechnology Journal*, *3*, 1232-1244. https://doi.org/10.1002/biot.200800067.

Honda, M., Kageyama, H., Hibino, T., Osawa, Y., Kawashima, Y., Hirasawa, K., & Kuroda, I. (2021). Evaluation and improvement of storage stability of astaxanthin isomers in oils and fats. *Food Chemistry*, *352* (February), 129371. https://doi.org/10.1016/j.foodchem.2021.129371

Khoo, K. S., Lee, S. Y., Ooi, C. W., Fu, X., Miao, X., Ling, T. C., & Show, P. L. (2019). Recent advances in biorefinery of astaxanthin from *Haematococcus pluvialis*. *Bioresource Technology*, *288*, 121606. https://doi.org/10.1016/j.biortech.2019.121606

Khoo, K. S., Chew, K. W., Yew, G. Y., Manickam, S., Ooi, C. W., & Show, P. L. (2020). Integrated ultrasound assisted liquid biphasic flotation for efficient extraction of astaxanthin from *Haematococcus pluvialis*. *Ultrasonics-Sonochemistry*, *67*, 105052. https://doi.org/10.1016/j.ultsonch.2020.105052

Khoo, K. S., Ooi, C. W., Chew, K. W., Foo, S. C., Lim, J. W., Tao, Y., Jiang, N., Ho, S. H., & Show, P. L. (2021). Permeabilization of *Haematococcus pluvialis* and solid-liquid extraction of astaxanthin by CO_2-based alkyl carbamate ionic liquids. *Chemical Engineering Journal, 411*, 128510. https://doi.org/10.1016/j.cej.2021.128510

Kollau, L. J. B. M., Vis, M., Van Den Bruinhorst, A., De With, G., & Tuinier, R. (2019). Activity modelling of the solid-liquid equilibrium of deep eutectic solvents. *Pure and Applied Chemistry*, *91*(8), 1341–1349. https://doi.org/10.1515/pac-2018-1014

Krichnavaruk, S., Shotipruk, A., Goto, M., & Pavasant, P. (2008). Supercritical carbon dioxide extraction of astaxanthin from *Haematococcus pluvialis* with vegetable oils as co-solvent. *Bioresource Technology*, *99*(13), 5556–5560. https://doi.org/10.1016/j.biortech.2007.10.049

Kruk, I., Michalska, T., Lichsztel, K., & Aboul-Enein, H.Y. (2000). The effect of thymol and its derivatives on reactions generatingreactive oxygen species. *Chemosphere*, *41*, 1059-1064. https://doi.org/10.1016/s0045-6535(99)00454-3.

Lee, Y. R., & Row, K. H. (2016). Comparison of ionic liquids and deep eutectic solvents as additives for the ultrasonic extraction of astaxanthin from marine plants. *Journal of Industrial and Engineering Chemistry*, *39*, 87–92. https://doi.org/10.1016/j.jiec.2016.05.014

Liu, X., McClements, D. J., Cao, Y., & Xiao, H. (2016). Chemical and physical stability of astaxanthin-enriched emulsion-based delivery systems. *Food Biophysics*, *11*, 302–310. https://doi.org/10.1007/s11483-016-9443-6

Liu, Z. W., Yue, Z., Zeng, X. A., Cheng, J. H., & Aadil, R. M. (2018). Ionic liquid as an effective solvent for cell wall deconstructing through astaxanthin extraction from *Haematococcus pluvialis. International Journal of Food Science & Technology*, *54*(2), 583-590. https://doi.org/10.1111/ijfs.14030

Liu, Z. W., Zeng, X. A., Cheng, J. H., Liu, D. B., & Aadil, R. M. (2018). The efficiency and comparison of novel techniques for cell wall disruption in astaxanthin extraction from *Haematococcus pluvialis. International Journal of Food Science & Technology*, *53*(9), 2212-2219. https://doi.org/10.1111/ijfs.13810

Machmudah, S., Shotipruk, A., Goto, M., Sasaki, M., & Hirose, T. (2006). Extraction of astaxanthin from *Haematococcus pluvialis* using supercritical CO_2 and ethanol as entrainer. *Industrial and Engineering Chemistry Research*, *45*(10), 3652–3657. https://doi.org/10.1021/ie051357k

Martins, M. A. R., Crespo, E. A., Pontes, P. V. A., Silva, L. P., Bülow, M., Maximo, G. J., Batista, E. A. C., Held, C., Pinho, S. P., & Coutinho, J. A. P. (2018). Tunable hydrophobic eutectic solvents based on terpenes and monocarboxylic acids. *ACS Sustainable Chemistry and Engineering*, *6*(7), 8836–8846. https://doi.org/10.1021/acssuschemeng.8b01203

Miao, F., Lu, D., Li, Y., & Zeng, M. (2006). Characterization of astaxanthin esters in *Haematococcus pluvialis* by liquid chromatography-atmospheric pressure chemical ionization mass spectrometry. *Analytical Biochemistry*, *352*(2), 176–181. https://doi.org/10.1016/j.ab.2006.03.006

Padilha, C. E. A., Damasceno, K. S. F. S. C., Leite, P. I. P., Freitas, P. R., Cordeiro, A. M. T. M., & Assis, C. F. De. (2021). Astaxanthin recovery from shrimp residue by solvent ethanol extraction using choline chloride:glycerol deep eutectic solvent as adjuvant. *Journal of the Brazilian Chemical Society*, *32*(5), 1030–1039. https://doi.org/10.21577/0103-5053.20210005

Pontes, P. V. A., Crespo, E. A., Martins, M. A. R., Silva, L. P., Neves, C. M. S. S., Maximo, G. J., Hubinger, M. D., Batista, E. A. C., Pinho, S. P., Coutinho, J. A. P., Sadowski, G., & Held, C. (2017). Measurement and PC-SAFT modeling of solid-liquid equilibrium of deep eutectic solvents of quaternary ammonium chlorides and carboxylic acids. *Fluid Phase Equilibria*, *448*, 69–80. https://doi.org/10.1016/j.fluid.2017.04.007

Praveenkumar, R., Lee, K., Lee, J., & Oh, Y. K. (2015). Breaking dormancy: an energy-efficient

means of recovering astaxanthin from microalgae. *Green Chemistry*, *17*, 1226-1234. https://doi.org/10.1039/C4GC01413H

Rodrigues, L. A., Pereira, C. V., Leonardo, I. C., Fernández, N., Gaspar, F. B., Silva, J. M., Reis, R. L., Duarte, A. R. C., Paiva, A., & Matias, A. A. (2020). Terpene-based natural deep eutectic systems as efficient solvents to recover astaxanthin from brown crab shell residues. *ACS Sustainable Chemistry and Engineering*, *8*(5), 2246–2259. https://doi.org/10.1021/acssuschemeng.9b06283

Rowlinson, J.S. (1970). Molecular thermodynamics of fluid-phase equilibria: Prausnitz, J. M. Prentice-Hall: Englewood Cliffs, New Jersey. *J. Chem. Thermodyn*., *2*(1), 158-159. https://doi.org/10.1016/0021-9614(70)90078-9.

Ruberto, G., & Baratta, M. T. (2000). Antioxidant activity of selected essential oil components in two lipid model systems. *Food Chemistry*, *69*(2), 167–174. https://doi.org/10.1016/S0308-8146(99)00247-2

Samorì, C., Mazzei, L., Ciurli, S., Cravotto, G., Grillo, G., Guidi, E., Pasteris, A., Tabasso, S., & Galletti, P. (2019). Urease inhibitory potential and soil ecotoxicity of novel "polyphenols-deep eutectic solvents" formulations. *ACS Sustainable Chemistry and Engineering*, *7*(18), 15558–15567. https://doi.org/10.1021/acssuschemeng.9b03493

Samorì, C., Pezzolesi, L., Galletti, P., Semeraro, M., & Tagliavini, E. (2019). Extraction and milking of astaxanthin from: *Haematococcus pluvialis* cultures. *Green Chemistry*, *21*(13), 3621-3628. https://doi.org/10.1039/c9gc01273g

Shah, M. M. R., Liang, Y., Cheng, J. J., & Daroch, M. (2016). Astaxanthin-producing green microalga *Haematococcus pluvialis*: From single cell to high value commercial products. *Frontiers in Plant Science*, *7*, 531. https://doi.org/10.3389/fpls.2016.00531

694 Silva, J. M., Pereira, C. V, Mano, F., Silva, E., Reis, R. L., Sa, I., Paiva, A., Matias, A. A., &
695 Duarte, A. R. C. (2019). Therapeutic role of deep eutectic solvents based on menthol and
696 saturated fatty acids on wound healing. *ACS Applied Biomaterials*, *2*, 4346–4355.
697 https://doi.org/10.1021/acsabm.9b00598
698 Silva, J. M., Reis, R. L., Paiva, A., & Duarte, A. R. C. (2018). Design of functional therapeutic
699 deep eutectic solvents based on choline chloride and ascorbic acid. *ACS Sustainable*
700 *Chemistry and Engineering*, *6*(8), 10355–10363.
701 https://doi.org/10.1021/acssuschemeng.8b01687
702 Solovchenko, A. E. (2015). Recent breakthroughs in the biology of astaxanthin accumulation by
703 microalgal cell. *Photosynthesis Research*, *125*(3), 437–449. https://doi.org/10.1007/s11120-
704 015-0156-3
705 Tan, Y., Ye, Z., Wang, M., Manzoor, M.F., Aadil, R.M., Tan, X., & Liu, Z. (2021). Comparison
706 of different methods for extracting the astaxanthin from *Haematococcus pluvialis*: chemical
707 composition and biological activity. *Molecules*, *26*, 3569. https://doi.org/10.3390/
708 molecules26123569
709 Tiecco, M., Cappellini, F., Nicoletti, F., Del Giacco, T., Germani, R., & Di Profio, P. (2019). Role
710 of the hydrogen bond donor component for a proper development of novel hydrophobic deep
711 eutectic solvents. *Journal of Molecular Liquids*, *281*, 423-430.
712 https://doi.org/10.1016/j.molliq.2019.02.107
713 Van Osch, D. J. G. P., Dietz, C. H. J. T., Van Spronsen, J., Kroon, M. C., Gallucci, F., Van Sint
714 Annaland, M., & Tuinier, R. (2019). A search for natural hydrophobic deep eutectic solvents
715 based on natural components. *ACS Sustainable Chemistry and Engineering*, *7*(3), 2933–2942.
716 https://doi.org/10.1021/acssuschemeng.8b03520

717 Vechio, H., Mariano, A. B., & Vieira, R. B. (2021). A new approach on astaxanthin extraction via
718 acid hydrolysis of wet *Haematococcus pluvialis* biomass. *Journal of Applied Phycology*, *33*,
719 2957–2966. https://doi.org/10.1007/s10811-021-02495-z
720 Zhang, H., Tang, B., & Row, K. H. (2014). A green deep eutectic solvent-based ultrasound-
721 assisted method to extract astaxanthin from shrimp byproducts. *Analytical Letters*, *47*(5),
722 742–749. https://doi.org/10.1080/00032719.2013.855783
723