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

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

Walter Pitacco,[a] Chiara Samorì,[a,b] Laura Pezzolesi,*[b,c] Virgina Gori,[c] Antonio Grillo,[d] Matteo Tiecco,[d] Martina Vagnoni,[a] Paola Galletti [a,b]*

[a] Dipartimento di Chimica "Giacomo Ciamician", Università di Bologna, via Sant'Alberto 163, Ravenna, Italy

[b] CIRI-FRAME, Università di Bologna, via Sant'Alberto 163, Ravenna, Italy

[c] Dipartimento di Scienze Biologiche, Geologiche ed Ambientali, Università di Bologna, via Sant'Alberto 163, Ravenna, Italy

[d] Dipartimento di Chimica, Biologia e Biotecnologie, Università di Perugia, via Elce di Sotto 8, Perugia, Italy

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

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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).

2

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 CO_2, 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.*

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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

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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:

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- 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

6

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 ≥ 98%). Free astaxanthin, canthaxanthin, and lutein

139   standards (purities ≥ 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   CO_2/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    $$ln(\chi_i \cdot \gamma_i) = \frac{\Delta_m h_i}{R} \cdot \left(\frac{1}{T_{m,i}} - \frac{1}{T}\right) + \frac{\Delta_m C p_i}{R} \cdot \left(\frac{T_{m,i}}{T} - ln\frac{T_{m,i}}{T} - 1\right) \qquad (1)$$

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

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

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

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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 m^2 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-phosphatidylcoline:oleic acid

201   in a weight ratio 95:5;

202 ii)   at 60°C with TAO;

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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) (Samorì,

216 Pezzolesi, et al., 2019). The model used was 101-PAM (H. Walz, Effeltrich, 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 (ΦPSII), calculated from the following equation (4):

223 $\Phi PSII = \frac{F_m - F_0}{F_m}$ (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 µmol m^{-2} s^{-1}). Then, a short saturating pulse of 3000 µmol

10

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

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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):

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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).

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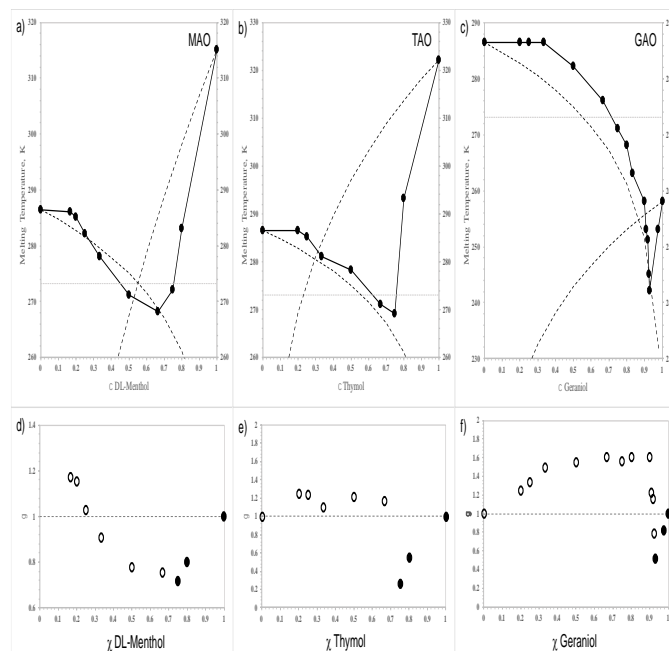
Fig. 1. Eutectic profiles, experimental melting points (dots) and theoretical curves (dashed lines) *vs* molar ratio of the DESs (a-c); experimental activity coefficients γ of the DESs (d-f).

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

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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-phosphatidylcoline: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).

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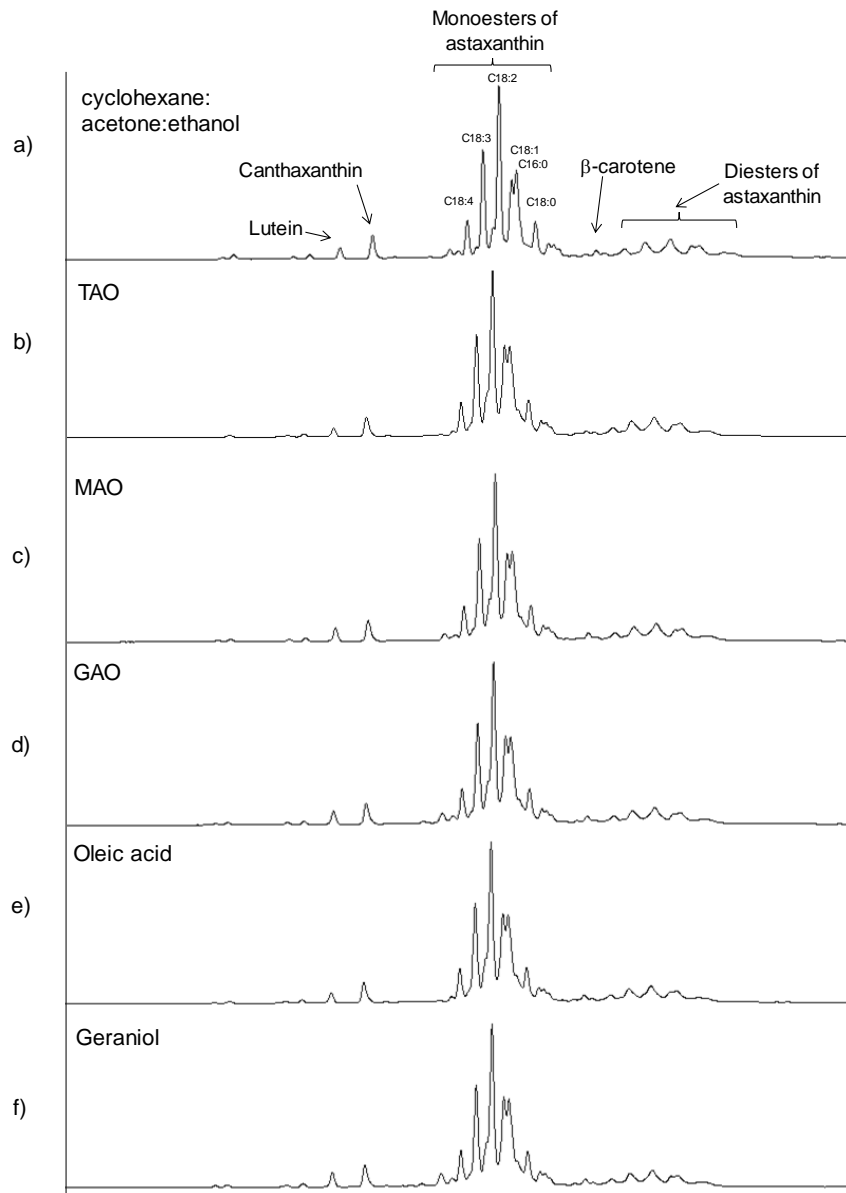
Fig. 2. Carotenoid profile obtained with a) cyclohexane:acetone:ethanol mixture 2:1:1 v/v/v; b)

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

16

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

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-phosphatidylcoline and oleic acid (95:5 ratio

375 by weight), known as OSMOS^{TM} 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).
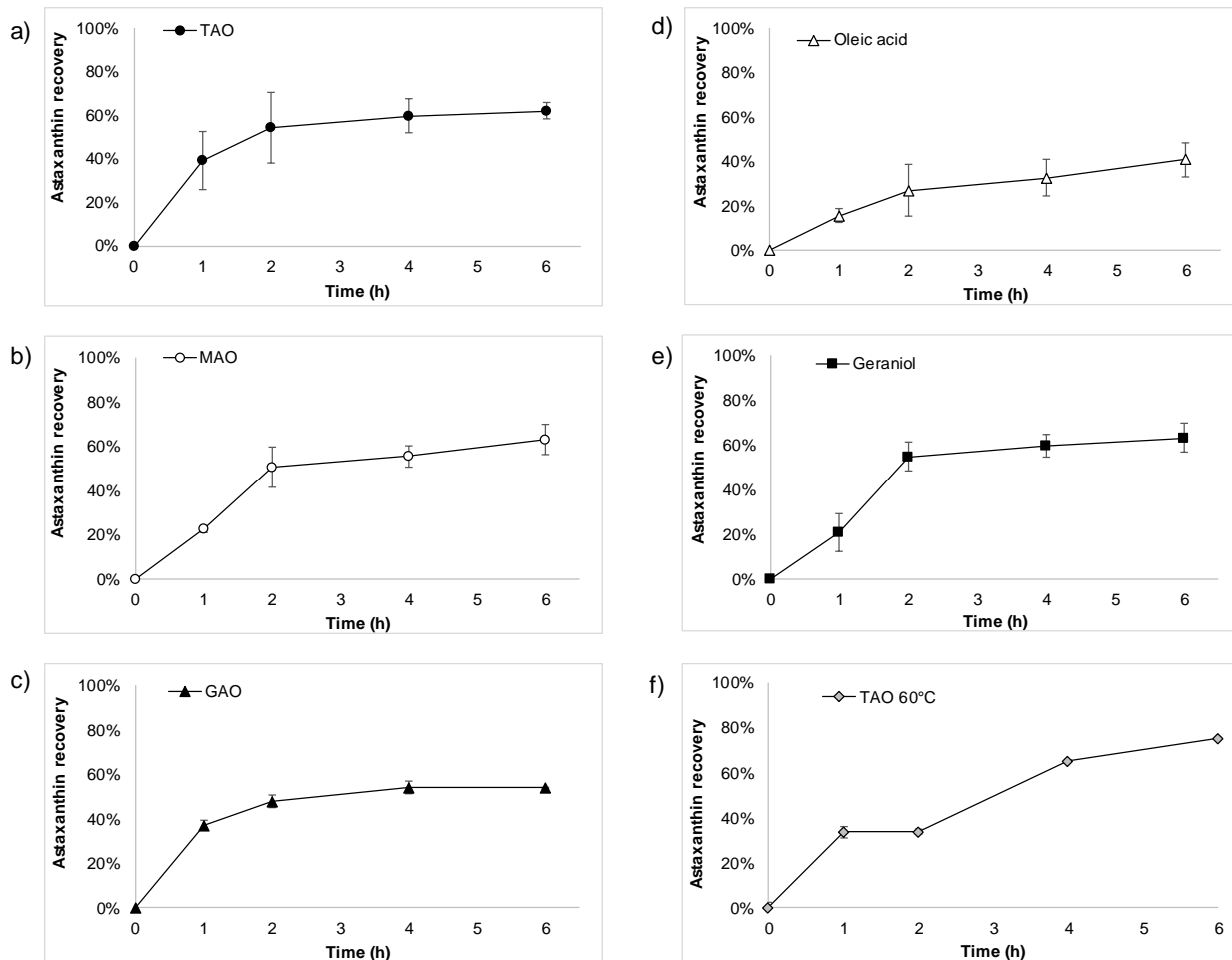
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Fig. 3. Astaxanthin recovery from *H. pluvialis* freeze-dried biomass with a) thymol:oleic acid

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

19

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 (Samorì,

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

20

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.

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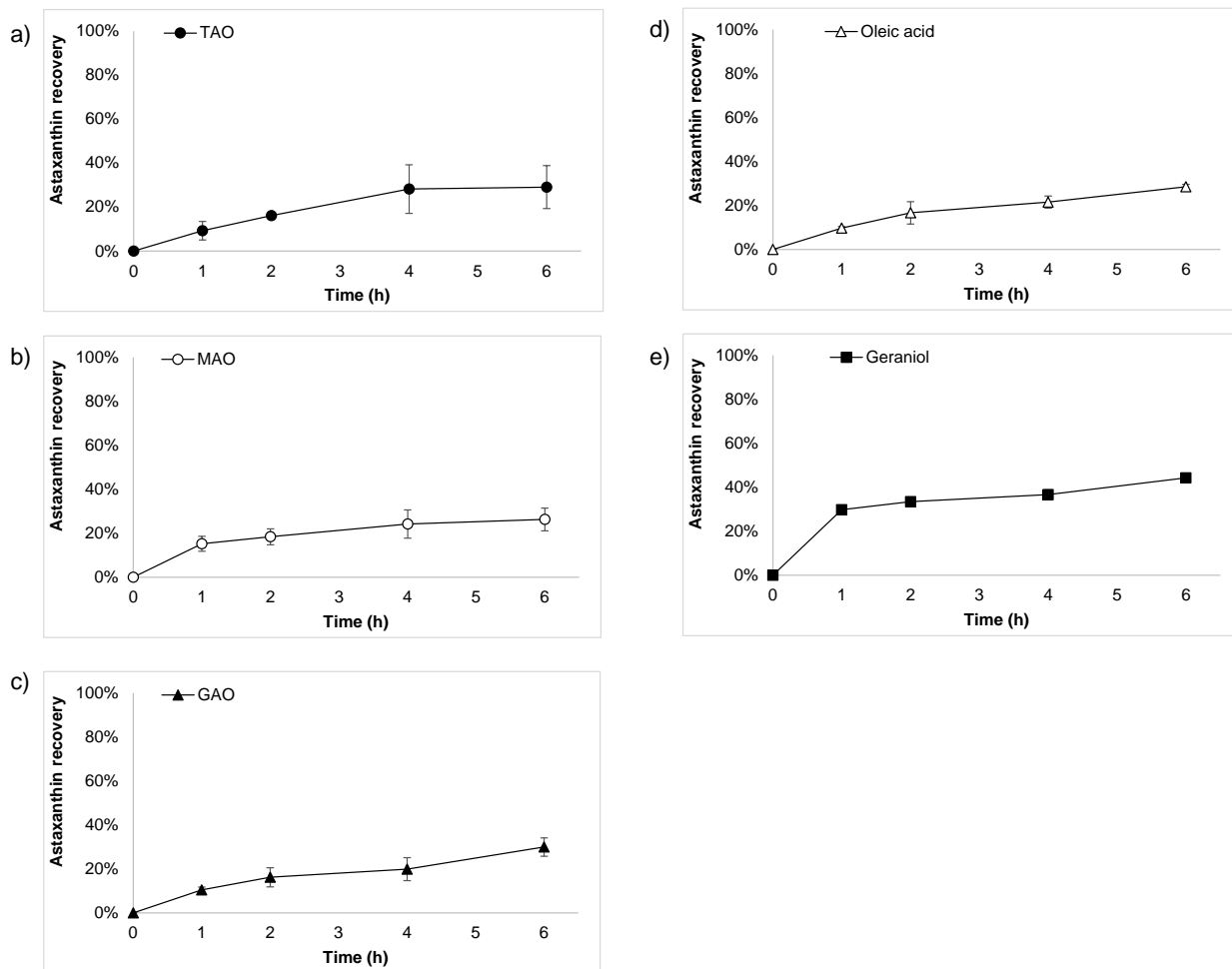
Fig. 4. Astaxanthin recovery from *H. pluvialis* cultures with a) thymol:oleic acid 3:1, TAO; b)

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 ± standard deviation of two independent experiments on different algal biomass.

431

21

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 (Samorì,

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).

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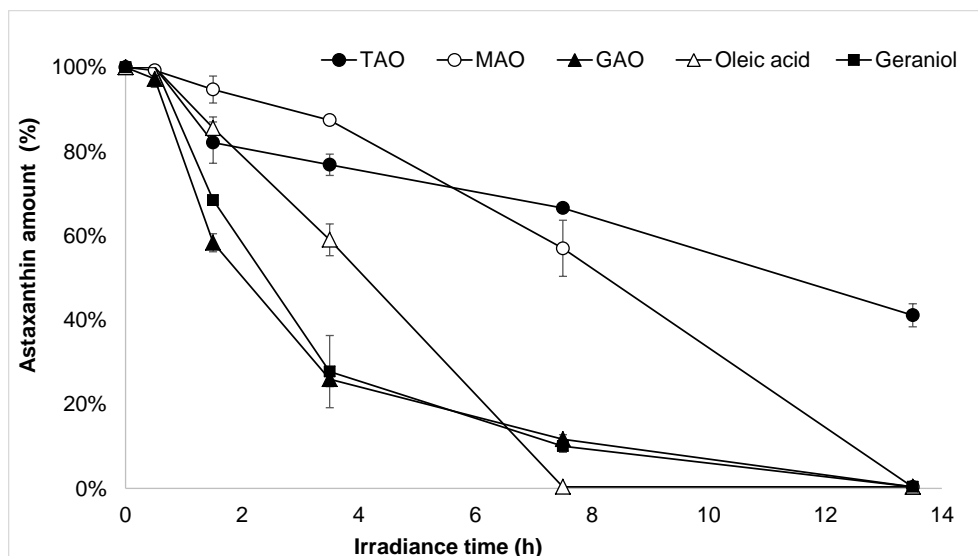
466   **Fig. 5**. Effect of light as oxidative factor on astaxanthin contained in DESs, oleic acid, and

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

23

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 $^1O_2$ 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.

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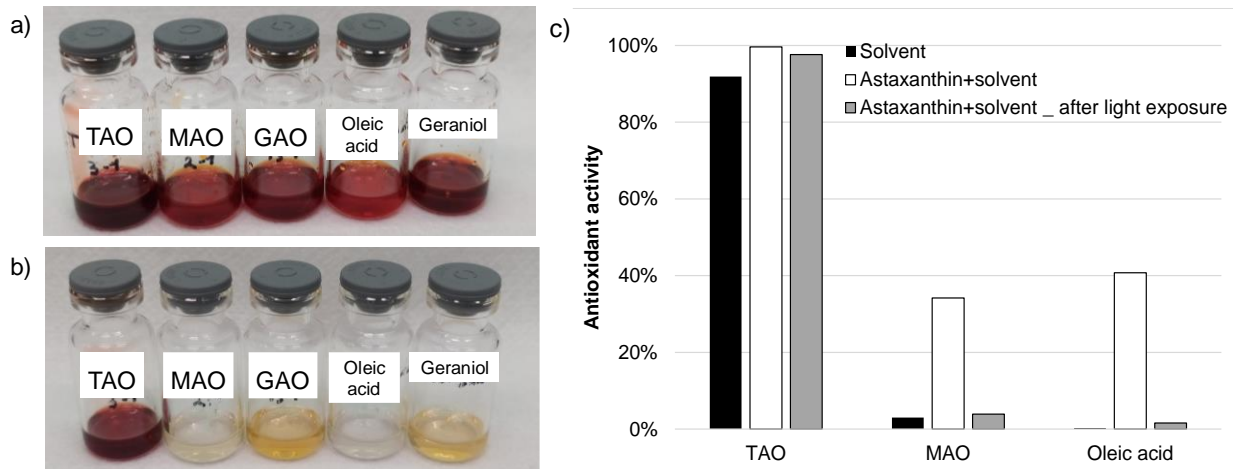
Fig. 6. Extracts of astaxanthin in TAO, MAO, GAO, oleic acid, and geraniol after a) the extraction from *H. pluvialis* freeze-dried cells, and b) 13.5 h of light irradiance. Antioxidant activity of TAO, MAO, and oleic acid tested alone or as extracts of astaxanthin (c).

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

25

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. ^{1}H-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 ^{1}H 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. ^{1}H NMR and ^{13}C

528 NMR spectra of TAO before and after the extraction of freeze-dried biomass.

529

530 AUTHOR INFORMATION

531 **Corresponding Author**

532 * Chiara Samorì, email address: chiara.samori3@unibo.it

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533 * Laura Pezzolesi, email address: laura.pezzolesi@unibo.it

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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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