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

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

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

16

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

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

31

32 1. INTRODUCTION

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

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

53 The recovery of natural astaxanthin has been accomplished with a variety of solvents, from
54 hazardous traditional organic compounds to safer solvents (e.g. ethyl acetate, acetone, ethanol,
55 Vechio et al., 2021), to unconventional (but sometimes highly-costly) alternatives (de Souza
56 Mesquita et al., 2021) like supercritical CO₂, vegetable oils, ionic liquids, or deep eutectic
57 solvents (Chandra Roy et al., 2021; Desai et al., 2016; Gao, You, et al., 2020; Krichnavaruk et
58 al., 2008; Machmudah et al., 2006; Rodrigues et al., 2020; Samorì, Pezzolesi, et al., 2019).

59 Regardless of the kind of solvent, the dewatering of the algal biomass, its pre-treatment to
60 weaken the cell walls (i.e. cell disruption), the solvent removal to recover astaxanthin, and the
61 color fading and loss of biological activity are unavoidable bottlenecks.

62 Ionic liquids have been widely used in the recovery of astaxanthin from natural matrices
63 including *H. pluvialis* (Khoo et al., 2019), as “weakening” agents to increase the cell membrane
64 permeability (Bi et al., 2010; Choi et al., 2019; Desai et al., 2016; Liu, Yue, et al., 2018; Liu,
65 Zeng, et al., 2018) and as real lipophilic solvents (Fan et al., 2019; Gao, Fang, et al., 2020; Gao,
66 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 CO₂-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

90 hydrophilic and water-soluble eutectic mixtures of choline chloride and diols/carboxylic acids
91 act as “adjuvants” for weakening algal cell wall and enhancing a subsequent astaxanthin
92 extraction, hydrophobic DESs can work as real solvents for astaxanthin itself (Florindo et al.,
93 2019). The only class of hydrophobic DESs exploited so far as solvents for extracting
94 astaxanthin are terpene-based mixtures, characterized by a low viscosity in comparison to
95 hydrophilic DESs and other phosphonium-based hydrophobic DESs (Lee & Row, 2016;
96 Rodrigues et al., 2020): perillyl alcohol, camphor, eucalyptol, and menthol were used mixed with
97 myristic acid, and the mixture menthol-myristic acid was the most efficient one in the extraction
98 of astaxanthin from crab waste (Rodrigues et al., 2020).

99 A common feature to both hydrophilic and hydrophobic DESs is their low or absent volatility: if
100 this property confers an intrinsic safety for the operators and the, it is also true that this strictly
101 influences their application since DESs are inseparable from the compounds they dissolve
102 (Samorì, Mazzei, et al., 2019). For this reason, the use of eutectic mixtures composed of
103 inherently safe components is mandatory for developing “bioactive compounds-DES”
104 formulations exploitable in applications for humans. Menthol-fatty acids hydrophobic DESs
105 meet this criterion (Silva et al., 2019) with the additional benefit of being Therapeutic DESs
106 (THeDES, Silva et al., 2018) since
107 both menthol and fatty acids are anti-inflammatory and antimicrobial compounds (Silva et al.,
108 2019; Van Osch et al., 2019).

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

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

129 Therefore, in the present paper, the possibility of extracting, conveying, and stabilizing
130 astaxanthin through oleic acid-based edible DES has been explored and demonstrated, trying to
131 meet the concept of “Green Food Processing” by reducing the use of hazardous solvents and
132 chemicals, minimizing the production of waste, and using renewable compounds (Khoo et al.,
133 2020).

134

135 2. MATERIALS AND METHODS

136 2.1 Chemicals

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

140

141 2.2 Solid-Liquid phase determination and Deep Eutectic Solvents (DESs) preparation

142 All the hydrophobic DESs (DL-menthol:oleic acid, thymol:oleic acid, geraniol:oleic acid)
143 characterization was based on the comparison between the experimental and the
144 theoretical solid-liquid phase diagrams. The experimental solid-liquid phase curves were
145 obtained by measuring the melting points of the different samples at the different molar
146 ratios with a thermometer via immersion of the samples in an ice/NaCl mixture or solid
147 CO₂/acetone mixture in a Dewar. The melting temperatures were evaluated in triplicate to
148 avoid any kinetic effect on the melting of the mixtures.

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

151
$$\ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T} \right) + \frac{\Delta_m C p_i}{R} \cdot \left(\frac{T_{m,i}}{T} - \ln \frac{T_{m,i}}{T} - 1 \right) \quad (1)$$

152 where χ_i is the mole fraction of component i, γ_i is its activity coefficient in the liquid phase,
153 $\Delta_m h_i$ and $T_{m,i}$ are its melting enthalpy and temperature, respectively, $\Delta_m C p_i$ is its heat capacity
154 change upon melting, R is the ideal gas constant, and T is the absolute temperature of the system.

155 This equation can be simplified by considering the heat capacity change upon the melting of a
156 substance as negligible, therefore equation (2) was used:

157
$$\ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T} \right) \quad (2)$$

158 The theoretical melting temperatures were determined from the theoretical curves by considering
159 the activity coefficients $\gamma_i = 1$. 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:

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

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 phosphatidylcholine: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 $\text{m}^2 \text{s}^{-1}$ and a 16 h light:8

181 h dark cycle. Under these conditions, the cells were kept in a vegetative phase until a dry weight
182 of 0.7 g L⁻¹ was reached. Then, the cultures were stressed under high light intensity (450-500
183 μmol of photons per m² s⁻¹) and nutrient starvation by 3-times dilution of the algal culture
184 (Samorì, Pezzolesi, et al., 2019). When mature aplanospores (red cysts) were obtained,
185 astaxanthin was extracted through two different procedures: extraction from freeze-dried algal
186 biomass (see Section 2.4) and from algal culture (see Section 2.5).

187

188 *2.4 Extraction of astaxanthin from freeze-dried H. pluvialis biomass*

189 Algal culture (100 mL) with an astaxanthin content of 1.6 wt% was collected and centrifuged at
190 2550 x g for 10 min at 4°C. The supernatant was removed, the algal pellet was freeze-dried and
191 then extracted at rt for 6 h with DESs, oleic acid, and geraniol (50 mg of biomass with 2 mL of
192 solvent, i.e. 2.5 wt%). At the end of such time, the extracts were centrifuged at 2550 x g for 10
193 min to separate the extracted biomass from the liquid phases, then recovered by pipetting.
194 Aliquots of the recovered liquid phases (0.02 mL) were withdrawn at specific time frames (1, 2,
195 4, and 6 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV vis
196 at 470 nm, as described below to determine the astaxanthin content. The same extraction
197 procedure was also applied varying specific conditions to evaluate their effect on the kinetics and
198 overall extraction performances:

- 199 i) at rt with three non-eutectic mixtures of oleic acid: i) thymol:oleic acid in a molar ratio
200 1:1, ii) geraniol:oleic acid in a molar ratio 2:1, and iii) L- α -phosphatidylcholine:oleic acid
201 in a weight ratio 95:5;
202 ii) at 60°C with TAO;

203 iii) at 60°C with two other biomass/TAO ratios: 50 mg biomass/1 mL TAO (i.e. 5 wt%), and
204 50 mg biomass/0.5 mL TAO (i.e. 10%).

205

206 2.5 Extraction of astaxanthin from *H. pluvialis* culture

207 Algal culture (3 mL) with a cell density of 1.3 g L⁻¹ and an astaxanthin content of 2.7 wt% was
208 put in contact with DESs, oleic acid, and geraniol (1 mL) and gently stirred with a magnetic bar
209 at 50-100 rpm for 6 h. At the end of such time, the biphasic mixtures were centrifuged at 2550 x
210 g for 10 min to separate the algal cultures from the liquid solvents, lastly recovered by pipetting.
211 Aliquots of the recovered solvent phases (0.02 mL) were withdrawn at specific time frames (1, 2,
212 4, and 6 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by HPLC-UV vis
213 at 470 nm, as described below to determine the astaxanthin content. *H. pluvialis* vitality before
214 and after the extraction experiments was evaluated through pulse-amplitude modulated (PAM)
215 fluorometry measurements in terms of kinetics and parameters of Photosystem II (PSII) (Samori,
216 Pezzolesi, et al., 2019). The model used was 101-PAM (H. Walz, Effeitrich, Germany)
217 connected to a PDA-100 data acquisition system, high power LED Lamp Control unit HPL-C
218 and LED-Array-Cone HPL-L470 to supply saturated pulses, US-L655 and 102-FR to provide
219 far-red light and light measurement, respectively. Before and after the extraction experiments,
220 aliquots of algal cultures were placed in cuvettes (10 × 10 mm) mounted on an optical unit ED-
221 101US/M. Measurement of the photosynthetic efficiency was derived from the maximum
222 quantum yield of PSII (Φ_{PSII}), calculated from the following equation (4):

$$223 \quad \Phi_{PSII} = \frac{F_m - F_0}{F_m} \quad (4)$$

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

226 $\text{m}^{-2} \text{s}^{-1}$ for 0.8 s induced the maximal fluorescence yield (F_m). Photosynthetic activity (%) was
227 calculated by dividing the maximum quantum yield of PSII (Φ_{PSII}) after the extraction by the
228 maximum quantum yield of PSII (Φ_{PSII}) 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: H₂O (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 $\mu\text{g mL}^{-1}$). 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, $m/z = 100\text{--}3000$ amu, fragmentor 30 V). The

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

255

256 *2.7 Light-stability test and DPPH assay*

257 Oxidation tests were performed on the extracts obtained from freeze-dried *H. pluvialis* biomass
258 to evaluate the potential of the different hydrophobic solvents here used to stabilize and preserve
259 astaxanthin. Samples were exposed to light radiation under controlled conditions employing sun
260 simulating OSRAM ULTRA-VITALUX 300W UV-A lamp (220-230 μE m⁻² s⁻¹, OSRAM spa,
261 Milan, Italy). Aliquots of solvent (0.02 mL) were withdrawn at specific time frames (0.5, 1.5,
262 3.5, 7.5 and 13.5 h), diluted in DMSO (0.08 mL) and methanol (0.4 mL), and analyzed by
263 HPLC-UV vis at 470 nm, as described above to determine the astaxanthin content. The
264 astaxanthin stability was expressed as the percentage of astaxanthin amount at specific ageing
265 time with respect to the astaxanthin content in the corresponding unaged sample.

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

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

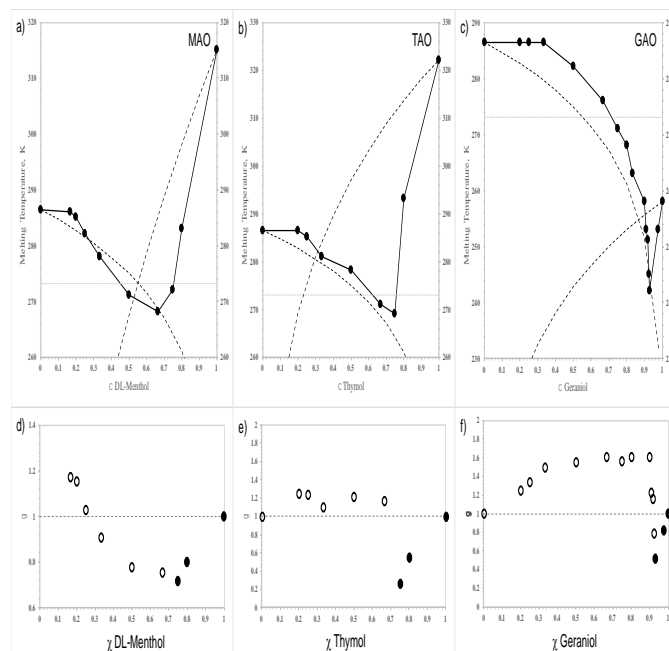
275 Where: A_S indicates the absorbance of the sample, A_C is the absorbance of the control (prepared
276 by diluting 500 μL of DPPH solution in 500 μL of ethanol) and A_B is the absorbance of blank
277 (prepared by mixing 500 μL of sample solution with 500 μL of ethanol).

278

279 3. RESULTS AND DISCUSSION

280 *3.1 Hydrophobic DESs preparation and solid-liquid phase diagrams*

281 Three oleic acid-based mixtures composed of natural and edible components approved by the
282 Flavor and Extract Manufacturers Association (FEMA) as GRAS flavor ingredients (oleic acid:
283 FEMA N° 2815; DL-menthol: FEMA N° 2665; thymol: FEMA N° 3066; geraniol: FEMA N°
284 2507) were here prepared with the aim of providing an improvement towards the use of
285 hydrophobic DESs (Martins et al., 2018). The solid-liquid phase diagrams of the three mixtures
286 were initially defined and compared with the theoretical melting curves (Figure 1) (Kollau et al.,
287 2019). This approach was necessary to define the identity of the liquid mixtures; in this case, this
288 was particularly important since oleic acid itself is a liquid (m.p. = 16°C), so the resulting
289 mixtures could be solutions rather than eutectic systems. Moreover, a shift of the eutectic point
290 from the theoretical one, in terms of both molar ratio of the components and melting point, is
291 necessary to define the mixtures as “deep eutectic solvents” (Pontes et al., 2017). This shift
292 indicates that the interactions occurring between the different molecules have intensities like the
293 ones occurring between the same species; therefore, the mixtures have a non-ideal behavior
294 (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).

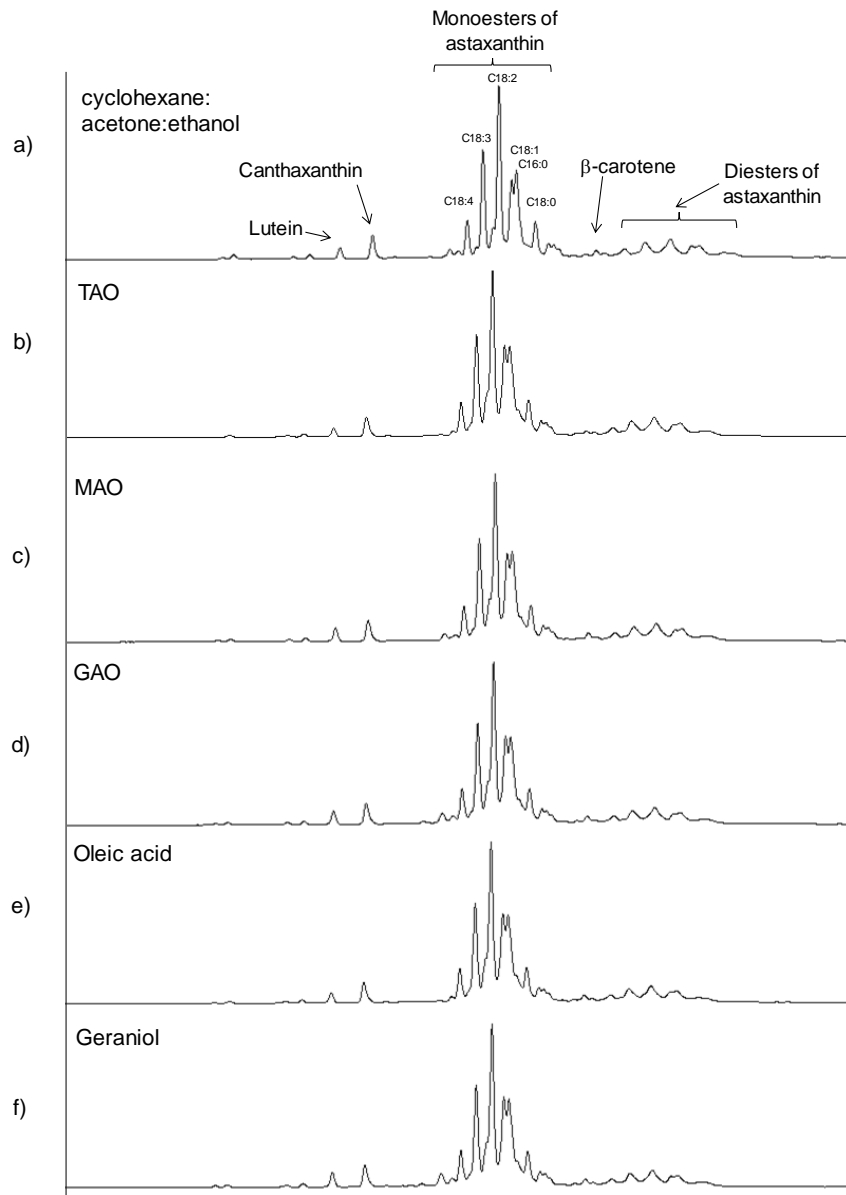
298
 299 All the three mixtures can be considered as DESs as the eutectic points observed differed from
 300 the theoretical curves both in terms of eutectic ratio and melting points (Abdallah et al., 2021).
 301 MAO had a eutectic point at 2:1 (DL-menthol:oleic acid) molar ratio with a melting point 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

310 activity ($\gamma = 1$) in correspondence to the eutectic points. If MAO showed the fewest difference
311 from the ideal curve in the melting point profile, TAO and GAO showed high differences from
312 the ideal behavior. All these liquid systems can be considered as Type V DESs because they are
313 composed of non-ionic molecules. Moreover, they are hydrophobic because both the components
314 are scarcely soluble in water (<0.01 mM oleic acid; <3 mM DL-menthol; 6 mM thymol; 5 mM
315 geraniol) and their water content (i.e. 2.9 wt% for TAO, 0.94 wt% for MAO, and 2.6 wt% for
316 GAO) was in agreement with literature data on hydrophobic DESs (Tiecco et al., 2019).
317 Mixtures of terpenes (such as geraniol, thymol, and menthol) with carboxylic acids have been
318 already reported and discussed in the literature (Martins et al., 2018); however, in those mixtures,
319 only small shifts of the experimental melting points from the theoretical ones are reported.
320 Differently, in all the oleic acid mixtures here reported, larger differences were observed; this
321 suggests that the hydrogen-bonding networks established in oleic acid-based mixtures are
322 significantly different in intensity to the ones reported for other carboxylic acids. Three other
323 non-eutectic but liquid mixtures of oleic acid were also prepared to understand whether the
324 “eutecticity” could give superior extraction performances: i) thymol:oleic acid in a molar ratio
325 1:1, ii) geraniol:oleic acid in a molar ratio 2:1, and iii) L- α -phosphatidylcholine:oleic acid in a
326 weight ratio 95:5.

327

328 *3.2 Extraction of astaxanthin from freeze-dried H. pluvialis biomass*

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



335

336 **Fig. 2.** Carotenoid profile obtained with a) cyclohexane:acetone:ethanol mixture 2:1:1 v/v/v; b)

337 thymol:oleic acid 3:1, TAO; c) DL-menthol:oleic acid 2:1, MAO; d) geraniol:oleic acid 13:1,

338 GAO; e) oleic acid, f) and geraniol.

339

340 All the chromatograms obtained by HPLC UV-Vis at 470 nm were predominantly characterized

341 by the peaks of astaxanthin monoesters, identified by LC-MS on the basis of the molecular

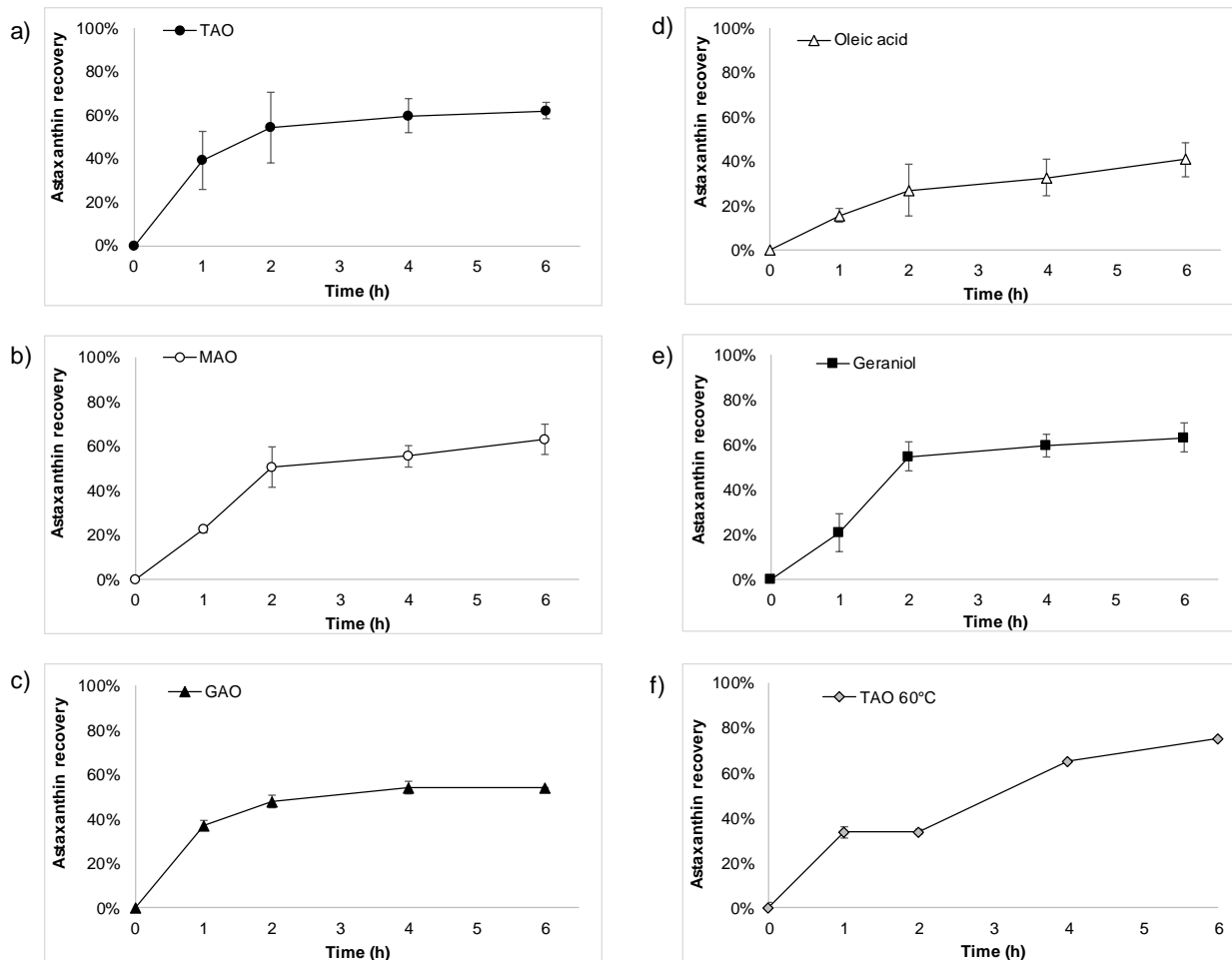
342 weight: monoesters of linoleic (C18:2) and linolenic acid (C18:3) were the most abundant peaks,
343 followed by oleic (C18:1) and stearidonic acid (C18:4) monoesters (Figure 2). The monoester
344 with palmitic acid (C16:0) was the only C16-ester detected, in line with previous findings
345 (Samorì, 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 ($83 \pm 13\%$), followed by MAO ($74 \pm 4\%$), and GAO ($66 \pm 6\%$). Geraniol tested alone behaved
364 similarly to GAO, while oleic acid was the worst hydrophobic phase among the tested ones

365 (41±7% of recovery after 6 h), even after prolonged extraction times (24 h, 64±10%). This
366 suggests that the combination of oleic acid in a DES mixture with all the three terpenes here used
367 effectively improves its extraction ability, probably due to a reduction of oleic acid viscosity or
368 to an increase in “affinity” for astaxanthin, thanks to π - π stacking interactions between the
369 conjugated systems of terpenes (but not DL-menthol) and that of astaxanthin. On the other hand,
370 the extraction performances of the non-eutectic mixtures of oleic acid here prepared (see Table
371 1S in ESI with the data for thymol:oleic acid in a molar ratio 1:1 and geraniol:oleic acid in a
372 molar ratio 2:1) were worse than the corresponding DESs (TAO and GAO) over a long period; in
373 particular, the recovery of astaxanthin with TAO was 1.4 times higher than a non-eutectic
374 mixture of oleic acid and thymol. The mixture L- α -phosphatidylcholine and oleic acid (95:5 ratio
375 by weight), known as OSMOSTM solvent, was the worst solvent among the ones tested (52%
376 after 24 h), presumably because of higher viscosity than the terpenes mixtures.

377 The effect of the temperature on the extraction performances was evaluated on TAO, the best
378 solvent among the tested ones. Increasing the extraction temperature from rt to 60°C improved
379 the kinetics and the overall performances, giving an astaxanthin recovery of 75±0.7% after 6 h,
380 1.2 times higher than what was obtained at rt and higher than all the solvents here tested.

381 Therefore, this temperature was chosen to investigate two other biomass/TAO ratios (i.e. 5 and
382 10 wt%, see Figure 3S in ESI). The recovery after 6 h did not substantially change, regardless of
383 the used ratio (70.9±2.8% at 10 wt% and 84.9±3.7% at 5 wt%), underlying that it is possible to
384 minimize the amount of solvent used without changing the extraction performances (higher
385 ratios were not tested since the viscosity of the “biomass-TAO” solution hampered an efficient
386 separation of the extracted biomass by centrifugation).



387

388 **Fig. 3.** Astaxanthin recovery from *H. pluvialis* freeze-dried biomass with a) thymol:oleic acid
 389 3:1, TAO; b) DL-menthol:oleic acid 2:1, MAO; c) geraniol:oleic acid 13:1, GAO; d) oleic acid,
 390 e) geraniol, and f) thymol:oleic acid 3:1, TAO at 60°C. Data are expressed on the basis of the
 391 percentage of astaxanthin content in *H. pluvialis* cells, as mean \pm standard deviation of two
 392 independent experiments on different freeze-dried algal biomass.

393

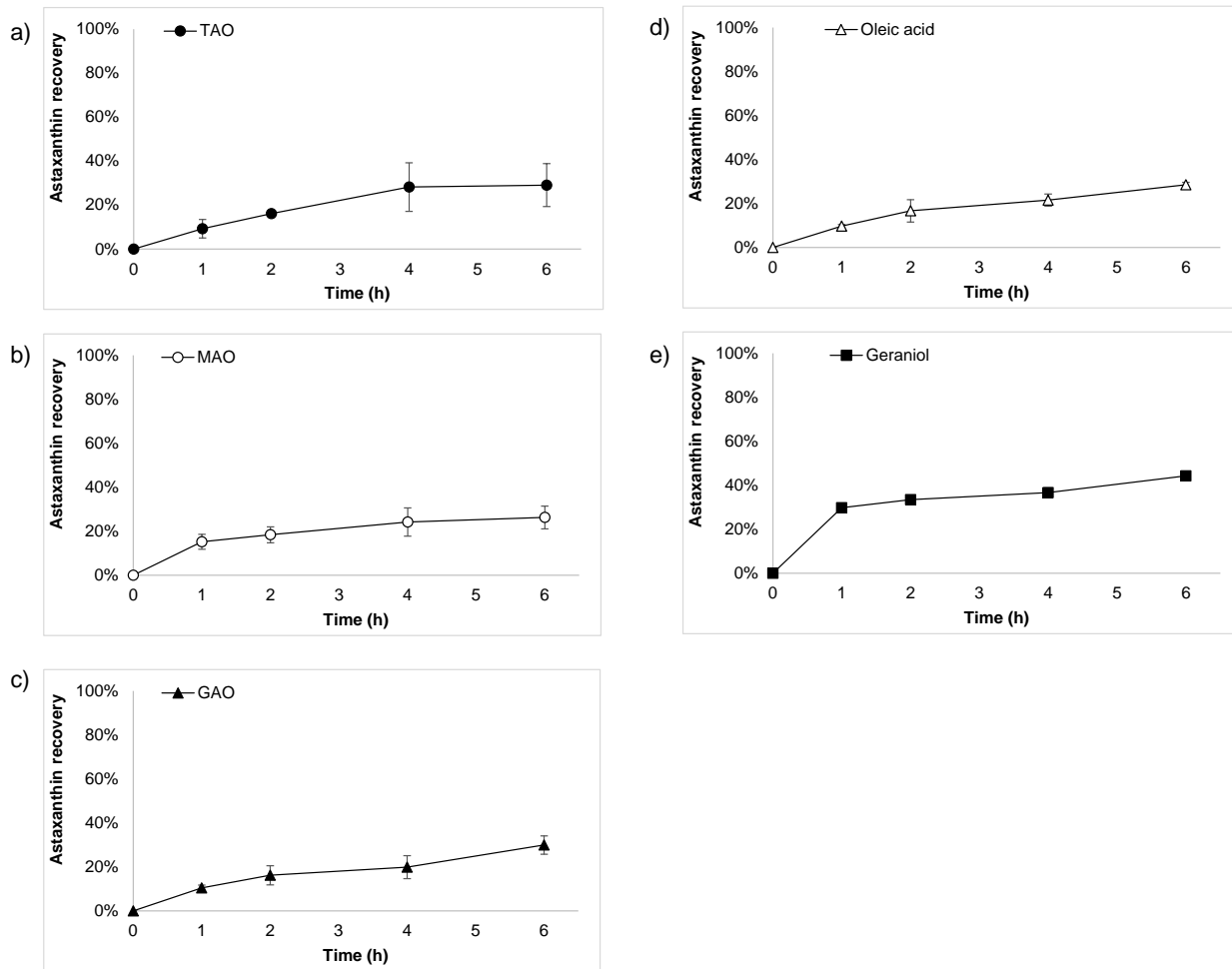
394 3.3 Extraction of astaxanthin from *H. pluvialis* cultures

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

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

407 All the three DESs (Figure 4 a-c) followed the same kinetics of extraction: the recovery of
408 astaxanthin increased from values of about 10% achieved in 1 h, up to 30% after 6 h; the
409 “hampering” effect created by water to the contact between solvent and algal cells was evident
410 since the recovery, in this case, was half of what achieved from *H. pluvialis* pellet in the same
411 time frame (Figure 3 a-c). After 48 h, the recovery of astaxanthin reached values of 56, 58 and
412 68% with MAO, TAO, and GAO, respectively. Diversely from what occurred in the extraction
413 of astaxanthin from algal pellets, oleic acid showed the same extraction pattern of DESs, while
414 geraniol gave the best performance under these conditions (three-times higher astaxanthin
415 recovery than DESs in 1 h, and 44% of recovery in 6 h). Qualitatively, the extracts recovered
416 from algal cultures showed some differences with the extracts obtained from algal pellets (see
417 Figure 5S in ESI): the chromatograms were dominated by the signal of astaxanthin monoesters,
418 but lutein and β -carotene were almost undetectable. The ratio between astaxanthin monoesters
419 and diesters (6.0 ± 0.2), was higher than what was observed in the extracts from freeze-dried
420 biomass (4.8 ± 0.3), but it is known that several biological factors related to algal growth and

421 physiology (like the cultivation period, cysts age, growth medium composition, Grewe and
 422 Griehl, 2008) influence this number and in the present case *H. pluvialis* cultures used for
 423 obtaining the pellet came from a different batch than the ones used for the liquid-liquid
 424 extraction.
 425



426
 427 **Fig. 4.** Astaxanthin recovery from *H. pluvialis* cultures with a) thymol:oleic acid 3:1, TAO; b)
 428 DL-menthol:oleic acid 2:1, MAO; c) geraniol:oleic acid 13:1, GAO; d) oleic acid, and e)
 429 geraniol. Data are expressed based on the percentage of the astaxanthin content in *H. pluvialis*
 430 cells, as mean \pm standard deviation of two independent experiments on different algal biomass.
 431

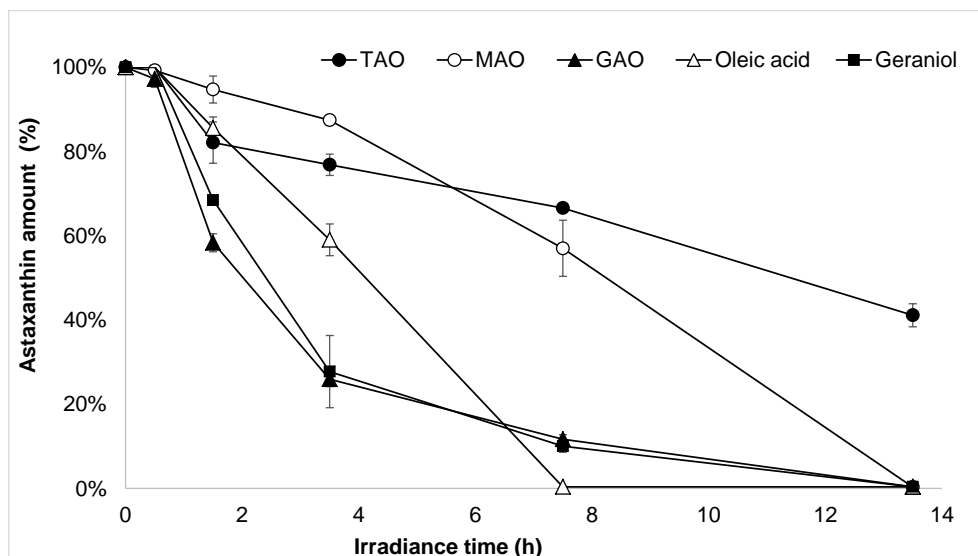
432 Even if after 1 and 4 h of contact algal cells seemed intact and still rich in astaxanthin or empty
433 under a light microscope (see Figure 10S in ESI), after 1 h no photosynthetic activity was
434 observed for the culture put in contact with TAO, GAO, and geraniol, while the viability of cells
435 extracted with MAO was 50% for the first hour of extraction, before dropping down to 0% after
436 4 h (see Figure 4S in ESI). In analogy to what was already observed for vegetable oils (Samori,
437 Pezzolesi, et al., 2019), oleic acid was the most algae-compatible compound, maintaining 80% of
438 algal viability within the first hour of extraction and about 30% even after 6 h of extraction (see
439 Figure 4S in ESI). NMR spectroscopy analysis of the algal cultures after 6 h of contact with the
440 various hydrophobic solvents here tested helped in explaining such behavior: in the case of the
441 three DESs, the presence of the terpenic component of the eutectic mixtures was detected
442 (geraniol >> thymol ~ DL-menthol, Figures 6S for TAO, 7S for MAO and 8S for GAO in ESI),
443 while oleic acid (tested alone or as a component of the DESs) was almost undetectable and
444 largely below the molar ratio of the mixtures used in the extraction step; this suggested that the
445 hydrophobic DESs here used were not completely water-stable (Florindo et al., 2017).
446 Therefore, the algal cell mortality could be related to the toxicity towards algae of each terpene
447 (the growth inhibition of DL-menthol, thymol, and geraniol on freshwater algae after 72 h of
448 exposition is reported to be in the range of 0.1 mM). These data demonstrated that preserving the
449 viability of algal cells after contact with solvents is even more challenging than extracting algal
450 metabolites directly from algal culture.

451

452 *3.4 Light-stability test and antioxidant activity*

453 The instability of astaxanthin to light, oxygen, and self-oxidation is a serious issue that can affect
454 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

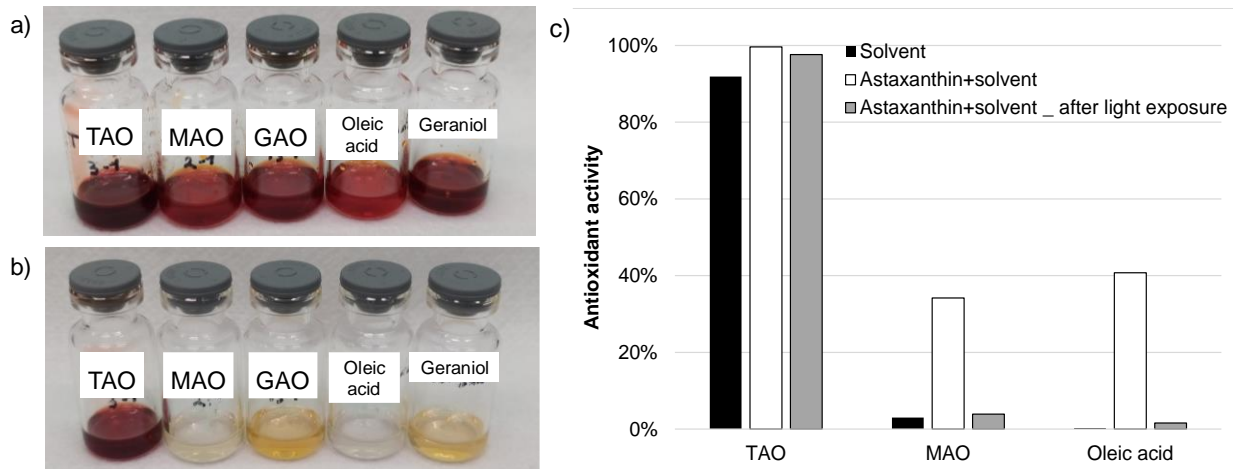


465
 466 **Fig. 5.** Effect of light as oxidative factor on astaxanthin contained in DESs, oleic acid, and
 467 geraniol.

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

471 astaxanthin content that rapidly decreased (after 3.5 h, more than 70% of the initial astaxanthin
472 content was degraded). In oleic acid a decrease with a constant rate was observed, reaching a
473 complete degradation after 7.5 h of exposure to light. MAO and TAO extracts performed the
474 best, maintaining the astaxanthin amount above 50% after 7.5 h. After 13.5 h in TAO 40% of the
475 initial astaxanthin content was maintained (Figure 6b), demonstrating TAO superior potential to
476 stabilize astaxanthin due to the antioxidant properties of thymol, higher than those of geraniol
477 and menthol (Ruberto & Baratta, 2000). The antioxidant activity of TAO alone was 30-times
478 higher than that of MAO (Figure 6c, black bars), while oleic acid did not have any antioxidant
479 activity at all. This finding can be attributed to the unique antioxidant properties of thymol, a
480 well-known $^1\text{O}_2$ quencher and anti-lipid peroxidation agent, suggested as a valid natural
481 replacement for synthetic antioxidant food additives (Aeschbach et al., 1994; Alam et al., 1999;
482 Kruk et al., 2000). Moreover, it is known that a whole carotenoid extract that contains
483 astaxanthin is more antioxidant than astaxanthin alone, thanks to the synergism that occurs in the
484 extract between astaxanthin and the polyunsaturated lipidic droplets strictly associated with
485 astaxanthin itself; moreover, astaxanthin monoester has a stronger total antioxidant capacity than
486 astaxanthin in the free form (Tan et al., 2021). This could explain the large increase of the
487 antioxidant potential of all the tested solvents (black bars) observed after the extraction process
488 (white bars). However, after the exposition to light, only TAO was capable to maintain such
489 property (grey bars), suggesting TAO as the most promising extractant, carrier and stabilizer of
490 natural astaxanthin among the tested DESs, useful for the development of food additives.

491



492

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

496

497 4. CONCLUSIONS

498 Three novel DESs based on oleic acid and thymol (TAO), DL-menthol (MAO) and geraniol
 499 (GAO) have been here prepared for the first time and applied to the extraction of astaxanthin
 500 from *H. pluvialis*. All of them gave good recovery percentages without any thermal, mechanical
 501 or chemical pre-treatment; the extraction of dried biomass gave an astaxanthin recovery of about
 502 60% in 6 h, independently from the DES used and significantly higher than the recovery of 40%
 503 achieved with oleic acid alone. Increasing the extraction temperature increased the recovery up
 504 to 75% under the same time frame, while the performances did not vary with the biomass/solvent
 505 ratio used.

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

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

517

518 ASSOCIATED CONTENT

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

529

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534

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539

540 ABBREVIATIONS

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

543

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