



HAL
open science

NaDES-based biorefinery of *Spirulina* (*Arthrospira platensis*): A new path for sustainable high value-added metabolites

Soukaina Hilali, Louise van Gheluwe, Mervé Yagmur, Laura Wils, Myriam Phelippe, Barbara Clément-Larosière, Bénédicte Montigny, Johan Jacquemin, Emilie Thiery, Leslie Boudesocque-Delaye

► To cite this version:

Soukaina Hilali, Louise van Gheluwe, Mervé Yagmur, Laura Wils, Myriam Phelippe, et al.. NaDES-based biorefinery of *Spirulina* (*Arthrospira platensis*): A new path for sustainable high value-added metabolites. *Separation and Purification Technology*, 2024, 329, pp.125123. 10.1016/j.seppur.2023.125123 . hal-04213157

HAL Id: hal-04213157

<https://hal.science/hal-04213157>

Submitted on 21 Sep 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 NaDES-based biorefinery of *Spirulina (Arthrospira platensis)*: a new 2 path for sustainable high value-added metabolites 3

4 Soukaina Hilali,^a Louise Van Gheluwe,^a Mervé Yagmur,^{a,c} Laura Wils,^a Myriam Phelippe,^b Barbara Clément-
5 Larosière,^b Bénédicte Montigny,^c Johan Jacquemin,^d Emilie Thiery,^a and Leslie Boudesocque-Delaye^{a,*}

6 *a. EA 7502 SIMBA, Université de Tours, Faculté de Pharmacie, 31 avenue Monge 37200 Tours, France*

7 *b. Aqua Eco Culture, 7 rue d'Armor Maroué, 22400 Lamballe, France*

8 *c. EA 6299 PCM2E, Université de Tours, Faculté des Sciences et Techniques, Bât J, Avenue Monge, 37200 Tours, France*

9 *d. MSN Department, Mohammed VI Polytechnic University, Lot 660, Hay Moulay Rachid, Ben Guerir 43150, Morocco*

10 **Correspondence: leslie.boudesocque@univ-tours.fr; +33(0)2.47.36.71.75.*

11

12

13 **Abstract**

14 To meet the criteria of modern extraction, it is essential to use solvents that simultaneously reach
15 the standards of green chemistry and allow the extraction of a wide spectrum of bio-compounds.

16 Natural Deep Eutectic Solvents (NaDES), as a green solution, meet this difficult task and represent a
17 satisfactory alternative to organic solvents. Microalgae are recognized as a sustainable resource to

18 produce polar and non-polar metabolites, such as carotenoids, free fatty acids (FFA) and

19 phycobiliproteins. Among them, *Spirulina (Arthrospira platensis)* is one of the most studied, due to its

20 strong potential for food, health and cosmetic. In this work, two biorefinery scenarios were

21 investigated using polar and non-polar NaDES. The first one is a combination of a polar/non-polar

22 sequential solid/liquid extraction together with a liquid-liquid extraction of the polar extract. The

23 second scenario implements an innovative triphasic solid/liquid/liquid approach, based on the

24 simultaneous use of polar and non-polar NaDES in the solid/liquid extraction step. Several NaDES

25 were screened in this study to identify the best polar/non-polar NaDES pair. According to the results,

26 the use of the triphasic approach allowed an increase of the productivity by a factor 4 for

27 chlorophylls and FFA, and by a factor 2 for carotenoids. It was also interesting to note that the

28 phycocyanin-enriched polar fraction achieves much higher levels of purity than the corresponding

29 single-phase extraction for both applied biorefinery scenarios. These results pave the way for the
30 first time for a microalgae biorefinery entirely based on the use of NaDES and industrially credible.

31

32 **Keywords**

33 Natural deep eutectic solvents; microalgae; phycobiliprotein; free fatty acids; biorefinery; Spirulina

34

35 **1. Introduction**

36 Since the last decade, many studies have been conducted on deep eutectic solvents (DES) for
37 organic synthesis, biomass dissolution, formulation of pharmaceuticals or extraction of
38 biocompounds among others [1]. Based on their unique physico-chemical properties, DES
39 have been considered as a new class of alternative solvents suitable to substitute
40 petrochemical solvents, which are very well-known for their toxicity and negative impacts on
41 the environment [2]. However, the formulation of DES was not always fully satisfying these
42 ecological and toxicological criteria, and in fact, more natural alternatives were then sought.

43 Inspiring by extremophiles plants, the concept of Natural Deep Eutectic Solvents (NaDES) was
44 first described in 2013 by Dai *et al.*[3] in which NaDES were considered as the third biological
45 liquid in plants beside water and lipids. NaDES were formed using abundant natural
46 molecules, such as sugars, amino-acids, organic acids, polyols, etc.[3,4] Briefly, DES and
47 NaDES exhibit a lower melting point than their pure components taken separately, due to
48 hydrogen bond, van der Waals and/or electrostatic interactions.[3,5] NaDES, because of their
49 great potential, have focused the attention of a large number of scientists working in very
50 different fields and disciplines, ranging from thermodynamics, pharmacology to extractive
51 sciences. The definition of the term NaDES is still intensely debated, especially within the
52 thermodynamics community.[6] Indeed, if the abbreviation NaDES is commonly used in the
53 field of extractive sciences to designate mixtures based on natural compounds, which in a
54 well-defined ratio are liquid at room temperature. Of course, such a description does not
55 really correspond, in fact, to a proper definition of a eutectic point.[7] Nevertheless, to

56 facilitate understanding between disciplines, we have chosen to use the term NaDES to
57 designate the selected formulations described therein, even if it is not certain that each
58 formulation corresponds to a eutectic (invariant) point in the strict sense. However, from an
59 environmental point of view, such alternative solvents represent therefore an interesting and
60 attractive alternative to conventional organic solvents, especially by considering their natural
61 origin, low toxicity and high biodegradability.[1-3] In the literature, the most common used
62 compounds for the formulation of NaDES are choline chloride, glycerol, ethylene glycol and
63 sugar but organic acids, fatty acids, and amino-acids were also reported.[8]

64 Most of the NaDES described in the literature are hydrophilic, even if hydrophobic NaDES
65 have also recently emerged.[5] Among all hydrophobic NaDES, those based on menthol
66 appear as the most popular. This abundant and cheap molecule is generally extracted from
67 *Mentha* species (~€60/kg),[5] and was used for the formulation of NaDES in numerous
68 studies for the extraction of different bio-compounds from different biomasses, including
69 microalgae.[9-11]

70 Microalgae, as renewable resources of molecules with high added value, have focused the
71 interest of both academic and industrial researchers. These photosynthetic organisms have
72 the ability to adapt to various environmental conditions, being able to be oriented to
73 facilitate the synthesis of high value-added compounds in substantial quantities.[12,13]

74 Microalgae are also recognized as a rich source of polyunsaturated fatty acids (PUFA) of
75 pharmaceutical or nutraceutical interest, such as eicosapentaenoic acid, docosahexaenoic
76 acid, linoleic acid and γ -linolenic acid.[12,13] One of these microalgae with high potential is
77 the cyanobacterium *Spirulina* (*Arthrospira platensis*). It was an excellent source of nutrients,
78 which ranks first in the microalgae market and it is also considered as "super food".[14]

79 Therefore, this microalgae is of major commercial interest in the cosmetic, pharmaceutical,
80 food and nutraceutical fields. This popularity is essentially due to its richness in proteins (60-

81 70% dry matter) and in pigments, mainly in carotenoids, chlorophylls and phycocyanin. [15-
82 18]

83 Phycocyanin is a hydrophilic pigment characterized by its intense blue color and has been
84 broadly referred to as a valuable dye due to its protein-based structure and uncommon
85 natural blue color. The biological activities of this pigment include antioxidant, antiviral,
86 anticancer and antitumor properties. [13] Carotenoids, another abundant pigments found in
87 spirulina, are lipid-soluble pigments that are accountable for the yellow, orange, and red
88 coloration in numerous vegetables, plants and micro-organisms. Both Humans and animals
89 are unable to synthesize the carotenoids and get them through alimentation. They are used
90 for coloring and flavouring in food and feed, and for nutraceutical application as a provitamin
91 A source. [15-17] Many researches were carried out in order to isolate and purify the
92 phycocyanin; however, in most cases the lipophilic bio-compounds, such as carotenoids,
93 chlorophylls, and fatty acids were generally wasted with the residual biomass. [19]

94 Chlorophylls are also known for their masking and pigmenting properties. Free fatty acids
95 (FFA) are of great interest in the cosmetics field, acting as regulators of the cutaneous
96 microbiome (reactive or acne-prone skin) or as anti-aging agents. [11]

97 The extraction of various bioactive compounds has been commonly carried out using
98 conventional extraction methods, such as maceration and Soxhlet extraction techniques.
99 However, such methods come with numerous drawbacks including high time and solvent
100 consumption with low extraction yield. Consequently, researchers have shifted their
101 attention toward novel extraction techniques, like microwave assisted extraction,
102 supercritical fluid extraction, ultrasound assisted extraction (UAE). Among those techniques,
103 UAE stand out as an inexpensive and quite straight forward substitute to conventional
104 extraction processes. [20-22] This process uses acoustic cavitation to disrupt the cell wall
105 allowing the penetration of the solvent into the plant material, increasing thus both the
106 contact surface area and the mass transfer. [17-22] Consequently, UAE increases the

107 extraction yield, rate of extraction and reduces the extraction time without the need of
108 applying high temperatures.[21] Numerous applications of UAE for the extraction of lipids,
109 proteins, carotenoids, and phycocyanin are already reported in the literature.[11,15,21,23]
110 Although UAE gives many advantages, there is no report available on biphasic extraction from
111 *Spirulina* using this technique, to date. In fact, almost all the methods reported in the
112 literature for the purification of phycocyanin require numerous of expensive and time-
113 consuming steps, such as precipitation, centrifugation, dialysis, ion-exchange
114 chromatography, gel filtration chromatography.[24-26] Moreover, their purification cost
115 ranges between 50–90% of the entire production.[27] Hence, the development of more
116 effective and cost-efficient techniques that allow both high purity and recovery is consider of
117 high priority.

118 To echo this need, herein, a novel extraction approach is proposed. This approach is based on
119 a biphasic extraction allowing, for the first time, both the extraction and purification of
120 phycocyanin, as well as, the extraction of lipophilic compounds in a single extraction step.
121 Furthermore, recently our group highlighted the high potential of glycerol based-NaDES for
122 phycocyanin recovery and stabilization using UAE.[15] Especially glycerol/glucose (2:1,
123 mol/mol) with 20% of water was highlighted as the most performant medium considering the
124 extraction and stability enhancement of phycocyanin.[15] Additionally, menthol-based NaDES
125 have been described as an interesting alternative option for FFA and pigments extraction
126 from *Spirulina*,[11] even if its total recovery was lower than those of fatty acids based NaDES,
127 as the selectivity towards polyunsaturated FFA (PUFA) could be dramatically improved using
128 them. During this study, these particular NaDES, *i.e.* those based on polar glycerol/glucose
129 and non-polar menthol-based systems, were selected to build up sustainable biorefinery
130 scheme, in order to propose a new sustainable path for *Spirulina* pigments and FFA
131 valorisation.

132 Therefore, the main objective of this work is to investigate and compare two biorefinery
133 scenarios (sequential coupled with liquid-liquid extraction and triphasic solid/liquid/liquid
134 (SLL) extraction) with different NaDES formulations to reach high phycocyanin extraction and
135 purification. More importantly, this pioneer work was performed to pave the way toward a
136 time and energy saving green process for the valorisation of numerous bio-compounds from
137 Spirulina.

138 **2. Material and methods**

139 *2.1 Raw Material.*

140 Spirulina (*Arthrospira platensis*) biomass was kindly provided by Aqua Eco Culture (Lamballe,
141 France). The fresh biomass (20.16% dry matter) was frozen just after harvesting at -20 °C and
142 was stored at the same temperature prior to be used. All extractions were performed
143 between January and May 2022, in SIMBA Laboratory (Tours, France). Octanoic acid 98%, α -
144 D-(+)-glucose 99%, lauric acid 98%, 1,3-propanediol 98%, 1,2-octanediol 98% were purchased
145 from Acros Organics (Geel, Belgium). Glycerol 99% was purchased from Fisher Scientific SAS
146 (Illkirch, France). Ascorbic acid and Menthol 99% were purchased from COOPER (Melun,
147 France). Water was purified using a Milli-Q system (Millipore Corporation, Bedford, MA, USA).
148 Ethanol 96% analytical grade and methanol HPLC grade were purchased from Carlo Erba (Val
149 de Reuil, France). Fatty acids standard kits (saturated and unsaturated) was purchased from
150 Sigma Aldrich (St Louis, MO, USA).

151 *2.2. NaDES preparation and analysis.*

152 *2.2.1 NaDES preparation*

153 NaDES were prepared by mixing a fixed ratio of hydrogen bond donor (HBD) and hydrogen
154 bound acceptor (HBA) as shown in Table 1. The mixture was stirred and heated at 80°C for
155 hydrophilic NaDES and at 50°C for hydrophobic NaDES until a colorless solvent was obtained.

156 *Insert here Table 1*

157 *2.2.2. ¹H and ¹³C NMR*

158 ^1H NMR spectra were recorded on a Bruker[®] Avance 300 (300 MHz) NMR spectrometer, using
159 DMSO- d_6 as internal solvent and reference ($\delta_{\text{H}} = 2.50$ ppm). ^{13}C NMR was recorded at 75 MHz
160 on the same instrument, using the DMSO- d_6 solvent, with the reference peak at $\delta_{\text{C}} = 39.52$
161 ppm.

162 The NMR tubes were prepared by diluting 20 or 50 μL of NaDES in 480 or 450 μL of DMSO- d_6 ,
163 respectively.

164 *2.2.3. COSMO-RS component calculations*

165 During this work, the sigma profile and the sigma surface of each species were directly
166 visualized by using the COSMOthermX software (version BIOVA 2021) along with their BP-
167 TZVP COSMO files when available into the BIOVA COSMObase 2021 database. However, if
168 such files are not already available into this database, like in the case of the α -D-glucose and
169 L-menthol, the following calculations were realized to obtain suitable 3D descriptors for a
170 wide range of conformers for each investigated molecule.

171 In such a case, the structure of the species was firstly optimized, with a convergence criterion
172 of 10^{-8} Hartree in the gas phase, by using DFT calculations within the Turbomole 7.5.1
173 program package[28] utilizing the BP68 functional with the def-TZVP basis set,[29]and
174 combining the Resolution of Identity (RI) approximation.[30] Each resultant optimized 3D
175 structure was then used as an input for the generation of the COSMO file within the sigma
176 profile and the sigma profile of each conformer of each species using the COSMOconfX
177 program (version BIOVA 2021). [31]

178 The sigma profile and the sigma surface of the most stable conformer (i.e. presenting the
179 lowest energy conformer) was used to represent the 3D charge distribution onto the surface
180 of each molecule (i.e. reflecting their sigma profile and sigma profile) to localize HBA and HBD
181 sites, as the sigma surface represents the charge distribution onto the surface of each
182 molecule.

183 In fact, within the COSMO-RS method, HBA areas are represented as negative surfaces
184 (shown in red color in Fig. S1 of the ESI), while HBD are expressed as positive surfaces, shown
185 in blue color (Fig. S1 of ESI). The green area represents the non-polar part of the molecule,
186 mainly corresponding to Van der Waals-type interactions.

187 Similarly, on each graph presenting a sigma profile, a peak localized with a positive surface
188 charge density (σ , e/Å²) corresponds to HBA groups, while a peak observed in negative values
189 corresponds to HBD groups. A peak observed at around 0 e/Å² mainly corresponds to
190 nonpolar moieties causing hydrophobic van der Waals-type interactions.

191 During this work, both sigma profile and sigma surface were used as descriptors to provide
192 information on molecule's association driven by their apolar (hydrophobic) and polar HBAs and
193 HBDs moieties.

194 *2.3. Extraction process.*

195 The ultrasound-assisted extraction (UAE) was carried using an ultrasonic bath RK-100H
196 (Bandelin electronic, Berlin, Germany).

197 Each extraction was performed in triplicate. Two strategies were investigated herein, as displayed in
198 Figure 1.

199 *Insert here figure 1*

200 The first one (Figure 1A) is based on a sequential extraction (SE) of Spirulina. First, biomass
201 was extracted with hydrophilic NaDES using UAE at room temperature for 90 min at a given
202 biomass/solvent ratio (1:5, w/w). The resulting polar extract was then centrifuged during 20
203 min at 16 200 g (Rotanta 460R, Hettich, Kirchleingern, Germany). After its centrifugation, the
204 supernatant was submitted to a liquid-liquid extraction (LLE) using a non-polar NaDES (1:1,
205 vol. ratio) and the pellet (algae) was reused for a second 90 min UAE using the same non-
206 polar NaDES.

207 The second scenario, specially developed for this work, is based on a biphasic extraction (Figure 1B).
208 Herein, 3 g of hydrophilic and 3 g of hydrophobic NaDES were simultaneously used along with 0.6 g
209 of microalgae (1:5, w/w ratio per NaDES) for a 90 min UAE at ambient temperature. After each 30
210 min, samples were vortex for 2 min. The resulting extract was then centrifuged during 20 min at 16
211 200 *g*, resulting, thus, in two separated phases, which were then further analyzed. To highlight the
212 potential interest to mix these two NaDES into this single extraction step, reference monophasic
213 extraction processes were carried under the same condition, *i.e.* for 90 min UAE, by mixing 3 g of a
214 particular NaDES with 0.6 g of biomass (1:5, w/w ratio).

215 2.4. Extract analysis.

216 2.4.1. Free Fatty acid (FFA) analysis

217 FFA qualitative and quantitative were determined by liquid chromatography-mass
218 spectroscopy (LC-ESI-MS), according to Wils *et al.*[25] These analyses were performed using
219 an Acquity H-Class coupled with an SQD detector (Waters, Saint Quentin in Yvelines, France).
220 The system was fitted with a BEH C18 (50 mm × 2.1 mm; 1.7 μm). The column oven was set at
221 60°C. The mobile phases were (A) 0.01% formic acid (aq.) containing 0.2 mM ammonium
222 formate and (B) 50% isopropanol in acetonitrile containing 0.01% formic acid (aq) and 0.2
223 mM ammonium formate. The flow rate was 0.24 mL/min and gradient was set as follows:
224 initial solvent B content was 50%, raised to 98% in 16 min and maintained for 4 min. The
225 column was then re-equilibrated to initial conditions. ESI was in negative mode and
226 performed with cone voltage at 50 V with capillary voltage of 2.8 kV. The NaDES extracts
227 required a prior pre-treatment, which was carried by solid-phase extraction (SPE) using C18-
228 silica gel (Ait, 40–60 μm Fischer Scientific, Waltham, MA, USA). The elution gradient ranged
229 from 50 to 100% water:methanol. Herein, only the 100% methanolic fractions were
230 analyzed.[25] Samples were all prepared in methanol. Nervonic acid (C 20:4) at 10 μg/mL was
231 added as internal standard. All FFAs were identified by comparison of retention time and
232 mass spectra using commercial standards. FFA titration was performed using a calibration

233 curve of each standard ranging from 2.5 µg/mL to 12.5 µg/mL (1 µL injection) and using
234 nervonic acid as internal standard. Linear regression and calculation were performed of SIR
235 chromatogram using Targetlynx (Waters).

236 2.4.2. Pigment titration of non-polar extract

237 The pigment titration was performed according to Wils *et al.*[25] Briefly, 100 mg of the
238 extract were diluted by 0.8 mL of methanol; 290 µL of the resulting solute were used for both
239 the carotenoid and chlorophyll titration. The absorption spectra were measured using UV–vis
240 microplates spectrophotometer reader (Multiskan GO, Thermo Fisher Scientific, SAS) and the
241 carotenoids (C_{Ca}) and chlorophyll (C_{Ch}) contents were then calculated using the absorbances
242 at 450, 645 and 663 nm by applying the following equations 1 and 2:

$$C_{Ch} = [(20.2 \times OD_{645}) + (8.2 \times OD_{663})] \times FD \quad \text{Eq. 1}$$

$$C_{Ca} = (4 \times OD_{450}) \times FD \quad \text{Eq. 2}$$

243

244 where C_{Ch} (µg/ml) is the chlorophyll concentration, C_{Ca} (µg/ml) the carotenoids concentration,
245 FD the dilution factor, and OD_{645} , OD_{663} , and OD_{450} the extract optical density at 645, 663 and
246 640 nm, respectively.

247 2.4.3. Pigment titration of polar extract

248 The pigment titration was performed according to Wils *et al.*[25] Briefly, 100 mg extract was
249 diluted by 0.8 mL of distilled water; 250 µL of the resulting solute was used for the
250 phycocyanin titration. The absorption spectra were measured using UV–vis microplates
251 spectrophotometer reader (Multiskan GO, Thermo Fisher Scientific, SAS). The phycocyanin
252 (C_{PC}) contents was obtained using the absorbances at 620 and 652 nm by applying the
253 equation 3 as proposed by Bennett *et al.*[27]

$$C_{PC} = \frac{OD_{620} - (0.474 \times OD_{652})}{5.34} \quad \text{Eq. 3}$$

254

255 where, C_{PC} (mg/mL) is the phycocyanin concentration, and OD_{620} and OD_{652} is the extract
256 optical density at 620 and 652 nm, respectively.

257 The phycocyanin yield (Y_{PC}) was then calculated using equation 4, as follows:

$$Y_{PC} = \frac{C_{PC} \times 10 \times V_{NaDES}}{M_{DM}} \quad \text{Eq. 4}$$

258 where V_{NaDES} is the NaDES volume, M_{DM} the mass of dried matter and Y_{PC} the extraction yield

259 expressed in milligram of phycocyanin per gram of dried matter (mg/g DM).

260 *2.4.4. Colorimetry in CIELAB color space.*

261 Colorimetric measurements were performed on the monophasic extract of GG and compared

262 to the biphasic extract of the same NaDES coupled with MC8. The equipment used is a

263 handheld chroma meter C400 (Konika Minolta, Tokyo, Japan) able to read translucent samples

264 in a cuvette. All the measurements were performed at ambient temperature and performed

265 in triplicates.

266 CIELAB coordinates (L^*a^*b) of all samples were measured and significant differences were

267 highlighted using equation 5.

$$\Delta E = (\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2 \quad \text{Eq. 5}$$

269

270 *2.4.5. Statistical analyses*

271 The statistical significance was evaluated by the one-way ANOVA. Differences were

272 considered to be significant at $p < 0.05$.

273 **3. Results and discussion**

274 *3.1. Smart NaDES selection strategy*

275 The selection of the raw materials used during the preparation of the so-called NaDES is a

276 crucial step in order to truly design a formulation which respects Green Chemistry principles

277 and could be then used as green solvents. Indeed, like all non-volatile solvents, the

278 solvent/metabolite separation requires purification or de-extraction steps using conventional

279 organic solvents, biobased or not, which drastically reduces the environmental interest of

280 using these matrices. One of the solutions frequently mentioned in the literature, and use in

281 industry, is the recycling and re-equipping of NaDES after the de-extraction of the metabolites

282 of interest.[32,33] However, this approach further complicates the extraction scheme by
283 multiplying the number of unit operations and in fact increases the overall cost and footprint
284 of the whole process.

285 Another approach, which we have applied herein, consists in selecting raw materials
286 biocompatible with the targeted application of the extract produced. In fact, by applying such
287 an approach, no further extraction scheme is requested as the NaDES will be further used as a
288 passive ingredient presents in final formulation containing the targeted and extracted
289 product. As in the present case, the targeted application being associated with the cosmetic
290 market, the raw materials were preselected according to the following criteria: i)
291 compatibility with the European and Chinese cosmetic regulations; and ii) availability of
292 biosourced resources. The main constraint of the cosmetic market concerning NaDES is the
293 prohibition of choline chloride, *i.e.* the most used ingredient for the preparation of this class
294 of solvents.[1]

295 Thus, all raw materials used during this work were firstly selected as they are compatible with
296 cosmetics, which allows their integration into the finished product. This singularity further
297 allows to deeply simplify the overall extraction process as separation units and steps including
298 metabolite recovery and solvent removal/recycling could be vanished. More importantly, this
299 possibility provides a real sense of the utilisation of NaDES, respecting thus Green Chemistry
300 principles by proposing, also, a sustainable solution with lower footprint than current
301 benchmarks.[34]

302 The ingredients of these NaDES are functional ingredients used in current cosmetic
303 formulation as emollient, solvent or texture agent. In fact, by keeping the NaDES in the
304 finished product further allows to decrease the use of additional formulation ingredients,
305 reinforcing the overall hypothesis that the proposed approach is greener and more
306 sustainable than conventional ones.

307 The biocompatible character of NaDES was also tested previously on keratinocyte cell culture
308 and all formulations do not show significant toxicity at tested concentrations.[11]
309 Among all NaDES described in the literature, GG and combination of Menthol and fatty acids
310 or thymol were in fact selected.[35-37] The choice of menthol partner in hydrophobic NaDES
311 was mainly guided thanks to the sigma profile of preselected biocompatible compounds (see
312 Figure S1 of the Electronic Supporting information). In fact, each decision made on either the
313 formulation or on a molecule selection, was also driven thanks to simulation calculations.
314 Herein, the COSMOthermX software was used to visualize the sigma profile and the 3D
315 mapping of the charges' distribution onto each preselected molecule. Both of them were
316 used therein as descriptors to further validate decisions made. In fact, these descriptors could
317 be used to depict the compatibility of the menthol with selected partners. Selected partners
318 are defined as : *i*) hydrophobic species mainly dominated by van der Waals-type interactions,
319 with a HBD group as observed with C8 and C12 fatty acids; *ii*) hydrotropes with variable chain
320 length to provide a modulated hydrophobic character along with both HBA and HBD moieties,
321 as observed with 1,3-propanediol and 1,2-octanediol; and, *iii*) a molecule mimicking the 3D
322 mapping and the sigma profile of the menthol like the thymol (see Figure S1 of the Electronic
323 Supporting information).
324 As reported in Table 1, another advantage for formulating hydrophobic NaDES based on
325 Menthol is related to their relatively low density data (on average around 0.9 g/mL) and their
326 low viscosity (on average between 20 and 70 mPa.s),[38] making these NaDES easy to handle.
327 In contrary, the tested glucose-based NaDES has a density and viscosity close to that of
328 glycerol, making its handling more tedious but still experimentally and industrially
329 feasible.[34]

330

331 *3.2. Reference extractions*

332 Reference extractions were performed with different NaDES; polar (GG) and non-polar (MC8, MC12,
333 MO, MP, and MT). Figure 2 displays the pigments (Fig. 2A) and FFA (Fig. 2B) content in the extracts
334 after each monophasic extraction.

335 *Insert here figure 2*

336 Regarding GG extract, the main pigment extracted was the water-soluble protein phycocyanin with
337 90.85 ± 2.50 mg/g DM. Although GG was considered as polar NaDES, a minority of chlorophylls and
338 carotenoids were extracted (respectively, 3.72 ± 0.09 , and 1.71 ± 0.04 mg/g DM). Regarding the non-
339 polar NaDES, the NaDES MO exhibited the highest selectivity toward non-polar pigments with $8.9 \pm$
340 0.1 mg/g DM of chlorophylls and 3.8 ± 0.1 mg/g DM of carotenoids.

341 Concerning the FFA content of reference extracts, the profile was substantially the same for each;
342 with: myristic acid (C14), palmitic acid (C16), palmitoleic acid (C16:1), stearic acid (C18), oleic acid
343 (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3). FFA profile was mostly dominated by PUFA.
344 The polar GG extract was surprisingly enriched in FFA with a total amount of 26.55 mg/g DM,
345 compared to 36.06 mg/g DM in MC12 extract. In fact, sugar-based NaDES are less polar than water
346 with polarity close to methanol.[37] Thus, belonging to the category of NaDES that exhibit solvent
347 duality (lipophilicity and hydrophilicity), explaining why GG can extract also a high quantity of FFA.

348 *3.3. Sequential extraction.*

349 Two strategies were initially considered: 1) an extraction with a polar NaDES followed by a
350 non-polar NaDES or 2) an extraction with a non-polar NaDES followed by a polar NaDES.

351 Regarding this last strategy, it was abandoned since no metabolite was extracted with polar
352 NaDES after the non-polar extraction (data not shown as its concentration was always lower
353 than the detection limit of the equipment used). Based on our observation, it appears that
354 the non-polar NaDES clogged the hydrophilic NaDES access points, limiting thus the extraction
355 of the targeted compounds. Subsequently, solely the strategy 1) was then used. First, polar
356 NaDES (GG) was used to extract metabolites, mostly polar, from Spirulina. After UAE extraction and

357 centrifugation, the polar extract exhibited a green-blue color due to the extracted pigment
358 phycocyanin (Fig. 2A). For sequential extraction, this green-blue extract has been set aside and the
359 pellet biomass (called GG pellet) was reused for the extraction of non-polar metabolites with non-
360 polar NaDES (MC8, MC12, MO, MP, and MT). Figure 3 displays the pigments (Fig. 3A) and FFA (Fig.
361 3B) content of non-polar extracts after the sequential extraction (abbreviated as Seq). These results
362 were compared with reference monophasic extractions (abbreviated as MonoP).

363 *Insert here Figure 3*

364 After the sequential extraction of pellet biomass with non-polar NaDES, the results showed a
365 decrease of non-polar pigments (chlorophylls and carotenoids) and FFA (especially linoleic
366 acid) compared to the references monophasic extraction. For example, regarding the
367 sequential extraction with the NaDES MO, the content of chlorophylls and carotenoids
368 decreased, respectively, by 71% and 78%, compared to the reference extract. Regarding FFA,
369 linoleic acid content was 17.74 ± 1.23 for the MO reference extract and was zero for
370 sequential extract. In view of the FFA content in GG extracts (Fig2.B), one could point-out that
371 a significant percentage of these non-polar metabolites was already extracted via the polar
372 NaDES GG.

373 Therefore, the polar extract GG was submitted to a liquid-liquid extraction (LLE) with non-
374 polar NaDES intended for both non-polar compound extraction and phycocyanin purification.
375 The non-polar phase obtained was pooled with the non-polar extract from the sequential
376 extraction to form the so-called: pooled extract. Figure 4 displays the phycocyanin content
377 (mg/g DM) in the polar extracts after liquid-liquid extraction with non-polar NaDES.

378 *Insert here Figure 4*

379 Regardless of the non-polar NaDES used to perform liquid-liquid extraction on the polar
380 extract, the amount of phycocyanin in this phase remains approximately the same: *e.g.* 82
381 mg/g DM; *i.e.* a result close to the reference GG extract close to 90.8 ± 2.5 mg/g DM.

382 Therefore, it could be speculated that the liquid-liquid extraction using a menthol based
383 NaDES did not affect the polar composition of the polar fraction. Figure 5 displays the non-
384 polar pigment (Fig. 5A) and FFA (Fig. 5B) content in the extracts after a sequential extraction
385 (abbreviated as Seq), liquid-liquid extraction (abbreviated as LL), and in the non-polar extract
386 called "pooled". Regarding the pigment profiles of non-polar extracts, as shown in Fig. 5A,
387 one can see that using a liquid-liquid extraction it is possible to recover a large majority of the
388 chlorophylls and carotenoids pigments. For example, the recovery rate of chlorophylls varied
389 between 61% and 80% with MC12 and MO, respectively. However, in the case of carotenoids,
390 its recovery rate is between 26% and 35% using MT and MO, respectively.

391 *Insert here figure 5*

392 The recovery of the green chlorophyll and orange carotenoid pigments allowed the
393 purification of GG phase restoring thus the bright blue color related to the high phycocyanin
394 content as shown in Figure 6.

395 *Insert here figure 6*

396 In the case of FFA (Fig.5B), the collected results indicate that only 12%, 13%, 11%, 16%, and
397 17% of FFA were left in the GG hydrophilic extract after MC8, MC12, MO, MP, and MT liquid-
398 liquid extraction, respectively. NaDES composition in each liquid phase was determined after
399 the LLE extraction using ^1H and ^{13}C NMR. No significant variation of the NaDES concentration
400 in its rich phase was notified. More importantly, no trace of non-polar NaDES component
401 transfer to GG was observed (Figure S2 to S5, Electronic Supporting Information). It was
402 important to assess this point, as GG can solubilize non- polar metabolites, and could then
403 disturb the non-polar NaDES equilibrium during the LLE process. By using the sequential
404 extraction combined with liquid-liquid extraction, it was then possible to obtain selectively
405 two distinct extracts: *i)* the purified polar with mainly water-soluble phycocyanin, and *ii)* the
406 pooled non-polar extract composed of non-polar metabolites: chlorophylls, carotenoids and

407 FFA. Collected data could be used as a first proof of concept that LLE using solely NaDES
408 matrixes is in fact possible. This further open the way to multistep biorefinery or purification
409 procedure, using such promising alternative solvents. However, reference extractions with
410 hydrophobic NaDES remain better and less time-consuming than the sequential extraction
411 followed by liquid-liquid extraction.

412 Nevertheless, in the literature most of LLE processes including NaDES described a
413 NaDES/organic solvent or water systems, either for metabolites recovery from NaDES,[38-42]
414 or liquid-liquid chromatography.[43] Herein and for the first time, we described an extraction
415 process using solely NaDES-based solvents, generating two different enriched NaDES-based
416 extracts. However, in order to improve the productivity of the process, we then decided to
417 investigate a triphasic S/L/L approach.

418 *3.3. Solid/Liquid/Liquid strategy.*

419 An innovative solid/liquid/liquid (SLL) process was considered as an alternative biorefinery
420 scenario allowing the extraction of polar and non-polar compounds into two easily separated
421 fractions. Herein, the polar fraction (GG) was characterized by a bright blue color (rich in
422 phycocyanin) while the non-polar fraction with a greenish yellow coloration (chlorophylls,
423 carotenoids, and FFA). Figure 7 displays the phycocyanin content (mg/g DM) in each polar
424 extract (abbreviated as BiP GG) after a biphasic extraction using non-polar NaDES.

425 *Insert here figure 7*

426 Whatever the NaDES used, similar phycocyanin content was collected back, *i.e.* about 75
427 mg/g DM. Contrary to monophasic extract (Figure 6), after the biphasic extraction, the polar
428 phase exhibits a bright blue color. This might imply that it is possible to both extract and
429 purify the phycocyanin using this extraction technique.

430 In order to confirm these hypotheses, the content of non-polar pigments (Fig.8B) and FFA
431 (Fig.8B) was investigated in the polar fraction (abbreviated GG SLL) and in the non-polar
432 fraction (abbreviated as “non-polar NaDES” SLL).

433 *Insert here figure 8*

434 It was found that chlorophylls, carotenoids, and FFA concentrations were more than 80%
435 lower in the GG polar fraction of the biphasic extract than values collected using the
436 monophasic GG for all used couplings. After the biphasic extraction the contents of pigment
437 and FFA in the non-polar extract were either higher or comparable with data found using the
438 monophasic extraction, used herein as a reference. Based on data collected, MO was found
439 to be the best lipophilic NaDES for the extraction of non-polar metabolites.

440 Extraction performances of FFA were slightly improved for all NaDES compared to the
441 reference extraction and GG resulting extracts exhibited all a bright blue color. Thus, this
442 biphasic strategy appears to be a relevant scenario to produce in one step both polar and
443 non-polar enriched extracts.

444 The non-polar fraction from the LLE and SLL processes could well be incorporated into anti-
445 aging cosmetic preparations, thanks to the compatibility of NaDES with cosmetic regulations
446 and its richness in carotenoids and FFA of interest. The presence of menthol will not be a
447 limitation, as the active ingredient content of finished products is generally around 1%.

448 Cytotoxicity results against keratinocytes and cutaneous microbiota carried out on a panel of
449 NaDES, including Menthol-based NaDES, showed good safety at cosmetic doses.[11]

450 *3.4. Colorimetric analysis*

451 Here, it was assumed that the biphasic extraction allowed both the extraction and
452 purification of phycocyanin. This hypothesis was argued by both the blue color of the extracts
453 and the important decrease in the chlorophylls, carotenoids, and FFA contents in the polar
454 fraction. To further investigate this hypothesis, a colorimetry analysis using CIELAB color

455 system was performed using GG extracts as reference, GG coupled with MC8 as the biphasic
456 extracts, and GG after liquid-liquid extraction with MC8. The extracts were all diluted with
457 water to 1:20 ratio for a better reading of colorimetry parameters. The system is composed of
458 one channel for light (L^*) and two color channels (a^* and b^*). The L^* axis goes from 0 (black)
459 to 100 (white), the a^* from green ($-a^*$) to red ($+a^*$), and b from blue ($-b^*$) to yellow ($+b^*$). In
460 the case of the GG extract, *i.e.* the reference, collected values were: $a^* = 3.66 \pm 0.11$, $b^* = 1.12$
461 ± 0.01 , and $L^* = 23.43 \pm 1.34$, representing a greener blue. In the case of the biphasic extract,
462 the following values were collected: $a^* = -0.2 \pm 0.01$, $b^* = -7.06 \pm 0.01$, and $L^* = 27.29 \pm 0.02$
463 representing a pure light blue similar to the one found after the liquid-liquid extract (*e.g.* $a^* =$
464 0.38 ± 0.01 , $b^* = -6.78 \pm 0.01$, and $L^* = 27.06 \pm 0.02$). This was further confirmed thanks to the
465 calculation of ΔE , which was significant, *i.e.* >2 , in comparison with the selected references:
466 *e.g.* the sequential GG ($\Delta E = 96.7$) or biphasic GG ($\Delta E = 86.3$), but not significant when
467 comparing with the value collected for the Sequential and Biphasic extract ($\Delta E = 0.5$).

468 It was noted here that both biphasic and liquid-liquid extractions allow the purification of
469 phycocyanin as confirmed by the blue-sky color of the extract. Even if the biphasic extraction
470 has a lower yield than the monophasic extraction, it allows, for all the tested coupling, both
471 the extraction and purification of phycocyanin. This could potentially eliminate a further
472 purification step prior to the incorporation of the blue extracts in cosmetic, nutraceutical or
473 food products.

474 Moreover, as GG was highlighted as powerful stabilization media for phycocyanin, using this
475 biphasic approach extraction, purification and stabilization of the high value pigment could be
476 performed in one step.

477 *3.5. Sequential/LLE vs SLL approach*

478 Considering the polar extracts and therefore the phycocyanin content, similar performances
479 were obtained with Seq/LLE and triphasic SLL extraction. Nonetheless, the SLL process slightly
480 improved the extraction of non-polar compounds in comparison with the sequential

481 extraction followed by a liquid-liquid extraction. Moreover, this biphasic process has an
482 extraction time three times shorter than the other. Figure 9 summarizes the results obtained
483 for the NaDES MC12 and MO, which allows an easy comparison of the two tested approaches
484 in terms of their productivity.

485 *Insert here Figure 9*

486 As shown in Figure 9, the use of the biphasic process increases the productivity for all tested
487 metabolites. Indeed, with the sequential approach, solely 100 mg of chlorophyll could be
488 extracted per hour, while 250 and 500 mg/h could be extracted using the biphasic approach
489 with GG/MC12 and GG/MO, respectively. For the FFAs, ~0.5 g would be extracted per hour
490 for the sequential approach compared to more than 2 g/h for the biphasic, corresponding to
491 a four-fold increased productivity. Considering carotenoids, major high value-added
492 pigments, productivity was increased only of a factor 2, which is still a very good result.

493 Data collected therein using the NaDES extracts with ultrasonic extraction processes were
494 then compared with those reported in the literature, to further highlight their competitive
495 levels and thus the validity of the proposed strategy. In fact, the levels observed using
496 laboratory scale processes (0.26 mg/mL solvent) [44] or industrial ones (0.17 mg/ml solvent)
497 [45], are about ten times less concentrated than those achieved therein by following the
498 NaDES protocols (reaching 3.0 mg/mL NaDES on average). However, the non-volatile nature
499 of NaDES makes subsequent concentration steps very difficult, unlike aqueous extracts.

500 However, it is important to note that biological activities related to cosmetic applications
501 (tyrosinase, antioxidant, anti-inflammatory) have shown active phycocyanin concentrations
502 oscillating between 20 and 100 µg/mL [46] These concentrations would correspond to a use
503 of our extracts between 0.5 and 3% in a cosmetic product, which is in line with industrial
504 practices in this field.[47]

505

506 *3.6. Biorefinery approaches sustainability assessment.*

507 The SLL extraction process, appearing then as a major improvement in microalgae biorefinery
508 processes panel, was then analyzed according to the principles of green extraction as
509 established by Chemat *et al.*[48] This original and green charter in the form of the “six
510 principles of green extraction” allows an innovation that is not only limited to the process but
511 to all the solid-liquid extraction aspects. More recently, new indicators derived from
512 analytical chemistry have appeared, such as the ComplexGAPI method.[49] This approach
513 makes it possible to assess the greenness of an analytical process by considering the entire
514 workflow, including the steps preliminary to the analysis itself. This approach has been
515 specially developed on the basis of the GAPI and CHEM 21 approaches, to take into account
516 the specific features of emerging solvents such as ionic liquids or deep eutectic solvents.[49]
517 The ComplexGAPI approach simplifies access to green performance indicators through the
518 use of freely available online software. Rente *et al.* recently used this tool to assess the
519 comparative impact of extraction processes involving NaDES.[47] Minimal adaptation of some
520 parameters makes the tool very easy to use. We therefore applied the ComplexGAPI strategy
521 to both our sequential/LLE and SLL approaches. The parameters used to generate the
522 pictograms are summarized in Table 2, and the pictograms generated are shown in Figure 10.

523 *Insert here Table 2 & Figure 10*

524 Indicators of good environmental performance appear in green, those of average impact in yellow
525 and the most negative impacts in red.

526 The parameters grouped together under the term "pre-extraction procedure" represent the steps
527 involved in preparing NaDES prior to be used as an extraction solvent. In the pictogram, these
528 indicators are located in the center of the graph. Logically, the pictograms are identical for both
529 processes, as the NaDES involved are the same. Next come parameters relating to sample
530 preparation (left), solvents and reagents (center), then instrumentation (right). The major difference
531 between the two processes lies in energy consumption, which is drastically reduced in the SLL

532 approach, enabling the indicator to change from red to yellow. Interestingly enough, the
533 consumption indicators are suitable for sample analysis and can be quite low when considering an
534 extractive process. Nevertheless, the ComplexGAPI approach allows us to visually highlight the
535 environmental impact of our processes. Selecting NaDES using our Smart NaDES approach eliminates
536 the need for numerous extract processing steps saving, thus, energy and limiting waste generation.
537 Overall, both approaches have good environmental impacts, with the SLL process having a more
538 favorable impact. This greenness indicator, combined with increased productivity, makes the SLL
539 process a prime choice for the development of economically and environmentally sustainable
540 biorefinery schemes.

541 **4. Conclusions**

542 Whether sequential/LLE or SLL strategies, polar and non-polar metabolites could be extracted
543 from the microalgae *Spirulina* using exclusively NaDES. The SLL process fully fulfilled the eco-
544 extraction guidelines allowing a dramatic reduction of unitary operation combined with an
545 improved mass transfer and pre-purification of phycocyanin. When compared to the
546 sequential extraction, the productivity of this process increases four-fold and doubled the
547 content of FFA and carotenoids, respectively. In the light of collected results, the biphasic
548 approach assisted by ultrasound extraction could be seen as a promising approach for the
549 extraction, purification, and valorisation of *Spirulina* high value-added compounds, covering a
550 wide range of polarity.

551 These results describe for the first time a full NaDES-based biorefinery scheme of *Spirulina*,
552 opening the way to alternative extractions solvent for developing promising biomasses
553 valorisation. Extension to other microalgae species and to plant biomasses is currently under
554 investigation, as well as, innovative alternative extraction processes.

555

556 **Author Contributions**

557 Conceptualization, L.B.D.; Methodology, L.B.D., E.T., J.J. and B.C.L.; investigation, S.H., L.V.G., E.T., L.W., and M.Y.;
558 resources, M.P. and B.C.L.; writing—original draft preparation, S.H., and L.V.G.; writing—review and editing, E.T.,
559 L.B.D., J.J. and B.M.; supervision, L.B.D., J.J. and B.M.; project administration, L.B.D.; funding acquisition, L.B.D. All
560 authors have read and agreed to the published version of the manuscript.

561 **Conflicts of interest**

562 There are no conflicts to declare

563 **Acknowledgements**

564 This work was supported by the French National Research Agency (ANR-19-CE43-0001-01), the Région Centre Val
565 de Loire (L. Wils, PhD Grant) and Université de Tours (M. Yagmur, PhD grant, les Rabelaisiennes).
566

567 **Supplementary material**

568 Supplementary material is available online at

569 **References**

- 570 [1] K. M. Jeong, J. Ko, J. Zhao, Y. Jin, D. E. Yoo, S. Y. Han, J. Lee, Multi-functioning deep eutectic
571 solvents as extraction and storage media for bioactive natural products that are readily applicable to
572 cosmetic products, *J. Clean. Prod.* 151 (2017) 87–95. <https://doi.org/10.1016/j.jclepro.2017.03.038>.
- 573 [2] B. Socas-Rodríguez, Á. Santana-Mayor, A. V. Herrera-Herrera, M. Á. Rodríguez-Delgado, Deep
574 eutectic solvents, in A. M. Inamuddin, S. K. Asiri (Eds.) *Green Sustainable Process for Chemical and*
575 *Environmental Engineering and Science*, Elsevier, 2020, pp. 123-177, [https://doi.org/10.1016/B978-0-](https://doi.org/10.1016/B978-0-12-817386-2.00005-6)
576 [12-817386-2.00005-6](https://doi.org/10.1016/B978-0-12-817386-2.00005-6)
- 577 [3] Y. Dai, G.-J. Witkamp, R. Verpoorte, Y. H. Choi, Natural Deep Eutectic Solvents as a New Extraction
578 Media for Phenolic Metabolites in *Carthamus tinctorius* L., *Anal. Chem.* 85 (2013) 6272–6278
579 <https://doi.org/10.1021/ac400432p>
- 580 [4] Y. H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I. W.C.E. Arends, G.-J. Witkamp, R.
581 Verpoorte, Are Natural Deep Eutectic Solvents the Missing Link in Understanding Cellular Metabolism
582 and Physiology?, *Plant Physiol.*, 156 (2011) 1701–1705, <https://doi.org/10.1104/pp.111.178426>

583 [5] B. D. Ribeiro, C. Florindo, L. C. Iff, M. A. Z. Coelho, I. M. Marrucho, Menthol-based Eutectic
584 Mixtures: Hydrophobic Low Viscosity Solvents. ACS Sustain. Chem. Eng. 3 (2015) 2469–2477.
585 <https://doi.org/10.1021/acssuschemeng.5b00532>

586 [6] B. B. Hansen, S. Spittle, B. Chen, D. Poe, Y. Zhang, J. M. Klein, A. Horton, L. Adhikari, T. Zelovich, B.
587 W. Doherty, B. Gurkan, E. J. Maginn, A. Ragauskas, M. Dadmun, T. A. Zawodzinski, G. A. Baker, M. E.
588 Tuckerman, R. F. Savinell, J. R. Sangoro Deep Eutectic Solvents: A Review of Fundamentals and
589 Applications, Chem. Rev. 121 (3) (2021) 1232-1285 <https://doi.org/10.1021/acs.chemrev.0c00385>

590 [7] G. Ah-lung, B. Flamme, F. Ghamouss, M. Maréchal, J. Jacquemin, Guidelines for designing highly
591 concentrated electrolytes for low temperature applications. Chem. Commun., 56 (2020) 9830-9833
592 <https://doi.org/10.1039/D0CC03963B>

593

594 [8] M. Benlebna, M. Ruesgas-Ramón, B. Bonafos, G. Fouret, F. Casas, C. Coudray, E. Durand, M. Cruz
595 Figueroa-Espinoza, C. Feillet-Coudray Toxicity of Natural Deep Eutectic Solvent Betaine:Glycerol in
596 Rats. J Agric Food Chem. 66(24) (2018) 6205-6212. <https://doi.org/10.1021/acs.jafc.8b01746>

597 [9] W. Pitacco, C. Samorì, L. Pezzolesi, V. Gori, A. Grillo, M. Tiecco, M. Vagnoni, P. Galletti. Extraction
598 of astaxanthin from Haematococcus pluvialis with hydrophobic deep eutectic solvents based on oleic
599 acid. Food Chem. 379 (2022) 132156. <https://doi.org/10.1016/j.foodchem>.

600 [10] C. Crampon, C. Nikitine, M. Zaier, O. Lépine, C. Dejoye Tanzi, M. Abert Vian, F. Chemat, E.
601 Badens, Oil extraction from enriched Spirulina platensis microalgae using supercritical carbon
602 dioxide. J. Supercrit. Fluids 119 (2017) 289–296 <https://doi.org/10.1016/j.supflu.2016.10.006>.

603 [11] L. Wils, C. Leman-Loubière, N. Bellin, B. Clément-Larosière, M. Pinault, S. Chevalier, C.
604 Enguehard-Gueiffier, C. Bodet, L. Boudesocque-Delaye Natural deep eutectic solvent formulations for
605 spirulina: Preparation, intensification, and skin impact. Algal Res. 56 (2021) 102317
606 <https://doi.org/10.1016/j.algal.2021.102317>

607 [12] X. Yang, Y. Li, Y. Li, D. Ye, L. Yuan, Y. Sun, D. Han, Q. Hu Solid Matrix-Supported Supercritical CO₂
608 Enhances Extraction of γ -Linolenic Acid from the Cyanobacterium *Arthrospira (Spirulina) platensis*
609 and Bioactivity Evaluation of the Molecule in Zebrafish. *Mar. Drugs* 17 (2019) 203
610 <https://doi.org/10.3390/md17040203>

611 [13] N. Seyidoglu, S. Inan, C. Aydin, A Prominent Superfood: *Spirulina platensis*. in N. Shiomi, V.
612 Waisundara (Eds.) *Superfood and Functional Food - The Development of Superfoods and Their Roles*
613 *as Medicine* ,InTech, 2017. <https://doi.org/10.5772/66118>.

614 [14] M. Raczyk, K. Polanowska, B. Kruszewski, A. Grygier, D. Michałowska, Effect of *Spirulina*
615 (*Arthrospira platensis*) Supplementation on Physical and Chemical Properties of Semolina (*Triticum*
616 *durum*) Based Fresh Pasta. *Molecules* 27 (2022) 355 <https://doi.org/10.3390/molecules27020355>

617 [15] S. Hilali, L. Wils, A. Chevalley, B. Clément-Larosière, L. Boudesocque-Delaye, Glycerol-based
618 NaDES as green solvents for ultrasound-assisted extraction of phycocyanin from *Arthrospira*
619 *platensis*—RSM optimization and ANN modelling. *Biomass Convers. Biorefinery* 12 (2022) 157–170
620 <https://doi.org/10.1007/s13399-021-02263-6>

621 [16] W.S. Park, H.-J. Kim, M. Li, D.H. Lim, J. Kim, S.-S. Kwak, C.-M. Kang, M.G. Ferruzzi, M.-J. Ahn Two
622 Classes of Pigments, Carotenoids and C-Phycocyanin, in *Spirulina* Powder and Their Antioxidant
623 Activities. *Molecules* 23 (2018) 2065 <https://doi.org/10.3390/molecules23082065>

624 [17] R. Sharma, B. Bhunia, A. Mondal, T. K. Bandyopadhyay, I. Devi, G. Oinam, R. Prasanna, G.
625 Abraham, O. N. Tiwari, Statistical optimization of process parameters for improvement of
626 phycobiliproteins (PBPs) yield using ultrasound-assisted extraction and its kinetic study. *Ultrason.*
627 *Sonochem.* 60 (2020) 104762 <https://doi.org/10.1016/j.ultsonch.2019.104762>

628 [18] L. Wils, S. Hilali, L. Boudesocque-Delaye, Biomass Valorization Using Natural Deep Eutectic
629 Solvents: What's New in France? *Molecules* 26 (2021) 6556
630 <https://doi.org/10.3390/molecules26216556>

- 631 [19] S. Marzorati, A. Schievano, A. Idà, L. Verotta, Carotenoids, chlorophylls and phycocyanin from
632 Spirulina: supercritical CO₂ and water extraction methods for added value products cascade. Green
633 Chem. 22 (2020) 187–196 <https://doi.org/10.1039/C9GC03292D>
- 634 [20] F. Chemat, N. Rombaut, A.G. Sicaire, A. Meullemiestre, A.S. Fabiano-Tixier, M. Abert-Vian
635 Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations,
636 protocols and applications. A review. Ultrason. Sonochem. 34, 540–560 (2017)
637 <https://doi.org/10.1016/j.ultsonch.2016.06.035>
- 638 [21] S. Dey, V. K. Rathod, Ultrasound assisted extraction of β -carotene from Spirulina platensis.
639 Ultrason. Sonochem. 20 (2013) 271–276 <https://doi.org/10.1016/j.ultsonch.2012.05.010>
- 640 [22] M. Jacotet-Navarro, N. Rombaut, A.S. Fabiano-Tixier, M. Danguien, A. Bily, F. Chemat.
641 Ultrasound versus microwave as green processes for extraction of rosmarinic, carnosic and ursolic
642 acids from rosemary. Ultrason. Sonochem. 27 (2015) 102–109
643 <https://doi.org/10.1016/j.ultsonch.2016.06.035>
- 644 [23] L. Vernès, M. Abert-Vian, M. El Maâtaoui, Y. Tao, I. Bornard, F. Chemat Application of ultrasound
645 for green extraction of proteins from spirulina. Mechanism, optimization, modeling, and industrial
646 prospects. Ultrason. Sonochem. 54 (2019) 48–60 <https://doi.org/10.1016/j.ultsonch.2019.02.016>
- 647 [24] F.S. Figueira, C. C. Moraes, S. J. Kalil, C-phycocyanin purification: multiple for different
648 applications. Braz. J. Chem. Eng. 35 (2018) 1117–1128 [https://doi.org/10.1590/0104-](https://doi.org/10.1590/0104-6632.20180353s20170160)
649 [6632.20180353s20170160](https://doi.org/10.1590/0104-6632.20180353s20170160)
- 650 [25] L. Wils, M. Yagmur, M. Phelippe, B. Montigny, B. Clément-Larosière, J. Jacquemin, L.
651 Boudesocque-Delaye, Alternative Solvents for the Biorefinery of Spirulina: Impact of Pretreatment on
652 Free Fatty Acids with High Added Value. Mar. Drugs 20 (2022) 600
653 <https://doi.org/10.3390/md20100600>

654 [26] G. Patil, S. Chethana, A. S. Sridevi, K. S. M. S. Raghavarao, Method to obtain C-phycoyanin of
655 high purity. J. Chromatogr. A 1127 (2006) 76–81 <https://doi.org/10.1016/j.chroma.2006.05.073>

656 [27] A. Bennett, L. Bogorad, Complementary chromatic adaptation in a filamentous blue-green alga.
657 J. Cell Biol. 58 (1973) 419–435 <https://doi.org/10.1083/jcb.58.2.419>

658 [28] TURBOMOLE User's Manual, Version 7.5.1; COSMOlogic GmbH & Co. KG: Leverkusen, Germany,
659 **2021.**

660 [29] A. Schäfer, C. Huber, R. Ahlrichs, Fully Optimized Contracted Gaussian Basis Sets of Triple Zeta
661 Valence Quality for Atoms Li to Kr. J. Chem. Phys., 100 (1994) 5829-5835
662 <https://doi.org/10.1063/1.467146>

663 [30] F. Weigend, M. Häser, RI-MP2: First Derivatives and Global Consistency. Theor. Chem. Acc., 97
664 (1997) 331-340 <https://doi.org/10.1007/s002140050269>

665 [31] BIOVIA COSMOconf 2021 User Guide, Version 2021; Dassault Systèmes Customer Support :
666 Vélizy-Villacoublay, France, 2021

667 [32] E. Rodríguez-Juan, C. Rodríguez-Romero, J. Fernández-Bolaños, M.C. Florido, A. Garcia. Phenolic
668 compounds from virgin olive oil obtained by natural deep eutectic solvent (NADES): effect of the
669 extraction and recovery conditions. J Food Sci Technol 58 (2021) 552–561
670 <https://doi.org/10.1007/s13197-020-04567-3>

671 [33] K.M. Jeong, M.S. Lee, M.W. Nam, J. Zhao, Y. Jin, D.K. Lee, S.W. Kwon, J.H. Jeong, J. Lee Tailoring
672 and recycling of deep eutectic solvents as sustainable and efficient extraction media, J. Chromatogr.
673 A, 1424, 10-17 (2015) <https://doi.org/10.1016/j.chroma.2015.10.083>

674 [34] E. Hendriks, G. M. Kontogeorgis, R. Dohrn, J.-C. de Hemptinne, I. G. Economou, L. F. Žilnik, V.
675 Vesovic Industrial Requirements for Thermodynamics and Transport Properties Ind. Engin. Chem.
676 Res. 49 (22) (2010) 11131-11141 <https://doi.org/10.1021/acs.iecr.0c05356>

677 [35] N. Schaeffer, D. O. Abranches, L.P. Silva, M. A.R. Martins, P. J. Carvalho, O. Russina, A. Triolo, L.
678 Paccou, Y. Guinet, A. Hedoux, J. A.P. Coutinho, Non-Ideality in Thymol + Menthol Type V Deep
679 Eutectic Solvents, ACS Sust. Chem. Engin. 9 (5) (2021) 2203-2211
680 <https://doi.org/10.1021/acssuschemeng.0c07874>

681 [36] T. Hložek, T. Bosáková, Z. Bosáková, P. Tůma, Hydrophobic eutectic solvents for endocrine
682 disruptors purification from water: Natural and synthetic estrogens study, Sep. Purif. Technol., 303,
683 122310 (2022) <https://doi.org/10.1016/j.seppur.2022.122310>

684 [37] D. van Osch, C. Dietz, S. Warrag, M. Kroon, The Curious Case of Hydrophobic Deep Eutectic
685 Solvents: A Story on the Discovery, Design, and Applications, ACS Sust. Chem. Engin. 8 (29) (2020)
686 10591-10612 <https://doi.org/10.1021/acssuschemeng.0c00559>

687 [38] Y. Dai, J. van Spronsen, G.-J. Witkamp, , Verpoorte, Y.H. Choi, Natural deep eutectic solvents as
688 new potential media for greentechnology, Analytica Chimica Acta, 766, 61– 68 (2013)
689 <https://doi.org/10.1016/j.aca.2012.12.019>

690 [39] Y. L. Kua, S. Gan, Natural Deep Eutectic Solvent (NADES) as a Greener Alternative for the
691 Extraction of Hydrophilic (Polar) and Lipophilic (Non-Polar) Phytonutrients. Key Eng. Mater. 797
692 (2019) 20–28 <https://doi.org/10.4028/www.scientific.net/KEM.797.20>

693 [40] D. Smink, S. R. A. Kersten, B. Schuur, Recovery of lignin from deep eutectic solvents by liquid-
694 liquid extraction. Sep. Purif. Technol. 235 (2020) 116127
695 <https://doi.org/10.1016/j.seppur.2019.116127>

696 [41] R. Verma, T. Banerjee, Liquid–Liquid Extraction of Lower Alcohols Using Menthol-Based
697 Hydrophobic Deep Eutectic Solvent: Experiments and COSMO-SAC Predictions. Ind. Eng. Chem. Res.
698 57 (2018) 3371–3381 <https://doi.org/10.1021/acs.iecr.7b05270>

699 [42] H. Ebadnezhad, M.R. Afshar Mogaddam, A. Mohebbi, M.A. Farajzadeh, M. Nemati, M. Torbati
700 Combination of temperature-assisted ternary phase homogenous liquid–liquid extraction with deep

701 eutectic solvent–based dispersive liquid–liquid microextraction for the extraction of phytosterols
702 from cow milk and cream samples. *J. Sep. Sci.* 44 (2021) 1482–1489
703 <https://doi.org/10.1002/jssc.202001012>

704 [43] J.-B. Chagnoleau, N. Papaiconomou, M. Jamali, D. O. Abranches, J. A. P. Coutinho, X. Fernandez,
705 T. Michel Toward a Critical Evaluation of DES-Based Organic Biphasic Systems: Are Deep Eutectic
706 Solvents so Critical? *ACS Sustain. Chem. Eng.* 9 (2021) 9707–9716
707 <https://doi.org/10.1021/acssuschemeng.1c01628>

708 [44] S. P. Kamble, R. B. Gaikar, R. B. Padalia, K. D. Shinde, Extraction and purification of C-
709 phycocyanin from dry Spirulina powder and evaluating its antioxidant, anticoagulation and
710 prevention of DNA damage activity. *Journal of Applied Pharmaceutical Science*, 3(08) (2013) 149–
711 153. <https://doi.org/10.7324/JAPS.2013.3826>

712 [45] Wachda, H. Hadiyanto, G. D. Harjanto, M. L. Huzain, R. W. Ajiet Production of antioxidant C-
713 phycocyanin using extraction process of Spirulina platensis in large scale industry IOP Conf. Ser.:
714 Mater. Sci. Eng. 633 (2019) 012025 <https://doi.org/10.1088/1757-899X/633/1/012025>

715 [46] R. Fernandes, J. Campos, M. Serra, J. Fidalgo, H. Almeida, A. Casas, D. Toubarro, A. Barros.
716 Exploring the Benefits of Phycocyanin: From Spirulina Cultivation to Its Widespread Applications.
717 *Pharmaceuticals* 16(4) (2023) 2023592. <https://doi.org/10.3390/ph16040592>

718 [47] D. Rente, M. Cvjetko Bubalo, M. Panić, A. Paiva, B. Caprin, I. Radojčić Redovniković, A. R. C.
719 Duarte, Review of deep eutectic systems from laboratory to industry, taking the application in the
720 cosmetics industry as an example, *J. Clean. Prod.* 380 (2022) 135147
721 <https://doi.org/10.1016/j.jclepro.2022.135147>

722 [48] F. Chemat, M. A. Vian, G. Cravotto, Green Extraction of Natural Products: Concept and Principles.
723 *Int. J. Mol. Sci.* 13 (2012) 8615–8627 <https://doi.org/10.3390/ijms13078615>

724 [49] J. Płotka-Wasyłka, W. Wojnowski, Complementary green analytical procedure index

725 (ComplexGAPI) and software. Green Chem. 23 (2021) 8657–8665

726 <https://doi.org/10.1039/D1GC02318G>

727

729 Table 1: Studied hydrophilic and hydrophobic NaDES compositions

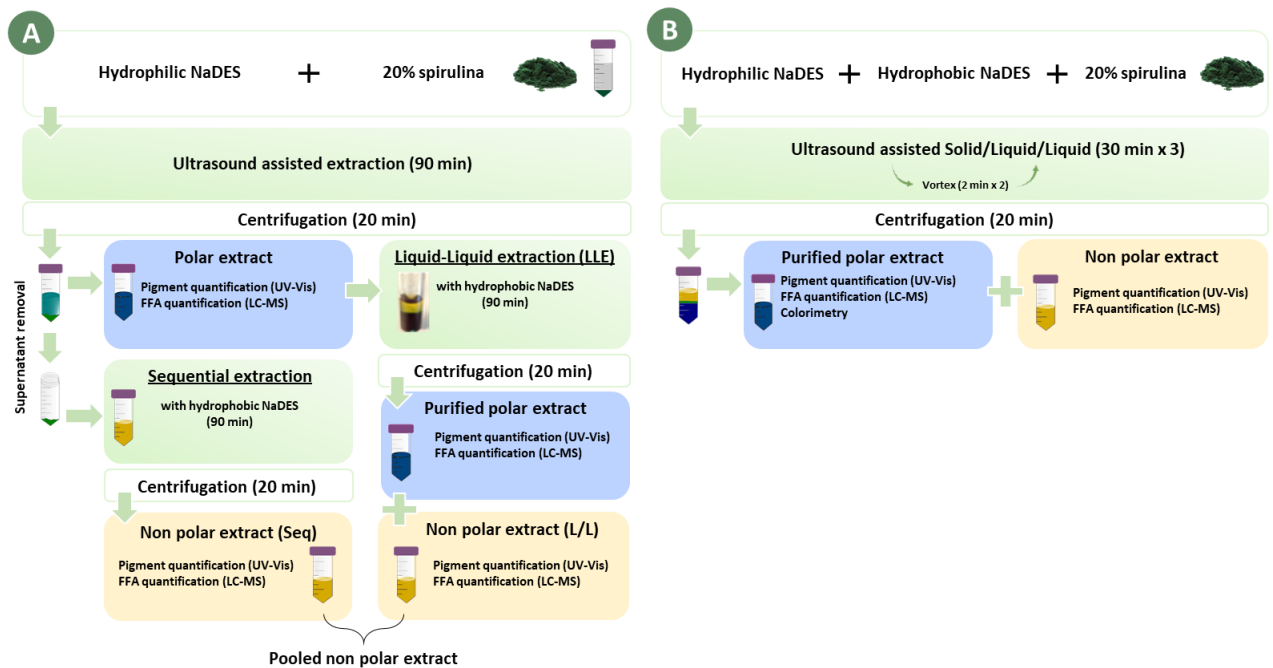
Eutectic mixtures		Component 1	Component 2	Molar Ratio	Water content (wt%)	Density (g/mL)
Polar	GG	Glycerol	Glucose	2:1	20%	1.303 ± 0.002
Non-polar	MC8	Menthol	Octanoic acid	5:1	-	0.885 ± 0.003
	MC12		Lauric acid	4:3	-	0.881 ± 0.003
	MO		1,2-Octanediol	1:1	-	0.907 ± 0.001
	MP		1,3-Propanediol	1:2	-	0.963 ± 0.005
	MT		Thymol	1:1	-	0.931 ± 0.002

730

731 Table 2: Complex GAPI indicator for sequential/LLE and SLL processes considering 1g of biomass

732 engaged

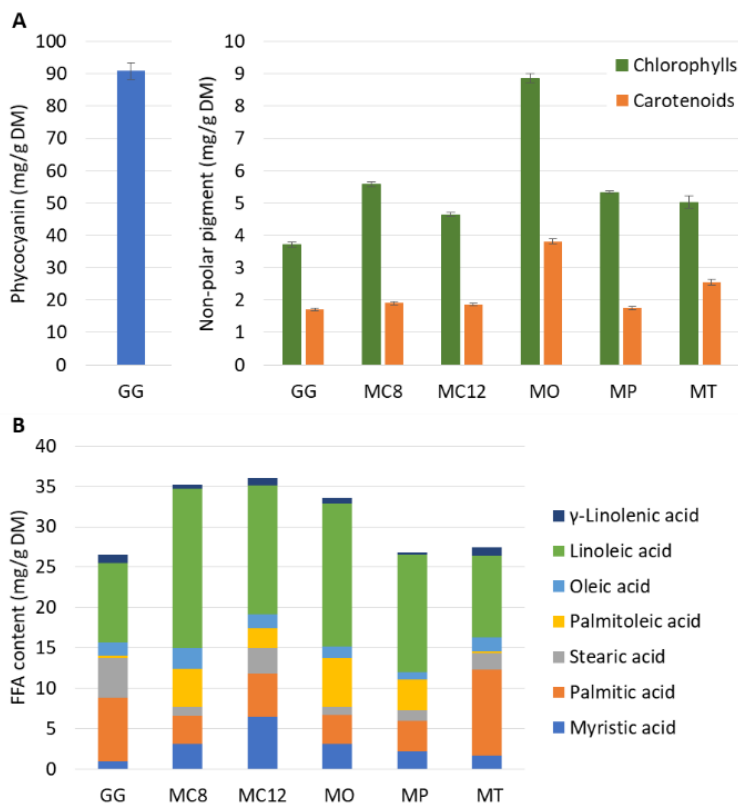
GAPI Parameter	Sequential /LLE	SLL
Pre-extraction processes		
<i>Yield and conditions</i>		
Yield	100 %	100 %
Temperature/time	max 1h 80°C	max 1h 80°C
<i>Relation to green economy</i>		
Number of rules met	5-6	5-6
<i>Reagents and solvents</i>		
Health hazard	No special hazards	No special hazards
Safety hazard	No hazard	No hazard
<i>Instrumentation</i>		
Technical set-up	Common set up	Common set up
Energy	0.5 kWh/sample	0.5 kWh/sample
Occupational hazard	Hermetization	Hermetization
<i>Work up and purification</i>		
End product work up purification	No work up	No work up
Purity	> 98%	> 98%
<i>E-factor</i>		
E-factor input	0	0
Sample preparation and extraction		
<i>Sample preparation</i>		
Collection	n.a.	n.a.
Preservation	None	None
Transport	None	None
Storage	Under normal conditions	Under normal conditions
Type of method	No sample preparation	No sample preparation
Scale of extraction	n.a.	n.a.
Solvent/reagent used	Green solvents	Green solvents
Additional treatment	none	none
<i>Reagents and solvents</i>		
Amount	< 10 g	< 10 g
Health hazard	No special hazards	No special hazards
Safety hazard	No hazards	No hazards
<i>Instrumentation</i>		
Energy	2.36 kWh per sample	0.79 kWh per sample
Occupational hazard	Hermetic	Hermetic
Waste	No waste	No waste
Waste treatment	n.a.	n.a.
<i>Method type</i>		
Type of analysis	n.a.	n.a.



734

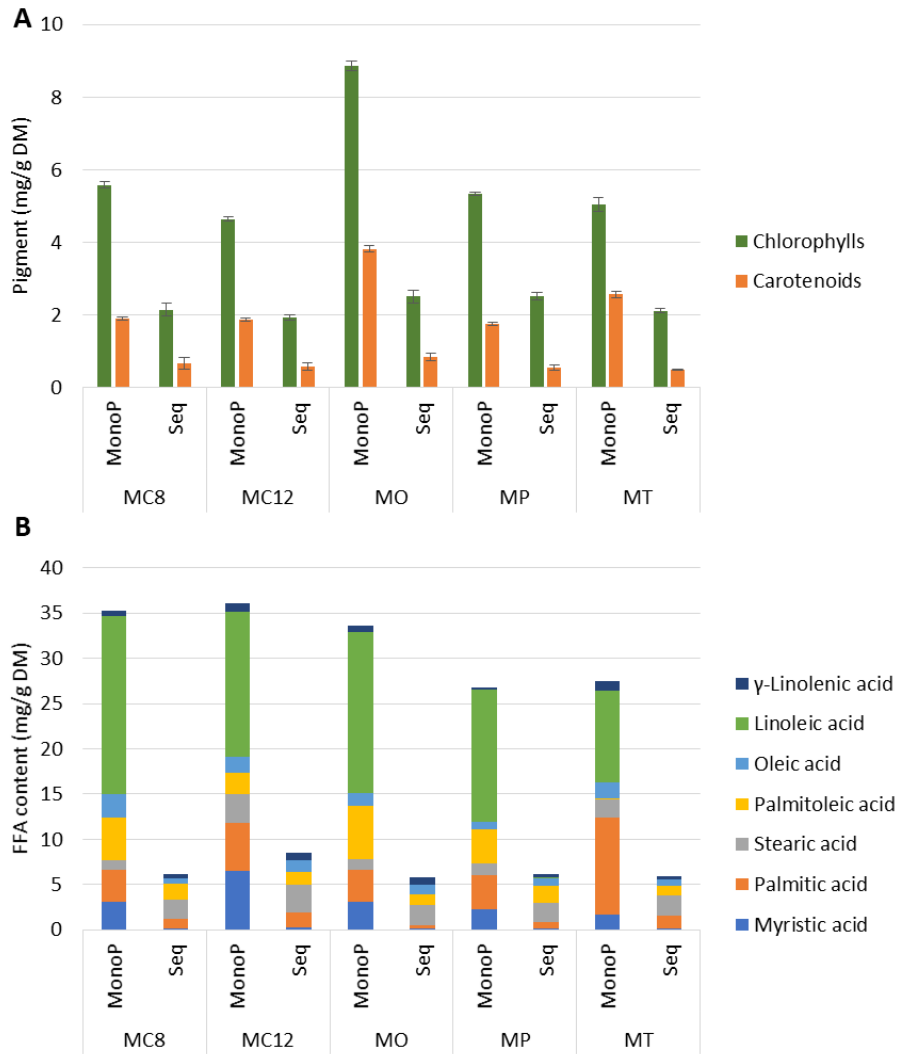
735 Figure 1: Extraction protocol for the sequential (A) and triphasic solid/liquid/liquid (B) extraction

736 strategy

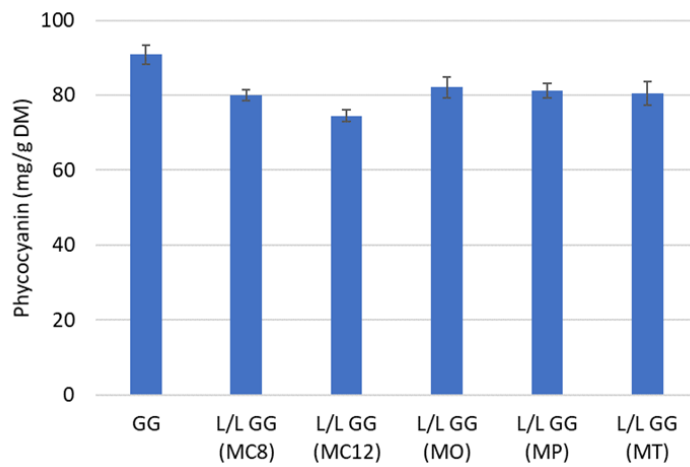


737 Figure 2: Pigment profile (A) and FFA profile (B) for reference extractions

738

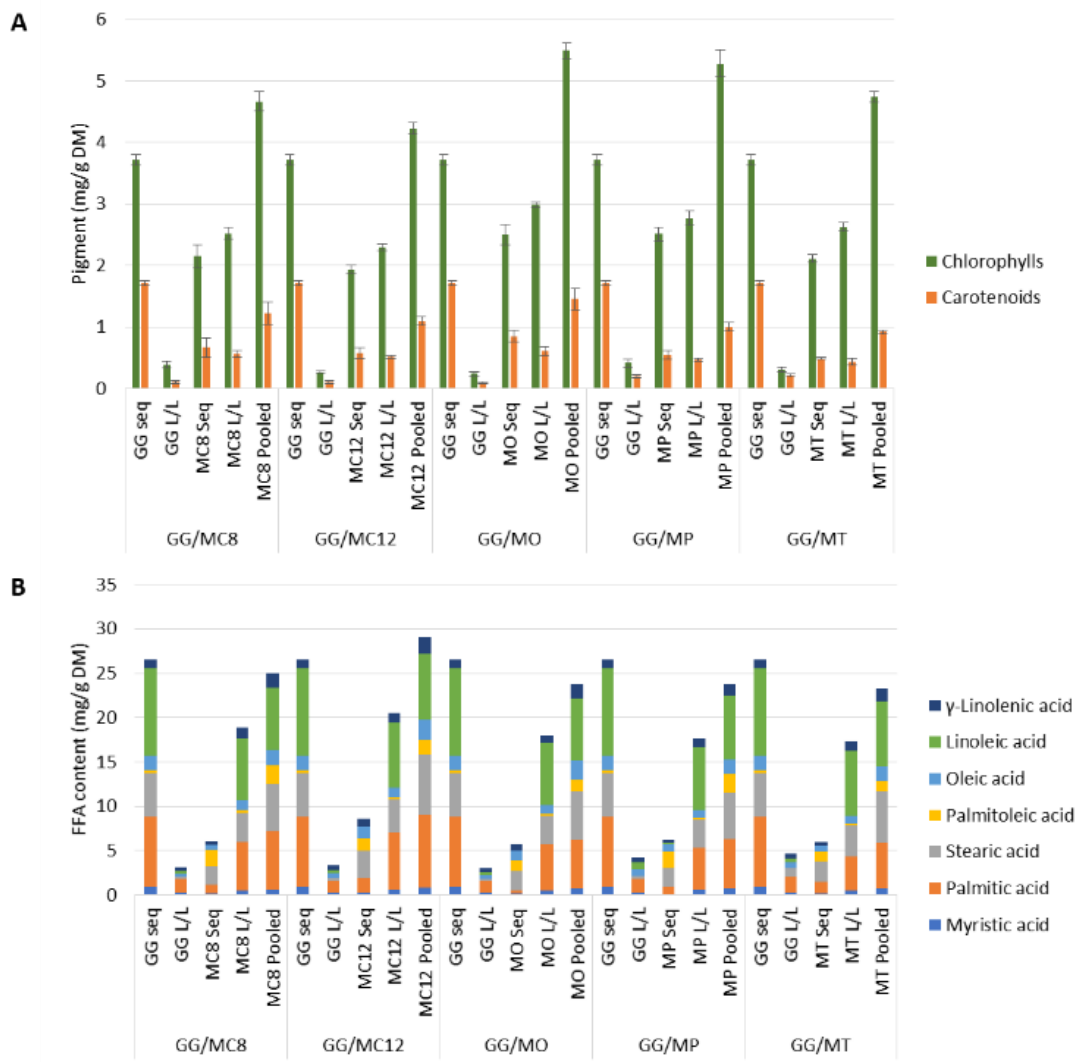


739 Figure 3: Pigment profile (A) and FFA profile (B) for reference solid/liquid extraction vs sequential



740 solid/liquid extraction of GG pellet

741 Figure 4: Phycocyanin content after liquid-liquid extraction of the polar phase GG



742

743 Figure 5: Pigment profile (A) and FFA profile (B) for sequential extraction compared to reference



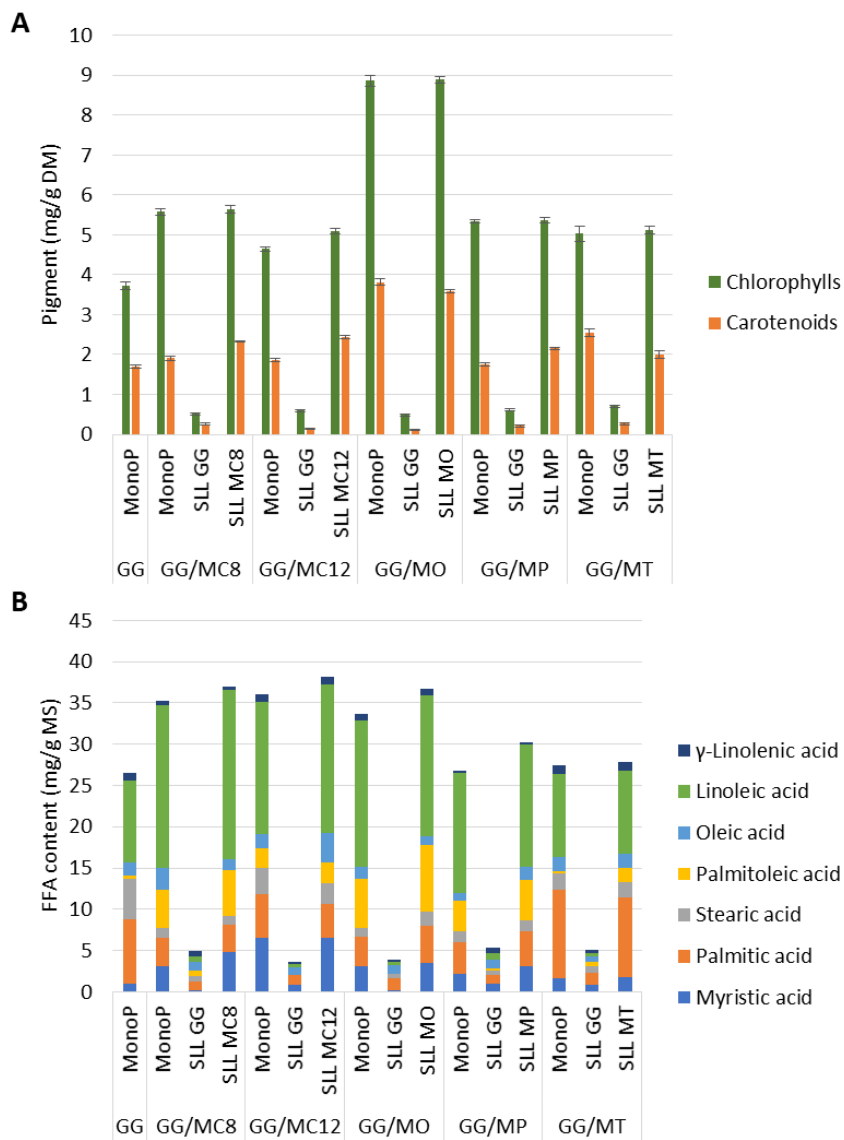
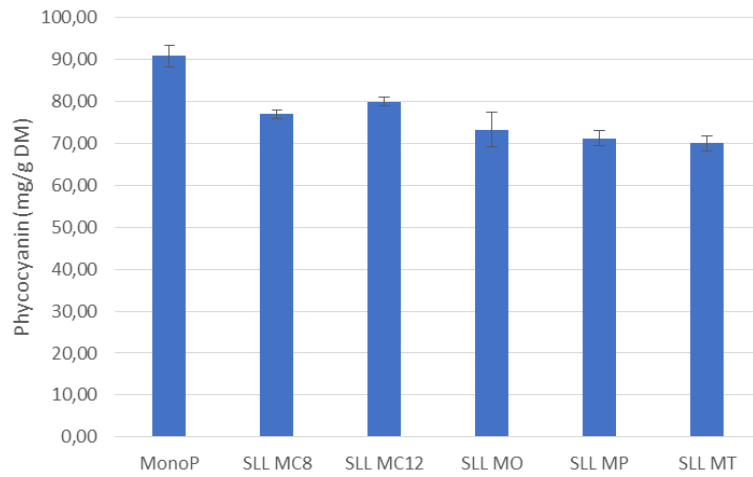
744 extraction

745 Figure 6: Typical color difference between monophasic, LLE and biphasic extracts.

746

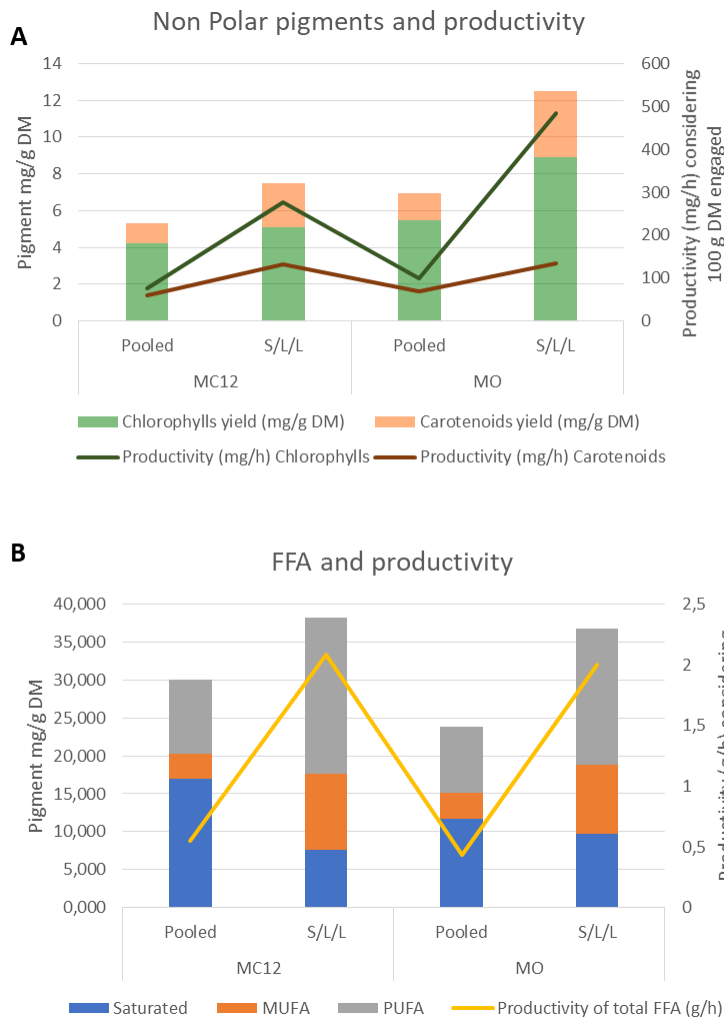
747

748



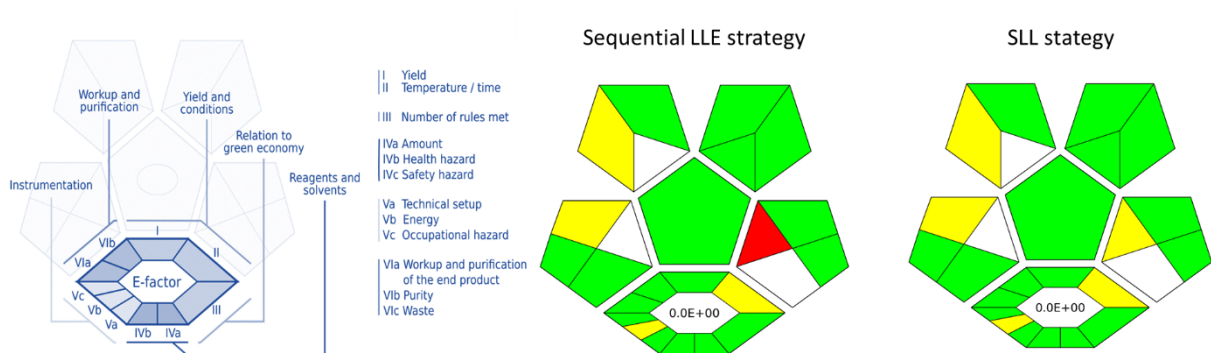
749 Figure 7: Phycocyanin content of the polar phase GG after SLL extraction

750 Figure 8: Pigment profile (A) and FFA profile (B) for biphasic extraction



751

752 Figure 9: Comparison of the sequential/LLE and SLL approach in terms of productivity



753

754 Figure 10: Sustainability analyses of sequential LLE strategy vs SLL approach using using ComplexGAPI
755 tool ⁴¹ Pictograms were drawn by a freeware software available at:
756 <https://mostwiedzy.pl/en/justyna-plotka-wasylnka,647762-1/complexgapi> (accessed on 06/30/2023)
757 (red fields indicate steps that may be hazardous to the environment, yellow fields indicate steps with
758 medium environmental impact, and green fields reflect eco-friendly steps).

759