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Chapter 4: Microalgal biomass of industrial interest: methods of characterization

Authors: Hugh D. Burrows¹, Maria G. Campos¹, Cédric Delattre², Catherine Dupré³, Telma Encarnação¹, Marilyne Fauchon⁴, Clément Gaignard², Claire Hellio⁴, Junko Ito⁵, Céline Laroche², Jack Legrand³, Philippe Michaud², Alberto A.C.C. Pais¹, Guillaume Pierre², Benoît Serive⁶, Makoto M. Watanabe⁵

⁽¹⁾ CQC, Department of Chemistry, University of Coimbra, Coimbra, Portugal.

⁽²⁾ Université Clermont Auvergne, CNRS, Institut Pascal, Polytech Clermont- Ferrand, 2 avenue Blaise Pascal, 63178 Aubière cedex, France.

⁽³⁾ GEPEA UMR 6144 CNRS, 37, Bd de l'Université BP 406, 44602 Saint-Nazaire cedex, France.

⁽⁴⁾ Biodimar, LEMAR UMR 6539, Institut Européen de la Mer, Université de Bretagne Occidentale, 29200 Brest, France.

⁽⁵⁾ Algae Biomass and Energy System R&D Center, University of Tsukuba, Japan.

⁽⁶⁾ USR 3151, CNRS Sorbonne Université, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff cedex, France.

Outline

4.1 Introduction (Jack Legrand)

4.2 Methods for biomass global characterization (Catherine Dupré)

4.3 Methods for protein determination in microalgae (Marilyne Fauchon, Claire Hellio)

4.4 Methods for polysaccharides determination in microalgae (Guillaume Pierre, Clément Gaignard, Cédric Delattre, Céline Laroche, Philippe Michaud)

4.5 Methods for lipids determination in microalgae (Junko Ito, Makoto M. Watanabe)

4.6 Methods for pigments determination in microalgae (Benoît Serive)

4.7 Methods for secondary metabolites determination in microalgae (Telma Encarnação, Alberto A.C.C. Pais, Maria G. Campos, Hugh D. Burrows)

4.8 References

48 **4.1 Introduction**

49 [Jack Legrand]

50

51 Microalgae represent a unique source of biomass and biodiversity in the living
52 world. There are several hundred thousand species colonizing almost the entire
53 globe. They represent the first link in the trophic chain, the main supplier of
54 oxygen and the first consumer of CO₂, some of whose storage is in the form of
55 calcareous sediments. The advantage of microalgae over higher plants is their
56 rapid growth. Their continuous cultivation makes it possible to direct their
57 metabolism, thus opening the way to a renewable chemistry which does not
58 encroach on the grounds reserved for agriculture and the forest.

59 Photosynthetic microorganisms include all unicellular organisms, both eukaryotic
60 and prokaryotic able to convert light radiant energy into chemical energy for the
61 synthesis of their cellular constituents. Some are defined photoautotrophic, as
62 they use inorganic carbon for growth. Depending on the level of complexity of
63 their intracellular structure, these organisms are either classified as
64 cyanobacteria (prokaryotes) or microalgae (eukaryotes). Microalgae show huge
65 diversity in terms of intracellular structure, morphology and life styles which can
66 lead to a wide range of biotechnological applications. Most studied groups of
67 microalgae include green algae (Chlorophyta and Charophyta), red algae
68 (Rhodophyta), diatoms (Bacillariophyceae), (Haptophyta) and Euglenozoa.

69 With only a few dozen species of cultivated microalgae, annual world production
70 is in the order of a few tens of thousands of tons. The last decades have shown
71 the presence of a great diversity of molecules potentially recoverable from the
72 biomass of microalgae and cyanobacteria. The algosourced molecules currently
73 used are essentially water-soluble (phycobiliproteins) or liposoluble (carotenoids)
74 pigments or lipids such as terpenes and terpenoids, lipids enriched with
75 polyunsaturated fatty acids from the omega-3 series, eicosapentaenoic acids
76 (EPA) and docosahexaenoic acids (DHA), which play an important role in
77 preventing cerebral and cardiovascular diseases. Other niche markets are
78 associated with cosmetic or nutraceutical applications, directly using biomass or
79 crude extracts. Microalgae valorisation must examine all cellular constituents
80 according to the biorefinery principle. The objective of biorefinery or algal
81 refining is to develop a process for exploiting microalgae, based on a series of
82 dedicated unit operations allowing the selective extraction of metabolites of
83 interest by integrating the recycling of waste and by-products, paving the way
84 for exploitation on an industrial scale of microalgae.

85 The wealth of microalgae is not limited to molecules with high added value, and
86 one of the major challenges of microalgal research is to enable developments on
87 a very large scale and at competitive costs. Microalgae can provide molecules of
88 varied interest in the field of Chemistry:

- 89 - lipid compounds, including triacylglycerols or hydrocarbons for use as
90 biofuels and various organic compounds (aromatics, esters, etc.). For
91 example, isomerization/methoxycarbonylation of unsaturated fatty acids
92 leads to diesters and then polycondensation to polyesters,
- 93 - polysaccharides (gelling agents, thickeners),
- 94 - proteins (for human food or animal feed).

95 The aim of the present chapter is to give a critical review of the analytical
96 methods used for the different classes of molecules of the algal biomass: global
97 biomass, proteins, polysaccharides, lipids, pigments and secondary metabolites.

98

99 **4.2 Methods for biomass global characterization**

100 [Catherine Dupré]

103 **4.2.1 Introduction**

104
105 Any analysis of biochemical composition as well as of process efficiency in
106 regards to a valorization of microalgae biomass should be based on accurate and
107 reproducible methods for determining its proximate composition. Indeed the
108 cellular components are usually expressed as a percentage on a weight basis or
109 as pg per cell. These data are used for mass balance calculations of the
110 bioprocess to understand the relations between the biochemical species produced
111 and the substrates consumed. Then proximate analysis of microalga biomass
112 includes the determination of the dry biomass, ash content but also elemental
113 composition. Some of characteristics could be taken into account in assessing the
114 effectiveness of some biorefinery process. Relatively recent documents detail
115 Standard Operating Procedure in order to minimize potential errors related to
116 such routine analysis [1,2].

117 According to the way the microalgae are produced, two preliminary studies
118 should be necessary. A quality control (QC) could be necessary in case of non-
119 application of good manufacturing practices and then that those cultures could
120 be suspected to be contaminated [3]. The method of microalga identification by
121 microscopical observations is based on the morphology [4]. Some database such
122 as AlgaeBase [5] or some microalgae identification guides are available [6].
123 However, this cytological approach, as it can be misleading, especially in the
124 case of species with few morphological features (e.g. coccoid cells), has been
125 associated with biochemical traits, mainly the carotenoid and/or the fatty acid
126 profiles [7-8]. Some authors have proposed the FTIR spectra of whole cells for
127 identification of the microalgae species [9]. This analysis requires few cells,
128 which have to be washed, as some components of the culture medium such as
129 nitrate could interfere with the infrared spectra. However, it was shown that
130 these spectra are changing as a function of metabolic activities [10]. The
131 application of FTIR analysis as QC is only suitable to continuous cultures at
132 steady state, to detect any deviation of the cell suspension. For industrial
133 production, DNA barcoding could be used for species identification, based on DNA
134 sequence similarity against a sequence database of a defined species [11]. The
135 Consortium for the Barcode of Life (CBOL) has recommended for microalgae DNA
136 barcodes the use of a two-step approach with the use of a universal pre-barcode
137 marker followed by the use of a more specific second marker [12]. DNA-based
138 identification should be particularly useful for identifying species with no or few
139 structural characters [13] DNA metabarcoding could be used to trace microalgae
140 of industrial interest, as already done for some plants of industrial interest [14].

141 The other preliminary study deals with the sample collection methods, which
142 should be adapted to the behavior of the microalga culture. By contrast with cell
143 suspensions cultivated in well-stirred photobioreactors, some species grow as
144 colonies, such as *Botryococcus braunii*, as cell aggregates, as a result of natural
145 palmelloid stage or flocculation. The sampling protocol has to take into account
146 the increase in the settling rate of such heterogeneous suspension to limit the
147 variability of the results. In some processes, microalgae are cultivated as biofilm
148 [15-16]. The sampling is done by scrapping biomass from a known area of the

149 support, which size depends on the regularity of the biofilm thickness on the
150 support [17-18].

151

152 **4.2.2 Dry weight method (DW)**

153

154 *5.2.2.1. Centrifugation or filtration*

155 The gravimetric measurement of DW is the reference method used for each
156 species to calibrate the other indirect methods. The Standard Operating
157 Procedures (SOPs) corresponding to this method are well described elsewhere
158 [2]. Accurate and precise values of microalgal dry weight are obtained thanks to
159 the use of appropriate protocols [19]. A precise volume (v) of the culture
160 (suspension cultures) or of the buffer with the homogenized biofilm sample has
161 to be filtered or centrifuged. The filtration method on glass filters, usually GF/C
162 or GF/F, is faster than the centrifuge method. It has to be used for buoyant
163 microalgae, either oleaginous or gas-vacuolated species, a part of the biomass
164 being lost in the supernatants. These filters retain particles down to 0.7 μm , just
165 enough for collecting cells of *Ostreococcus tauri* (0.8 μm). For picoplankton cells
166 (0.2–2 μm) and some cyanobacteria (size down to 0.5 μm) the use of membrane
167 filters with a 0.2 or 0.1 μm pore size is necessary. The filters inside an aluminum
168 cup and the centrifuge tubes should be pre-dried at 103-105 °C for 1 h followed
169 by incubation for 15 to 30 minutes in a desiccator to prevent moisture
170 absorption. Then the aluminum cup + the filter or the centrifuge tube are pre-
171 weighted (mass m_1).

172

173 *4.2.2.2 Washing the biomass*

174 Biomass of marine species as well as from (photo)heterotrophic strains grown in
175 hypertonic culture medium as the result of high salt and/or organic substrates
176 concentrations have to be washed before drying [20]. The aim is to eliminate the
177 components of the culture medium to avoid overestimation of the biomass
178 concentration. However, rinsing with distilled water could induce some cell lysis,
179 as the result of the hypotonic shock, and then an underestimation of the DW. The
180 biomass should be rinsed with an isotonic solution. Fast, accurate and precise
181 values of the actual osmotic strength of the culture medium are obtained by
182 using freezing-point micro-osmometers; it needs very small samples (<100 μL).
183 Isotonic solutions of either ammonium formate or ammonium bicarbonate are
184 generally used. Both salts decompose to volatile compounds during the drying,
185 the first one at 105 °C and the second at 60 °C. If the biomass is presumed to
186 contain volatile components, ammonium bicarbonate should be used. Moreover
187 the dewatering could be done in a vacuum oven at 40 °C. When the pH of the
188 cell cultures is higher than pH 9, some protocols recommend a washing with
189 acidified solutions (5 to 10 mM HCl) to remove salts, mainly carbonates,
190 adsorbed on the cell walls. It works with distilled water for freshwater species but
191 also with ammonium formate solutions (adjusted to pH 5.5). However, such pH
192 adjustments may not be suitable for all the microalgae; the coccolithes calcified
193 microalgae could be affected by pH decrease, with an impact on the microalga
194 dry weight but not on the ash-free dry weight (AFDW).

195

196 *4.2.2.3 Biomass dewatering*

197 The filters removed from the holder of the vacuum filter system and back in the
198 aluminum cup or the centrifuge tubes are placed in an air oven at 104 °C
199 (ammonium formate) or 60 °C (ammonium bicarbonate). An alternative is the
200 use of a vacuum oven, for an efficient temperature of 40 °C to prevent volatile

201 organic compounds (VOCs) to escape, but also for a shorter period to reach
202 constant weight, indicative of the end of the drying kinetic. Fast drying is
203 obtained with halogen moisture balances, provided that, for reliable DW
204 measurements, the drying temperature is set to 105 °C. Indeed, too high
205 surface temperatures (> 400 °C) should be avoided [21].

206 The dried filters or tubes are quickly transferred in a desiccator for 30 minutes
207 minimum before the last weighing m_2 . The biomass concentration (g L^{-1}) is
208 deduced from the ratio of the dry biomass to the volume of the sample $(m_2 -$
209 $m_1)/v$. The biomass concentration X in attached cultures is expressed as biofilm
210 areal density (X , g m^{-2}).

211

212 **4.2.3 Ash and ash-free dry weight (AFDW) method**

213

214 The dried biomass contains both minerals and organic compounds. In diatoms,
215 ash contents can reach up to 59% of the DW, about two to five times the values
216 measured in non-silicified or non-calcified microalgae [22]. For accurate mass
217 balances of biological reaction within microalgae cultures, this mineral fraction
218 should be taken into account. Then ash free dry weight (AFDW) calculated as the
219 difference of the weights obtained after drying at 105 °C and after ashing at 575
220 °C should be preferred over total dry weight measurement when the presence of
221 mineral ash would induce a significant error in the estimation of organic content
222 dry weight. AFDW is an estimation of the organic weight. Data are expressed as
223 mg/liter AFDW and ash content as a % of total dry weight.

224

225 *4.2.3.1 Classical gravimetric method*

226 The classical gravimetric method corresponds to the determination of the
227 remaining ash obtained after further combustion of the dry biomass in a furnace
228 at high temperatures (> 550 °C). The difference gives the ash free dry weight
229 (AFDW), corresponding to the global organic fraction of the microalga biomass. It
230 is also named as the volatile suspended solids (VSS) fraction. The values of
231 biochemical fractions (lipids, proteins, sugars...) are higher when calculated on an
232 AFDW basis. Moreover the AFDW allows avoiding the biomass rinsing problems.
233 The samples are placed within porcelain, silica or platinum crucibles to prevent
234 loss; they are pre-weighed. Then, they are placed in a combustion furnace to
235 burn up to 700 °C with a recommended value of 575 °C and for a minimum of
236 four hours. The duration corresponds to the time needed to reach a constant
237 weight, defined as that obtained when mass variation is less than 0.3 mg after
238 one hour of re-heating the crucible. This time is a function of the initial mass and
239 the structure of the biomass to be treated. After its total oxidation in the muffle
240 furnace, the samples within their crucibles are placed in a desiccator to cool to
241 ambient temperature (1 hour recommended) before the final weighing.

242 The ash content and composition of the biomass is important in furnace
243 operations during combustion processes, but also during bio-refinery processes
244 [23]. When necessary, the ash composition (Si, Ca, Mg, K, Na, P...) could be
245 easily determined using inductively coupled plasma mass spectroscopy (ICP-MS)
246 or X-ray fluorescence (XRF).

247

248 *4.2.3.2 Thermogravimetric analysis*

249 Thermogravimetric analysis is a technique in which the mass of a substance is
250 measured as a function of temperature or time as the sample is subjected to a
251 controlled temperature program in a controlled atmosphere within a furnace. The
252 interest of thermogravimetric (TG) techniques has been proven for the study of

253 thermal characteristics of microalgae biomass during combustion and pyrolysis
254 [24]. Indeed the combustion behavior of algae is still far to be well understood
255 [25].

256 Microalgal ash content could be determined using an usual protocol that includes
257 a temperature gradient with a heating rate of 20 °C/min from ambient
258 temperature to 600 °C followed by an isothermal step a temperature of 600 °C
259 for 30 min under an air atmosphere for TG analysis. Indeed, at higher
260 temperatures, the ash content could be reduced by the loss of some volatile
261 minerals. The analysis of the peaks obtained by derivative thermogravimetric
262 (DTG) graph has been used for qualitative or semi-quantitative analysis of the
263 lipid, carbohydrate and protein fractions in microalgae by fitting peaks for
264 carbohydrates, protein and lipids [26]. The ash content can be quickly (about
265 one hour) and easily performed using only 5–10 mg of sample.

266

267 **4.2.4 Cell counts methods**

268

269 Different methods are used for measuring the cell populations, including
270 microscopic numeration with hemocytometers, impedance measurements or flow
271 cytometer.

272

272 *4.2.4.1 Hemocytometry*

273 The cell count method by hemocytometry is commonly used as it is easy to
274 implement and not expensive, although time consuming [27]. Some errors could
275 be introduced by improper use of the hemocytometer. However, this microscopy
276 analysis can help to detect any morphological changes or microbial
277 contamination in the cultures in contrast to gravimetric or optical methods. The
278 processing time is accelerated when the method associates an image processing
279 program (ImageJ with the plugin cell counter.jar). For later analysis, the
280 microalgae samples could be fixed with glutaraldehyde, lugol or formaldehyde
281 and stored at 4/6 °C [28]. The relations between dry weight and cell population
282 data depend not only of the species, but also of the eco-physiological conditions
283 [29].

284 The measured counts are total cells since it is impossible to differentiate between
285 live and dead cells.

286 Taking into account the autofluorescence of chlorophylls or phycobiliproteins,
287 epifluorescence microscopy is advantageously used for numeration of
288 photosynthetic microalgae. In complex culture media such property is used to
289 distinguish microalgae from inert particles. Moreover, some protocols include
290 fluorescence staining, such as Sytox green, for rapid dual fluorescence assays to
291 distinguish dead from live microalgae [30]. At least 300 cells in random fields
292 have to be counted in numeration and viability calculations for each assay.

293

294 *4.2.4.2 Flow cytometry*

295 The flow cytometric method (FCM) is considered as an efficient, although
296 expensive, substitute for the time-consuming direct methods (e.g. microscopic or
297 gravimetric methods). FCM represents an automated system, allowing precise
298 and fast determination of unicellular microalgae number in a culture [28-31].
299 Their auto-fluorescent properties, due to the presence of chlorophylls, allows
300 discrimination from other non-photosynthetic microorganisms and non-living
301 matter (excitation with 488 nm using the argon laser). Fresh unfixed samples are
302 preferably used to preserve their fluorescent properties. Some specific protocols
303 have to be used for colonial microalgae or filamentous cyanobacteria, aiming to
304 disrupt the filaments without cell lysis, such as through sonication with Triton-

305 X100 treatment [32]. In addition, FCM offers the possibility to distinguish the
306 smallest microalgae (picoplankton < 2 µm) from bacteria which is relatively
307 difficult by optical microscopy.

308 Imaging flow cytometry is a hybrid technology, integrating the capabilities of
309 flow cytometry with imaging features of microscopy. The combination of
310 fluorescence microscopy with flow cytometry (1000 cells s⁻¹) results in both
311 quantitative and qualitative data. Thanks to on-line image processing by
312 visualization software, each enumerated particle is linked to an image, with
313 access to size distribution, detection of any morphological changes, viability or
314 metabolic activity [33].

315

316 4.2.4.3 Image-based cytometry

317 Solid-phase cytometry (SPC) is an image-based technique for quick and accurate
318 enumeration of microorganisms such as microalgae with similar precision to flow
319 cytometry [34-35]. Microalgae are collected on a membrane filtration. The
320 scanning involves using either chlorophyll auto-fluorescence or labelling by a
321 fluorescent dye. The entire membrane filter surface is scanned and the
322 fluorescent light emitted by labeled cells is automatically detected and counted.
323 The software allow differentiating the target signal from electronic noise or non-
324 target particles [36]. The technique allows an accurate enumeration down to a
325 detection limit of one cell per filter.

326 Other automated cell counters are adapted to disposable cell counting chambers
327 (20 µL sample size) or even to standard hemocytometers. They also provide cell
328 sample analysis results, including cell count, concentration, diameter, images,
329 and viability, in less than 30 seconds.

330 After scanning, cells can be examined by epifluorescence microscopy. The dry
331 weight concentration could be estimated on the basis the single cell density and
332 the cell size distribution.

333

334 4.2.5 Elemental analysis

335

336 The mass balance of a microalga culture is now recognized as a valuable tool not
337 only for overall stoichiometry equations used in process control, e.g.
338 identification of nutrient limitations, but also for analytical data validation, e.g.
339 detection of measurement errors and/or unnoticed products [37]. For instance,
340 the determination of the main elemental components of a microalgal biomass,
341 carbon, nitrogen, hydrogen, oxygen, phosphorus and sulfur, could highlight the
342 presence of an S-enriched fraction, such as some sulphated polysaccharides.
343 Different instruments have been developed for measuring the elemental
344 composition. Most of them employ catalytic combustion with pure oxygen to
345 decompose the sample to nitrogen, water, carbon dioxide and sulphur dioxide,
346 which are then quantitatively determined by chromatography with flame
347 ionization or thermal conductivity detectors. Oxygen is determined by catalytic
348 conversion to carbon monoxide.

349 One advantage of elemental analysis is the relatively small sample weights
350 required. The main constraint is that samples should be dry and free of foreign
351 substances. Indeed, total mass of every main element (C, N, O, H, P and S) is
352 determined and reported to an exact global dry weight. The good practices
353 previously described for sample preparation for the dry weight determination
354 have to be scrupulously applied. Other elements which occur in smaller, but
355 significant quantities are sodium, potassium, calcium, magnesium and silicon.

356 Some of them have to be considered for characterizing biomass from some bio-
357 mineralized microalgae, such as diatoms or calcareous species [38].
358 The large biodiversity and chemodiversity of microalgae is of great interest in
359 tapping in never exploited natural molecules, thanks to recent and ongoing
360 screening studies. However, such diversity implies that the standard analytical
361 protocols for biomass characterization have to be critically examined for potential
362 interference.

363 **4.3 Methods for protein determination in microalgae**

364 [Marilyne Fauchon, Claire Hellio]

365

366

367 **4.3.1 Introduction**

368 Microbial biotechnology focuses more and more on the potential of microalgae as
369 a source of biomass, biofuels, carbohydrates, lipids, fishmeal replacement and
370 food, and, high value molecules [39,41]. The chemical compounds synthesized
371 by microalgae have several applications. The high-protein content of some algae
372 species is one of the main reasons to consider them as a non-conventional
373 source of proteins [39]. For example, *Chlorella* was amongst the first
374 microorganism species to be commercialized as a health food in Japan, Taiwan
375 and Mexico [42,43]. Microalgal protein has high potential for animal feed and
376 human consumption, and recombinant protein technology. The use of marine
377 strains could avoid conflicts with agriculture for freshwater supplies ; thus strains
378 such as *Nannochloropsis* are used both in fish-farm aqua-feed and large scale
379 biofuel production [44,45]. Moreover high-value products from microalgae are
380 usually produced within a biorefinery model, since the composition of the
381 microalgal cell allows for extraction of different co-products, and thus proteins
382 are valuable by-products of biofuel production [46]. Commercialization prices are
383 still high due to the process expenses associated to the extraction and
384 purification of intracellular metabolites. In microalgal biotechnological processes,
385 the downstream stage can account for 50–80 % of total production costs,
386 depending on the biochemical characteristics of the compound and the purity
387 ratio that needs to be achieved [47].

388 Proteins are large and complex naturally-produced molecules composed of one or
389 more long chains of amino acids, in which the amino acid groups are held
390 together by peptide bonds. They make up a large fraction of the biomass of
391 actively growing microalgae [48]. Depending on the microalgae strains, protein
392 extraction may be more or less easy. The composition of the cells walls has a
393 significant impact on the protein extraction [40].

394 Most microalgae exhibit a high protein content (**Table 4.3.1**) with the highest
395 content being found in *Chlorella pyrenoidosa* and *Chlorella vulgaris*
396 (Trebouxiophyceae) where protein content represents more than 50 % of dry
397 weight. It is important to note that protein content amongst the strains of a
398 species can vary significantly ; for example, for *Chlorella* species, there are
399 significant differences within protein content, from only 6.87 % in *Chlorella*
400 *spaeckii* and 10.97 % in *Chlorella ovalis* to 57 % in *C. pyrenoidosa*.

401

402 **Table 4.3.1:** Protein content (% dry weight basis), adapted from [49].

Alga	Class	Protein content (%)
<i>Chlorella pyrenoidosa</i>	Trebouxiophyceae	57
<i>Chlorella vulgaris</i>		53.3
<i>Chlorella ellipsoidea</i>		42.2

<i>Chlorella ovalis</i>		10.97
<i>Chlorella spaerckii</i>		6.87
<i>Dunaliella salina</i>	Chlorophyceae	57
<i>Dunaliella primolecta</i>		12.26
<i>Dunaliella tertiolecta</i>		11.4
<i>Scenedesmus obliquus</i>		48
<i>Scenedesmus almeriensis</i>		41.8
<i>Tetraselmis chui</i>	Chlorodendrophyceae	46.5
<i>Porphyridium cruentum</i>	Porphyridiophyceae	35
<i>Porphyridium aerugineum</i>		31.6

403

404

405 **4.3.1.2 Applications of algal protein**

406 **4.3.1.2.1 Human nutrition**

407 The nutritional quality of a protein is determined by the content, proportion and
 408 availability of its amino acids [50]. Four indices can be calculated to characterize
 409 the nutritional value of microalgae (**Table 4.3.2**):

- 410 • PER: Protein efficiency ratio, expressed in terms of weight gain per unit of
 411 protein consumed by the test animal in short-term feeding trials.
- 412 • BV: Biological value, a measure of nitrogen retained for growth and
 413 maintenance.
- 414 • DC: Digestibility coefficient
- 415 • NPU: Net protein utilisation (BV x DC), a measure of both the digestibility
 416 of the protein and the biological value of the amino acids absorbed from
 417 the food

418 Cell walls of microalgae consist of a polysaccharide and glycoprotein matrix
 419 providing the cells with a formidable defense against its environment. It
 420 represents about 10 % of the algal dry matter and as it is non-digestible for
 421 humans and non-ruminants animals, a post-harvesting treatment of the
 422 microalgal cells is necessary to make the proteins accessible for digestive
 423 enzyme and can affect the various parameters (BV, DC, NPU and PER) (**Table**
 424 **4.3.2**).

425

426

427

428

429

430

431 **Table 4.3.2:** Comparative data on biological value (BV), digestibility coefficient
 432 (DC), net protein utilisation (NPU) and protein efficiency ratio (PER) of selected
 433 processed microalgae (adapted from [51,52].
 434

Source	Processing	BV	DC	NPU	PER
<i>Scenedesmus obliquus</i>	Drum dried	75	88	67.3	1.99
	Sun dried	72.1	72.5	52.0	1.14
<i>Chlorella sp.</i>	Air dried	52.9	59.4	31.4	0.84
	Drum dried	76.6	89.0	68.0	2.10

435
 436 One of the quality criteria for a protein is its amino acid composition (specifically
 437 the essential amino acids which are important for human feed application). Some
 438 microalgal protein can compare with proteins derived from conventional sources
 439 such as egg and soybean (**Table 4.3.3**) and thus can be used as substitute for
 440 eggs or animal proteins.

441
 442
 443 **Table 4.3.3:** Essential Amino acid profile of different microalgae as compared
 444 with conventional protein sources (g 100 g⁻¹ protein) (adapted from [49]).

Source	Ile	Leu	Val	Lys	Met	Cys	Thr	His
Egg	6.6	8.8	7.2	5.3	3.2	2.3	5.0	2.4
Soybean	5.3	7.7	5.3	6.4	1.4	1.9	4.0	2.6
<i>Chlorella sp.</i>	4.4	9.2	6.1	8.9	2.2	0.4	4.7	2.4
<i>Dunaliella sp.</i>	4.5	9.3	6.0	6.2	2.5	4.0	5.0	2.5
<i>Scenedesmus sp.</i>	4.7	9.4	6.0	6.8	2.4	0.1	4.9	2.6

445
 446 Microalgae are usually consumed as a dietary supplement in the form of powder,
 447 pills or tablet [52]. Some microalgae can be also incorporated in the composition
 448 of industrial food, such as noodles, bread, biscuits, drinks, sweets and beer [53].
 449 *Chlorella* is one of the most consumed microalgae.

450 One of the quality criteria for a protein is its amino acid composition (specifically
 451 the essential amino acids which are important for human feed application). Some
 452 microalgal protein can compare with proteins derived from conventional sources
 453 such as egg and soybean (**Table 4.3.3**) and thus can be used as substitute for
 454 eggs or animal proteins.

455
 456
 457 **4.3.1.2.2 Industrial application**

458 **4.3.1.2.2.1 High value metabolites: phycobiliproteins**

459 Among functional ingredients from microalgae, natural pigments exhibit
 460 beneficial biological activities such as antioxidant, anti-carcinogenic, anti-
 461 inflammatory, anti-obesity and neuroprotective agents [39,55]. Phycobiliproteins
 462 are brilliant-coloured and water-soluble antennae-protein pigments which
 463 originate mainly from covalently bound prosthetic groups that are open-chain
 464 tetrapyrrole chromophores bearing A, B, C and D rings named phycobilins [56].
 465 Phycobiliproteins absorb energy in portions of the visible spectrum (450–650 nm)
 466 and function as accessory pigments for photosynthetic light collection [57] In
 467 microalgae, three main classes of phycobiliproteins are produced:
 468 allophycocyanin (APC, bluish green), phycocyanin (PC, blue) and phycoerythrin

469 (PE, purple) [56]. Due to its physical properties, Phycoerythrin can be used in
 470 clinical research and molecular biology [39]. Phycoerythrin can be used as label
 471 for biological molecules, as a reagent in fluorescence immunoassays, flow
 472 cytometry, fluorescence microscopy and diagnostics [58].

473
 474 Phycocyanin is found as a complex mix of monomers, trimers, hexamers and
 475 various oligomers, and thus its molecular weight ranges from 44 to 260 kDa
 476 [59]. Phycocyanin is a colorant commonly used in food and cosmetics. However
 477 its use is limited because phycocyanin is sensitive to heat treatment, which
 478 results in precipitation and fading of the blue colour. Phycocyanin can as well be
 479 used as a probe for immunodiagnostics thanks to its fluorescence properties
 480 [60]; it is an efficient scavenger of oxygen free radicals and therefore its
 481 therapeutic use is promising since many diseases are related to an excessive
 482 formation of reactive oxygen species (ROS) [61].

483
 484 **4.3.1.2.2 Functional food-ingredients**

485 Protein quality is determined by the essential amino acid content and
 486 bioavailability, which is defined as the proportion of a nutrient that can be
 487 absorbed and used. Indeed, during digestion, some proteins are not completely
 488 decomposed into free amino acids, but rather into peptides that can be absorbed
 489 and thus influence physiological processes. Thus, microalgae have been identified
 490 as valuable and sustainable sources of protein for the industrial production of
 491 peptide-based functional foods to prevent or treat cardiovascular disease
 492 [62,63]. Peptides that have antihypertensive (by inhibiting angiotensin
 493 converting enzyme (ACE), anti-obesity (by stimulating the hormones that
 494 regulate satiety) and antioxidant properties have been successfully produced
 495 from microalgae (**Table 4.3.4**) [62]. Such peptides are key for pharmaceutical
 496 industry as worldwide mortality from cardiovascular disease was estimated at 37
 497 % in [64]; 50 % of deaths from stroke and coronary artery disease are caused
 498 by hypertension. Moreover, oxidative stress has been identified as one of the key
 499 factors in the development of hypertension [65] because high levels of ROS can
 500 cause the oxidation of biological macromolecules, ultimately leading to
 501 pathological conditions. However, even if peptides give promising results in lab-
 502 assays, in most cases, the link with a beneficial health outcome in humans is
 503 currently theoretical [66].

504
 505
 506

507 **Table 4.3.4:** Examples of bioactive peptides from microalgae (adapted from
 508 [62]).

509

Source of peptide	Type of Treatment	Amino acid sequence	Activity
<i>Navicula incerta</i>	Hydrolysis with papain	Pro-Gly-Trp-Asn-Gln-Trp-Phe-Leu-Val-Glu-Val-Leu-Pro-Pro-Ala-Glu-Leu	Antioxidant
<i>Chlorella vulgaris</i>	Hydrolysis with pepsin	Val-Glu-Cys-Tyr-Gly-Pro-Asn-Arg-Pro-Glu-Phe	Antioxidant
<i>Chlorella vulgaris</i>		Phe-Ala-Leu	ACE inhibition

<i>Chlorella ellipsoidea</i>		Leu-Asn-Gly-Asp-Val-Trp	Antioxidant
<i>Chlorella ellipsoidea</i>	Hydrolysis with alcalase	Val-Glu-Gly-Tyr	ACE inhibition

510
511 *Navicula incerta* and *Chlorella ellipsoidea* are promising sources of protein for the
512 production of bioactive peptides [62]. The anti-hypertensive properties of
513 peptides derived from microalgae have been widely studied and it was
514 demonstrated that the short chain and low molecular weight peptides are
515 significantly more efficient than the larger peptides. This is partly due to their
516 bioavailability and their accessibility to the active sites [62].

517
518 **4.3.1.2.2.3 Animal feed**

519 The increase in the global population combined with the increase in income may
520 double the global demand for animal products by 2050, with a particular impact
521 on the most consumed meats in the world. This increased demand for meat will
522 be particularly dramatic for farmed agriculture, as maize and soybean food crops
523 are the two main conventional feeds. Microalgal biomass can provide natural
524 ingredients and supplements in animal diets to meet the growing demand for
525 protein and energy and replace synthetic additives in foods, as it has been
526 claimed that the average quality of most fractions of microalgae proteins is equal
527 to or even higher than that of conventional vegetable protein fractions [50]. Dry
528 *Chlorella* has maximal protein concentrations of between 50 and 60 % of dry
529 weight, similar in quality to yeast, soy flour and milk protein [67].

530
531 **4.3.1.2.2.3.1 Agriculture**

532 Supplementing poultry feed with microalgae as a protein source can improve
533 their health, productivity, and value. This has been demonstrated using a variety
534 of species, including *Chlorella sp.*, *Arthrospira sp.*, *Porphyridium sp.*, and
535 *Haematococcus sp.* [68-70]. The replacement of up to 33 % of soy proteins with
536 proteins from *Chlorella vulgaris* in pig feed has been reported as being suitable
537 without any adverse effects [71]. The cell wall of microalgae is largely
538 indigestible by monogastric animals. Therefore, it becomes imperative for the
539 feed industry to develop appropriate technologies to improve the bioavailability
540 of microalgae nutrients in animals [72]. Of all the animals evaluated for algae
541 supplementation, ruminants are the most promising in terms of digesting the
542 high fibre content for the greatest extraction efficiency of algal proteins. This is in
543 contrast to monogastric animals, for which it has been suggested that some form
544 of prior processing may be required in order for animals (and humans) to utilise
545 algal proteins more efficiently.

546
547 **4.3.1.2.2.3.2 Aquaculture**

548 Microalgae are the base of the aquatic food chain and thus are essential for the
549 artificial reproduction of many aquaculture species, such as molluscs, shrimps
550 and rotifers [50]. Filtering molluscs are the greatest consumer of microalgae
551 [73]. Due to the rapid global expansion of the aquaculture industry, access to
552 major foods (fishmeal and fish oil) is increasingly difficult due to limited wild fish
553 resources [74]. Fisheries are the most important sources of feedstock for
554 fishmeal. Only a small percentage of global fish production is indeed channeled
555 to human consumption, with the remainder being used for fish and animal feed.
556 Fishmeal is a protein-rich food, and sets the basis for any balanced formulation

557 used in commercial aquaculture. Nevertheless, microalgae can be considered as
558 a promising alternative that can replace fishmeal and fish oil as the chemical
559 composition of some commercially important microalgal species is comparable to
560 the available food ingredients used in the aquaculture feed industry. The
561 chemical composition of some commercially important microalgal species is
562 comparable to the available food ingredients used in the aquaculture feed
563 industry. The nutritional value of microalgae is determined primarily by the
564 protein content and, secondly, by the polyunsaturated fatty acid content
565 (eicosapentaenoic acid (EPA), arachidonic acid (ARA) and docosahexaenoic acid
566 (DHA)). Microalgae are a more reliable and less volatile source of protein, and
567 their availability is not dependent on fish captures. This provides industry with a
568 better control of their costs, and supports a potential for future investment due
569 to the reduction of risk in aquaculture farming operations [75]. Studies
570 highlighted that biomass of *Chlorella* sp., *Scenedesmus* sp., *Nanofrustulum* sp.
571 and *Tetraselmis suecica* can be used as additional protein sources or partial
572 substitutes for fish meal proteins in the diet of various species of omnivorous and
573 carnivorous fish [76].

574

575 **4.3.1.2.2.4 Recombinant proteins**

576 Microalgae show great potential as bioreactors for large-scale production of
577 recombinant proteins. Microalgae combine high growth rates like prokaryotic
578 cells with all advantages of eukaryotic expression systems, i.e. post-
579 transcriptional and post-translational modifications and the assembly of
580 multimeric protein complexes [77]. Monoclonal antibodies are important tools in
581 medical therapy, diagnostics and research and are mainly produced in
582 mammalian cell lines, since the establishment of hybridoma technology in 1975.
583 As cultivation of mammalian cells is expensive, alternative expression systems
584 are studied. A full-length IgG antibody was synthesized in the chloroplast of
585 *Chlamydomonas reinhardtii* demonstrating that antibody expression in an algal
586 system is feasible [78]. Following this study, Hempel et al. [77] published the
587 synthesis of a fully-assembled and functional antibody (CL.4mA) against the
588 Hepatitis B Virus surface protein in *P. tricornutum* and manage to obtain an
589 accumulation of 8.7 % of total soluble protein in two days of induction. When
590 expressed in *Nicotiana tabacum* the same antibody reached much lower
591 expression levels of only 0.2–0.6 % in several weeks [79]. Another frequently
592 reported problem with antibody expression in plants is rapid protein degradation
593 resulting in low antibody levels and many fragmented products [80]. However in
594 *P. tricornutum* no degradation products were detected [77]. Antibody purification
595 was carried out with protein A-Sepharose and ELISA assays demonstrated that
596 the algal-produced antibody is functional and binds the respective target antigen
597 (HBsAg) very efficiently *in vitro* [77]. The Hepatitis B surface protein was
598 functional and recognized by algae-produced and commercial antibodies.

599

600 **4.3.1.2.2.5 By-product of biofuel consumption**

601 Microalgae are considered as one of the most promising biobased feedstocks, as
602 their productivity in converting CO₂ into lipids exceeds that of oilseed crops [81].
603 Applying biorefinery, not only for oil, but as well for protein, may lead to the
604 sustainable and economical microalgae-based fuels. With this approaches, the
605 biomass treatment for protein extraction, alongside other compounds of interests
606 (lipids, carbohydrates), is different to when only proteins are extracted and may
607 affect the yield [82].

608

609 **4.3.2 Protein extraction and quantification**

610 **4.3.2.1 Pre-treatment prior to extraction**

611 Prior to protein extraction, most microalgae have to be pre-treated in order to
612 break or fragilise the cell wall to enhance the protein extraction yields. For this,
613 various treatment are available such as: hydrolytic enzymes or sodium dodecyl
614 sulfate (for chemical treatments), or milling a cell slurry in presence of glass
615 beads or fine ceramic particles can be efficient as well for the hardest cells.
616 Ultrasonication is another effective method for disrupting the cells but has the
617 inconvenient to sometimes damage the proteins. In a study by Gonzalez Lopez et
618 al. [48] several pre-treatments methods, prior to proteins analysis of
619 *Porphyridium cruentum* and *Muriellopsis sp.*, were compared. The following pre-
620 treatments were tested: suspension in lysis buffer, ultrasonication at high power
621 for 10 minutes in lysis buffer, or milling for 5 minutes with a pestle and mortar.
622 They concluded that depending on the species studied, different pre-treatment
623 must be used for optimisation of cell lysis. Moreover, the growth phase has also
624 an impact on the ease of cell disruption, with rapidly growing cells likely to be
625 less robust than slow growing or stationary-phase cells [83], therefore milling
626 with ceramic particles is expected to be critically important to maximise the
627 intracellular proteins released.

628 Extensive studies on cell disruption techniques have been conducted on *Chlorella*
629 *vulgaris* because it has a resistant cell wall, which limits the digestibility and
630 extraction of cellular contents. Multiple techniques for breaking the wall were
631 tested on *C. vulgaris* and are summarised in **Table 4.3.5**. Enzymatic treatment
632 is a promising technique that requires a thorough knowledge of ultrastructure
633 and cell wall composition to select the appropriate enzyme and lysis conditions.
634 According to Lee et al. [84] and Zheng et al. [85]. The best cell disruption
635 techniques of *C. vulgaris* are autoclaving, microwave treatment, enzymatic lysis
636 and liquid nitrogen grinding. The success of cell disruption techniques is generally
637 evaluated by performing microscopic observations or comparing the extraction
638 yield of a compound before and after the cell disruption treatment.

639
640 **Table 4.3.5:** Different cell disruption techniques carried out on *C. vulgaris*
641 (adapted from [86]).
642

Cell disruption	Time	Experimental set-up
Acid treatment	25 min	Hot Ac2O* + H ₂ SO ₄ (9:1, v:v)
Alkaline treatment	60 min	2 N NaOH
Autoclaving	5 min	125 °C + 1.5 MPa
Bead milling	20 min	Beads: 0.4 - 0.6 mm Rotational speed 1500 rpm
	5 min	Beads: 0.1 mm Rotational speed 2800 rpm
	2 min	Beads: 1 mm
Electroporation	not available	Electric field: 3 kV/cm Electrode 2 cm

Enzymatic lysis	60 min	Snailase (5 mg. L ⁻¹), 37 °C
	10 h	Cellulase or Lysozyme (5 mg.L ⁻¹), 55 °C
	not available	4 % Cellulase + 1 % others (w/v) 25 mM Sodium Phosphate buffer pH 7.0 0.5 M Mannitol
	10 h	4 % Cellulase + 1 % Macerozyme R10 + 1 % Pectinase (w/v) pH 6.0 25 mM Phosphate buffer 0.6 M Sorbitol/Mannitol (1:1)
	24 h	Cellulase 0.5 mg.L ⁻¹ 0.5 M Mannitol
French Press	not available	138 MPa
Manual grinding	1-10 min	With liquid nitrogen or quartz
	not available	With dry ice
Microwaves	5 min	100 °C, 2450 MHz
		40-50 °C, 2450 MHz
Osmotic shock	48 h	10 % NaCl
	60 min	2 N NaOH
Ultra-sonication	6 min	10 W
	20 min	600 W
	5 min	10 kHz

643 * acetic anhydride

644

645 **4.3.2.2 Protein extraction**

646 Ion-exchange chromatography, gel filtration chromatography, gel electrophoresis
647 and ultrafiltration have been employed for the fractionation, purification,
648 characterization and structural elucidation of proteins, peptides and amino acids
649 from microalgae [87]. The protein extraction procedure is technically the same
650 for all microalgae and is mainly carried out by solubilization of proteins in alkaline
651 solution (pH 10-12) in the presence of NaOH [88-90]. Further purification can be
652 achieved by precipitating the solubilized proteins with trichloroacetic acid (25 %
653 TCA) or hydrochloric acid (0.1 N HCl) [91,92].

654

655 **4.3.2.3 Protein content determination**

656 For a given algal species, the intracellular concentration of inorganic and organic
657 nitrogen depends on the growth phase [93]. Various methodologies have been
658 developed for proteins quantification and analysis. Main methods are based on
659 colorimetry (Coomassie, Lowry, BCA, Amido Black, Biuret), fluorescence
660 (fluorescamin, Nano-orange, CBQCA, OPA), immunology (Elisa) and spectroscopy
661 (amino acids analysis, UV, mass spectrometry) protocols [94].

662 For microalgae, commonly protein are quantified by elemental analysis, Kjeldahl,
663 Lowry assay, Bradford assay [95] or dye binding method [91]. However, the first
664 two analyses take into consideration total nitrogen present in the microalga,
665 multiplying it by the standard nitrogen to protein conversion factor (NTP) 6.25
666 which may lead to overestimation or underestimation of the true protein quantity
667 because microalgae contains high concentration of non-protein nitrogenous
668 substances such as pigments (chlorophyll or phycoerythrin for example), nucleic
669 acids, free amino acids and inorganic nitrogen (nitrate, nitrite, ammonia) [93]
670 whose presence makes the factor 6.25 unsuitable since it overestimates the real
671 protein content [96]. Several authors recommended to use a value of NTP lower
672 then the standard 6.25 [48,97]. Moreover, a study by Safi et al. [89] highlighted
673 that no universal conversion factor could be recommended due to the fact that
674 multiple factors, such as cell wall rigidity, growth conditions, growth media and
675 environmental uncertainty, can affect the NTP value. Gonzales Lopez et al. [48]
676 determined the NTP using a technique that correlates protein content (Lowry
677 assay) to total nitrogen content (Kjeldahl and elemental analysis). In addition,
678 Servaites et al. [98] quantified proteins of microalgae by staining the protein
679 isolate with Coomassie brilliant blue R-250 (CBB) on a paper and then eluting the
680 remaining stained proteins in 1 % sodium dodecyl sulphate (SDS) followed by
681 measuring the absorbance at 600 nm. On the other hand, the colorimetric
682 method of Lowry [99] was also considered as one of the most accurate methods
683 to quantify proteins, but with time this method showed to only quantify hydro-
684 soluble proteins [89], which represents the major part of proteins. Lowry assay is
685 considered as more acceptable then Bradford assay because the latter does not
686 react with all amino acids present in the extract and thus giving lower protein
687 concentrations [91].

688 General methodologies can be used but it is important to state that some protein
689 may lead to challenges depending on its structure and mode of action. So far, no
690 single method of quantification can be used for the determination of the true
691 protein concentration for all proteins in all kinds of buffers to serve as a "gold
692 standard" [94]. This can be explained by the fact that proteins can have a very
693 large variety of structures and physicochemical properties. Thus, the choice of
694 the right quantification method is crucial. The different assays available for
695 protein quantification have various characteristics that must be considered before
696 choosing the right protocol depending on the type of experimental work planned.
697 Many different methodologies to assay proteins have been developed; the
698 purpose of this chapter is not to review all but describe the most commonly
699 applied and/or appropriate assays.

700

701

701 **4.3.2.3.1 Total nitrogen content**

702 Techniques that can be used to measure protein content rapidly in lyophilized
703 material include Dumas-based combustive methods of elemental analysis and
704 Kjeldahl to measure N-content [40]. In both methods, the total nitrogen in the
705 sample is liberated at high temperature. In the Kjeldahl method, the nitrogen is
706 released into a strong acid and the content is measured after neutralization and
707 titration. In the Dumas method, the nitrogen is liberated in a gaseous form and

708 is determined with a thermal conductivity detector, after removal of carbon
709 dioxide and water aerosols. The Kjeldahl method was chosen as an example of
710 this analytical principle as it is still recognized as the official method for food
711 protein determination by the AOAC International [87].

712 For microalgae, measurements can be done using 200 mg samples of the dry
713 biomass. The biological material is hydrolyzed with 3 mL concentrated sulfuric
714 acid (H₂SO₄) containing one copper catalyst tablet in a heat block (for example,
715 Kjeltex system 2020 digester, Tecator Inc., Herndon, VA, USA) at 420 °C for 2 h.
716 After cooling, H₂O was added to the hydrolysates before neutralization and
717 titration. Following the nitrogen determination, crude protein content is
718 estimated using a conversion factor (see 4.3.2.3).

719

720 **4.3.2.3.2 Colorimetric methods**

721 Colorimetric assays rely on the appearance of a chromophore as a consequence
722 of either the binding of a dye to a protein or the protein being involved in a redox
723 reaction. Such methods are sensitive to interferences and their accuracy depends
724 significantly on the methods used for pre-treating the samples. Indeed, for
725 microbial proteins to be measured accurately, the cells must be pretreated in
726 order to fully release the intracellular proteins [48]. For better results, Barbino
727 and Lourenço [91] suggested to start the protein extraction from 50 mg of
728 freeze-dried material. The most common treatments involved physical or
729 chemical disruption of the cell wall (see 4.3.2.1 pre-treatment).

730 Prior to protein quantification by Bradford, BCA or Lowry methods, it is necessary
731 to proceed to a protein precipitation step. For this a commonly used procedure is
732 the Berges et al. [100] methodology which is based on the use of trichloroacetic
733 acid (TCA) to allow for protein precipitation. The advantage of this method is that
734 the pellet containing the proteins from microalgae can be stored at -20 °C until
735 further analysis. Prior to colorimetric analysis, precipitated proteins are
736 respectively suspended in 0.5 mL 1.0 N NaOH for the Bradford assay and 2.0 mL
737 for the Lowry assay (when starting from 50 mg freeze dried biomass before pre-
738 treatment).

739 A study by Berges et al. [100] stated that even if it remains unclear which
740 spectrophotometric assay is the most accurate (between Lowry, BCA and
741 Coomassie), it appears that Coomassie assay is faster and simpler, and, has the
742 advantage of being less affected by non-protein compounds found in marine
743 phytoplankton. The reactivity of either of the assays to a particular protein will
744 be a function of that protein's composition as well as any other compound which
745 might oxidize the Folin phenol reagent or bind the Coomassie dye. The
746 bicinchoninic acid (BCA) assay, similarly to the Lowry assay, measure the
747 conversion of Cu²⁺ to Cu⁺ under alkaline conditions, and, both assays have
748 similar sensitivity but BCA is stable under alkaline conditions.

749

750 **4.3.2.3.2.1 Lowry assay**

751 The Lowry method has been widely used for protein determination for many
752 decades, due to its simplicity and availability. However, besides aromatic amino
753 acids, a wide range of other compounds react with the Folin-Ciocalteu reagent
754 [101]. The Lowry assay detects protein through a copper-catalysed reduction of
755 Folin phenol reagent [99] under alkaline conditions. The reactions result in a
756 strong blue color with a maximum absorbance at 750 nm. This reaction will
757 detect peptide bonds, but it is also highly sensitive to specific amino acids such
758 as tyrosine and tryptophan, and to a lesser extent on cystine/cysteine and
759 histidine contents. The method is sensitive to about 0.01 mg of protein/mL within

760 a range of 0.01 to 1.0 mg protein/mL. However, some caution should be taken
761 as the Lowry assay is subjected to interference from many substances including
762 buffers, detergents, EDTA, nucleic acids and sugars [48].

763

764 *Example of experimental protocol:*

765 Step 1 : Pre-treatment of microalgal samples : for example, 20 mg of aliquots of
766 microalgal freeze-dried biomass suspended for 20 min in 10 mL of lysis buffer

767 Step 2: An aliquot of this suspension is diluted with the lysis buffer such that the
768 protein concentration is within the range of 0 and 1000 mg L⁻¹.

769 Step 3: 0.1 mL SDS and 1 mL of reagent C is added to 0.1 mL of the above
770 solution, the solution is then vortexed.

771 Step 4: 10 min later 0.1 mL of Folin reagent is added and well mixed. The
772 sample is then kept in the dark to avoid the degradation of the Folin reagent.

773 Step 5: after 30 min the absorbance of the sample is measured at 750 nm (a
774 blank is prepared is prepared without the algal extract).

775 Step 6: The spectrophotometric absorbance is converted to protein concentration
776 using a calibration curve established with bovine serum albumin (BSA) dissolved
777 in lysis buffer. The protein content of the biomass is calculated using the
778 formula:

$$779 \text{ Protein (\% ; w=w) } = (CVD/m) \times 100$$

780 with C the protein concentration (mg L⁻¹) obtained from the calibration curve, V
781 the volume (L) of the lysis buffer used to resuspend the biomass, D is the
782 dilution factor and m is the amount of biomass (mg).

783

784

785

786 **4.3.2.3.2.2 Coomassie (Bradford) Bradford assay**

787 The Bradford [95] protein assay is used to measure the concentration of total
788 protein in a sample. The principle of this assay is that the binding of protein
789 molecules to Coomassie dye (G-250) under acidic conditions results in a color
790 change from brown to blue which has an absorbance maximum at 595 nm. The
791 quantity of proteins can thus be estimated by determining the amount of dye in
792 the blue ionic form by measuring the absorbance of the solution at 595 nm. The
793 method is sensitive within a range 0.01–1.0 mg protein mL⁻¹. Coomassie Brilliant
794 Blue dye is bound by protein, primarily by arginine residues, but also to a lesser
795 degree by histidine, lysine, tyrosine, tryptophan and phenylalanine [102]. The
796 binding of the dye is very rapid and the protein-dye complex remains soluble and
797 stable for 1 h. The presence of SDS even at low concentrations can interfere with
798 protein-dye binding, moreover this assay is not suitable for quantifying free
799 amino acids or peptides smaller than 3,000 Da as the dye will not bind to them
800 [102].

801

802 *Example of experimental protocol:*

803 The Quick Start™ Bradford protein assay is a simple and accurate procedure for
804 determining the concentration of protein in solution. The assay supplies ready-
805 to-use dye reagent at 1x concentration and two protein standards at seven
806 prediluted concentrations (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg mL⁻¹).
807 Protein concentration is determined in one step, with no need to dilute
808 standards. Quick Start Bradford protein assay kits offer either bovine serum
809 albumin or bovine γ-globulin standard sets.

810 Step 1: Prepare calibration curves using either Bovin Serum Albumin (BSA) with
811 a range of 1,25-10 µg mL⁻¹ or bovine γ-globulin in the range of 1,25-20 µg mL⁻¹

812 Step 2: Pour 150 μ L of samples/standard in a microplate
813 Step 3: in each well of the microplate, add 150 μ l of 1X Dye Reagent and shake
814 manually
815 Step 4: incubate 5 minutes at room temperature
816 Step 5: Take absorbance readings at 595 nm against a blank
817
818

819 Note: In classical protein staining protocols using Coomassie Brilliant Blue (CBB),
820 solutions with high contents of toxic and flammable organic solvents (Methanol,
821 Ethanol or 2-Propanol) and acetic acid are used for fixation, staining and
822 destaining of proteins in a gel after SDS-PAGE. To speed up the procedure,
823 heating the staining solution in the microwave oven for a short time is frequently
824 used. This usually results in evaporation of toxic or hazardous Methanol, Ethanol
825 or 2-Propanol and a strong smell of acetic acid in the lab which should be
826 avoided due to safety considerations.

827 In a protocol published by Lawrence and Besir [103], an alternative composition
828 of the staining solution is described in which no organic solvent or acid is used.
829 The CBB is dissolved in bidistilled water (60-80 mg of CBB G-250 per liter) and
830 35 mM HCl is added as the only other compound in the staining solution. The
831 CBB staining of the gel is done after SDS-PAGE and thorough washing of the gel
832 in bidistilled water. By heating the gel during the washing and staining steps, the
833 process can be finished faster and no toxic or hazardous compounds are
834 evaporating. The staining of proteins occurs already within 1 minute after
835 heating the gel in staining solution and is fully developed after 15-30 minutes
836 with a slightly blue background that is destained completely by prolonged
837 washing of the stained gel in bidistilled water, without affecting the stained
838 protein bands.
839

840 **4.3.2.3.2.3 Bicinchoninic Acid (BCA) Microplate Assay**

841 The bicinchoninic acid (BCA) assay measures the conversion of Cu^{2+} to Cu^+ under
842 alkaline conditions. The amount of Cu^{2+} reduced is a function of protein
843 concentration that can be determined spectrophotometrically by a color change
844 of the sample solution into purple, which absorbs at 562 nm. The absorbance is
845 directly proportional to the amount of protein present in the solution and it can
846 be estimated by comparison with a known protein standard, such as bovine
847 serum albumin (BSA). This assay is also generally less affected than the Lowry
848 assay by interfering compounds such as some detergents and denaturing agents
849 (urea, guanidine chloride). However, the BCA assay is more sensitive to the
850 presence of reducing sugars and high level of lipids [104]. The effects of these
851 interferences can be eliminated or reduced through several strategies such as
852 removing the interfering substances through dialysis, gel filtration or if the
853 protein concentration is high enough, by diluting the sample [105]. Compared to
854 other methods BCA assay is one of the most sensitive (it can detect proteins at
855 concentrations as low as 5 $\mu\text{g mL}^{-1}$). It has less variability than others (i.e.,
856 Bradford assay), and it can be used to measure a wide range of protein
857 concentration [106].

858 *Example of experimental protocol* for samples containing 0.1–1 mg protein mL^{-1} :

859 Step 1: Prepare calibration curves with Bovin Serum Albumin (BSA) with a range
860 of 5-2,000 $\mu\text{g mL}^{-1}$.

861 Step 2: Prepare BCA working reagent (WR)

862 Step 3: Dilute the microalgal samples with distilled water (1:3, v/v).

863 Step 4: Pour 10 μL of distilled water with 10 μL of diluted samples/standard in a
864 microplate.

865 Step 5: Add 200 μL of working reagent.

866 Step 6: Cover the microplate and incubate for 30 min at 37 °C in the dark.

867 Step 7: Keep all tubes at room temperature for 15 min before measurement.

868 Step 8: Take absorbance readings at 562 nm against a blank (distilled water).

869

870 **4.3.2.3.3 Amino acids analysis**

871 Amino acid analysis is one of the analytical principles for protein determination.

872 The principle is that the proteins are broken down into their constituent amino

873 acids by hydrolysis of the peptide bonds. The liberated amino acid residues are

874 then determined, most often chromatographically, and protein content is

875 calculated as the sum of individual amino acid residues after subtraction of the

876 molecular mass of H_2O .

877 After sample preparation (hydrolysis of the protein with 1 mL of 6 N HCl in

878 vacuum-sealed hydrolysis vials at 110 °C for 22 h), sodium or lithium buffers

879 (depending on the amino acids present in the sample) are prepared for

880 separation of the amino acids by Ion Exchange Chromatography. The eluate from

881 the ion exchange column is passed through in a teflon coil placed in a boiling

882 water bath, or other heating apparatus. Before entering, the column effluent is

883 mixed with reduced ninhydrin reagent, which is dissolved in acetate buffer. The

884 ninhydrin reacts with amino acids forming a dye complex. The absorption is

885 determined in a flow photometer, and registered on the chart of a recorder or a

886 computer. The area under the peaks corresponds to the amounts of amino acids

887 present in the sample. The evaluation can be done manually or automatically

888 with an integrator or a computer. The circumstances of the analysis make it

889 possible to quantitate as little as one nanomol amino acid with a high degree of

890 accuracy.

891 Example of protocol (from Mæhre [107])

892 From microalgal crude samples: 50 mg of extracts are dissolved in 0.7 mL
893 distilled H_2O and 0.5 mL 20 mM norleucine (internal standard).

894 1. From protein extract samples: 500 μL extract is mixed with 50 μL 20 mM
895 norleucine (internal standard).

896 2. Subsequently, for all samples, concentrated hydrochloric acid (HCl, 12 M)
897 is added, to a final concentration of 6 M.

898 3. The sample mixture is flushed with nitrogen gas for 15 s in order to
899 minimize oxidation, before hydrolysis at 110 °C for 24 h.

900 4. Following hydrolysis, 100 μL aliquots of the hydrolysates are evaporated
901 under nitrogen gas until complete dryness and re-dissolved to a suitable
902 concentration in lithium citrate buffer at pH 2.2.

903 5. All amino acids are analyzed chromatographically using an ion exchange
904 column followed by ninhydrin post column derivatization on a Biochrom 30
905 amino acid analyzer (for example, Biochrom Co).

906 6. Amino acid residues were identified using the A_{9906} physiological amino
907 acids standard (Sigma).

908 7. Protein content was calculated as the sum of individual amino acid
909 residues (the molecular weight of each amino acid after subtraction of the
910 molecular weight of H_2O).

911

912 **4.3.2.3.4 Validation of protein quantification methodology**

913 Once a bioassay has been selected, it is of high importance to validate that assay
914 for use by running risk analysis techniques such as failure mode and effect

915 analysis (FMEA) [94]. The goal is to assess the influence that each experimental
916 step will have on the final result of an analysis and to produce a list of
917 parameters that must be evaluated to obtain a robust assay. Key validations
918 parameters consist of accuracy, precision, repeatability, intermediate precision,
919 specificity, detection limit, quantification limit, linearity and range of the assay.
920

921 **4.3.2.4 Extraction and purification of high value phycobiliproteins from** 922 **microalgae**

923 **4.3.2.4.1 Phycoerythrin**

924 On order to meet the standards of pharmaceutical and molecular biology fields,
925 this protein must be highly purified. Two methods are used to determine the
926 purity:
927

- 928 • the absorbance ratio A_{565}/A_{280} defines the relationship between the
929 presence of phycoerythrin and other contaminating proteins. A purity ratio
930 $A_{565}/A_{280} > 4$ corresponds to diagnostics and pharmaceutical grade
931 phycoerythrin [108].
- 932 • the absorbance ratio A_{615}/A_{565} determines phycoerythrin purity in relation
933 to phycocyanin, which is its closest contaminating protein [39].
934

935 The purification protocol requires three steps: protein extraction by cell
936 disruption, primary recovery and purification.

- 937 • Cell disruption: several methods can be used (sonication, mechanical
938 maceration or lysozyme treatment). From the literature, the best recovery
939 yields are obtained with sonication [108]. Jubeau et al. [109] evaluated
940 the extraction of B-phycoerythrin from *Porphyridium cruentum* by high-
941 pressure cell disruption varying the parameters of pressure (25–270 MPa)
942 and they proposed a two-step selective extraction with a first passage at
943 50 MPa (to eliminate the contaminating protein present in the cytoplasm)
944 in culture medium followed by a second passage at 270 MPa (to extract
945 the B-phycoerythrin) in distilled water, achieving a 0.79 purity ratio.
946
- 947 • Primary recovery: the most commonly, fast and inexpensive method used
948 for primary recovery is selective precipitation with ammonium sulfate.
949 Various concentration of ammonium sulfate is used depending of the
950 microalgal strains, for example 65 % for *Porphyridium cruentum* [110] or
951 a a two-step ammonium sulfate precipitation, at 20 % and 70 %
952 saturation for *Phormidium spp.* [111].
953
- 954 • Purification: Purification is typically achieved by chromatographic methods
955 like ion exchange chromatography, hydroxyapatite chromatography, gel
956 filtration and expanded bed adsorption chromatography [39].
 - 957 ➤ Parmar et al. [111] purified phycoerythrin from *Phormidium sp.*
958 A27DM with a single-step gel permeation chromatography using a
959 Sephadex G-150 matrix pre-equilibrated and eluted with a 10 mM
960 Tris-HCl buffer (pH 8.1) at a flow rate of 60 mL h⁻¹. This protocol
961 yielded a final purity ratio of 3.9.
 - 962 ➤ Bermejo et al. [110] purified phycoerythrin from *P. cruentum* using
963 an anionic chromatographic column of Diethylaminoethanol (DEAE)
964 cellulose. Elution was performed as a discontinuous gradient of
965 acetic acid-sodium acetate buffer (pH 5.5). The best results were
966 achieved with flow rate of 100 mL h⁻¹. Later, Bermejo et al. [112]

967 developed a method of purification of phycoerythrin by expanded
968 bed adsorption chromatography (EBA) using a DEAE adsorbent. The
969 authors focused on maximizing product recovery rather than purity,
970 since the process is intended to replace low-resolution methods. The
971 use of EBA chromatography allowed partial concentration of the
972 product and therefore works as a preparative method with little
973 product loss and is suitable to large scale production [113].
974

975 **4.3.2.4.2 Phycocyanin**

976 The purity ratio of the phycocyanin extract is determined by the A_{620}/A_{280} ratio.
977 Absorbance ratio ≥ 0.7 refers to food grade pigment, while reagent and analytical
978 grade correspond to 3.9 and ≥ 4.0 respectively [42].

979 General protocol for purification includes a first extraction in buffer solutions
980 (phosphate buffer) with sonication or ultrasound as cell disruption pretreatment
981 which should be as quick as possible in order to avoid proteins destabilization.
982 Later, proteins precipitation and recovery by ultracentrifugation or filtration are
983 carried.

984 Phycocyanin is water-soluble and thus can be easily extracted as a protein-
985 pigment complex. Sørensen et al. [114] evaluated different extraction techniques
986 for c-Phycocyanin extraction from *Galdieria sulphuraria* and obtained contents of
987 25–30 mg/g. Ammonium sulfate concentration above 1.28 mol L⁻¹ ensured only
988 C-Phycocyanin precipitation with purity of 0.7. In case of ultrafiltration, more
989 than 50 % was lost at 100 kDa tangential flow filter, while 79% was retained by
990 the 50 kDa filter. The authors also proposed to combine ammonium sulfate
991 fractionation with the other methodologies tested (anion exchange
992 chromatography, tangential flow filtration) in order to enhance purity of the
993 recoveries (3.5–4.5).
994

995 **4.3.3 Proteomics**

996 Numerous scientific publications report proteomic studies on microalgae [115-
997 118], mainly targeting on topics such as the response of microalgae to different
998 stress sources or environmental signals and issues related to taxonomy and
999 identification of potentially harmful species [119]. With the fast development of
1000 microalgal biofuel researches, the proteomics studies of microalgae have
1001 increased quickly [120]. Proteomic studies provide a platform for discovery of
1002 some yet unidentified genes and proteins [121]. Protocols used have significantly
1003 evolved with the development of two-dimensional electrophoresis (2-DE) which
1004 represents a powerful approach for high-throughput analysis of complex protein
1005 mixtures [121]. This technique is based on the integration of multi-dimensional
1006 chromatography and mass spectrometry (MS) with the proteomic informatics.
1007 Most experiments are broadly classified as either "gel-based proteomics" or "gel-
1008 free proteomics", depending on whether 2-DE is used for protein separation and
1009 quantification or not [121].

1010 Nowadays, 2-DE is the most common strategy for protein separation and
1011 quantification, enabling the separation, detection and quantification of different
1012 proteins from a single extract. With this method, information on small post-
1013 translational modifications (PMTs) and highly homologous isoforms can often be
1014 obtained directly, since these tend to shift the isoelectric point of a protein
1015 without extensively changing its molecular weight. However, technical difficulties
1016 such as gel-to-gel variation, limited linear dynamic range, limited throughput,
1017 and protein co-migration have been reported. This can be overcome by the
1018 utilisation of software such as Progenesis SameSpots (Nonlinear Dynamics),

1019 PDQuest (Bio- Rad Laboratories) and DeCyder (GE Healthcare) for the analysis of
1020 2-DE gels.

1021 Jia et al. [121] described 2-DE as an elaborate pre-fractionation step that
1022 precedes MS analysis in a typical proteomic workflow and, as such, some
1023 researchers prefer to omit this step and apply different strategies which scale up
1024 much better, thus gel-free strategies are used more and more, since they allow
1025 higher analytical throughput and deeper proteome coverage than gel-based
1026 methods.

1027
1028 The common workflow (gel-based and gel-free) analysis includes: 1) sample
1029 preparation; 2) protein separation and quantification; and, 3) Protein
1030 identification and characterization, and, is described below.

1031

1032

1033

1034 **4.3.3.1 Gel-based approach**

1035 **4.3.3.1.1 Sample preparation**

1036 The first step consists of protein extraction, since most analytical techniques
1037 used in proteomics require prior solubilization of proteins in an appropriate
1038 solvent (aqueous buffers, organic solvents). Commonly used aqueous extraction
1039 buffers often contain (besides buffering agents) detergents, chaotropes, reducing
1040 agents and protease inhibitors, ensuring that enzymatic activity is inhibited
1041 during extraction and that interactions between proteins are minimized,
1042 preventing aggregation. Fractionation prior to proteome can be done using
1043 chromatography, electrophoresis, differential solubility and/or centrifugation.

1044

1045 **4.3.3.1.2 Protein separation and quantification**

1046 Detection and quantification methods for 2-DE are based on Coomassie Brilliant
1047 Blue. Recently, the development of multiplex 2-DE ("difference gel
1048 electrophoresis" or DIGE), involves tagging the protein samples with different
1049 fluorophores prior to 2-DE, leading to two main advantages as several samples
1050 can be run on a single gel and improves gel-to-gel variability, by providing a
1051 common reference channel across all gels of an experiment [122].

1052

1053 **4.3.3.1.3 Protein identification and characterization**

1054 Most gel-based proteomic studies rely on digestion of detected proteins, followed
1055 by the analysis of the resulting peptides by MS for their identification and
1056 characterization. Instruments currently employed for this purpose include ESI-
1057 Ion Trap, MALDI-TOF/TOF and ESI-QTOF mass spectrometers to a lesser extent.
1058 Identification of proteins can be assessed either directly through its peptide mass
1059 fingerprint (PMF), for the case of organisms with fully sequenced genome, or by
1060 analysis of the fragmentation spectra of such peptides (PFF, peptide fragment
1061 fingerprinting or even de novo sequencing) obtained through tandem MS.

1062

1063 **4.3.3.2 Gel free approach**

1064 With this technic, proteins are digested from the start and analyses (separation,
1065 quantification, characterization) are done at the peptide level. Gel-free methods
1066 can be combined with fractionation methods, such as liquid-phase
1067 chromatography procedures (coupled to ESI-based mass spectrometers), in
1068 order to reduce the number of different peptides entering the mass spectrometer
1069 for maximization of the total number of distinct peptides detected over the
1070 course of a sample run.

1071 Multidimensional chromatographic separations are commonly used, as in the
1072 case of MudPIT, where peptides are separated by charge (SCX-HPLC) and
1073 hydrophobicity (RP-HPLC) prior to MS analysis. Most gel-free workflows rely on
1074 stable isotope labeling for peptide quantification, either by metabolic
1075 incorporation of radioactive amino acids in proteins (SILAC) or by post-extraction
1076 chemical modification (ICAT, TMT, iTRAQ). With stable isotope labeling, several
1077 samples can be analyzed in parallel on the same MS run and relative abundance
1078 can be estimated.

1079
1080

1081 **4.3.3.3 Obtention of peptides and identifying proteins from peptides**

1082 **4.3.3.3.1 Peptides obtention**

1083 Peptides are short-chain protein-linked amino acid residues linked by peptide
1084 bonds. They are produced by enzymatic cleavage of proteins during food
1085 digestion, microbial fermentation, food processing or exogenous enzymatic
1086 hydrolysis [63,123]. In order to isolate the bioactive peptides, the protein
1087 hydrolysates are subjected to several fractionation and purification techniques, in
1088 particular membrane ultrafiltration and ion exchange chromatography, affinity
1089 and gel permeation techniques. The choice of treatment techniques depends
1090 largely on the structural characteristic of the peptide of interest [61]. Peptides
1091 have shown interesting biological activities that would prevent hypertension,
1092 oxidative stress, cancer, diabetes, inflammation and immune disorders [63]. The
1093 bioactivity of peptides depends on several factors such as the molecular weight
1094 of the peptide, the amino acid composition, the molecular and surface
1095 hydrophobicity [63,125].

1096 For example, hydrolysis of *Chlorella vulgaris* with pepsin has been shown to
1097 produce a short-chain peptide (of 11 amino acids :Val-Glu-Cys-Tyr-Gly-Pro-Asn-
1098 Arg-Pro-Gln-Phe) with dose-inhibiting -dependent anti-hypertensive properties *in*
1099 *vitro* and *in vivo* [61,126]. Another peptide (Val-Glu-Gly-Tyr) derived from the
1100 hydrolysate of *Chlorella ellipsoidea* has also been reported to have ACE inhibitory
1101 activity *in vitro* and an *in vivo* effect of blood pressure reduction [61].

1102

1103 **4.3.3.3.2 Identifying proteins from peptides**

1104 Most proteomic studies attempt to identify proteins by analysing peptides, as
1105 large proteins constitute a challenge for MS-based methods. The classical
1106 method used ("peptide mass fingerprinting" or PMF) is based on *in silico*
1107 digestion of genomic/EST sequences following the pattern of a predictable
1108 endonuclease (usually trypsin) to obtain a list of peptide masses (or "mass
1109 fingerprint") for each database entry. Identification is performed by comparing
1110 experimentally obtained MS mass lists with those generated *in silico*, and
1111 choosing significantly similar matches.

1112 It is possible as well to tandem MS instruments in order to obtain a peptide mass
1113 list and information on their fragmentation mass spectra, leading to a fingerprint
1114 for each peptide that directly reflects its sequence. For this, "peptide fragment
1115 fingerprinting" (or PFF) involves starting with a genomic/EST database and
1116 performing *in silico* digestion with a standard endonuclease. Then, for each
1117 peptide, the masses of all likely fragments are deduced from prior models.
1118 Identification of peptides is deduced by comparison of experimentally obtained
1119 MS/MS spectra against all possible fragmentation spectra in the database. Since
1120 a fragmentation spectrum (unlike mass) is usually very specific for a certain
1121 peptide sequence, identification of proteins can often be attained from a single

1122 high-quality peptide match. However, this strategy works only for species for
1123 which there is genomic/EST data available or for highly conserved peptides.

1124
1125

1126 **4.3.4 Challenges and future perspectives**

1127 To produce quality microalgal biomass as a whole-feed ingredient, cultivation
1128 techniques should aim to balance the lipid profile and the protein content. An
1129 alternative and potentially more efficient approach is a biorefinery-type system
1130 where microalgal oils could be separated from the cell biomass and used as
1131 concentrated feed or food supplements [127]. Thus, oil production could be
1132 maximized, nitrogen consumption minimized, and the residual biomass used for
1133 other processes including energy production [128]. In order to achieve this,
1134 microalgae containing sufficient ω -3 fatty acids and protein are searched [127].

1135
1136 Microalgae biorefinery uses the overall composition of the cell, and next to lipids,
1137 including carbohydrates and proteins [81]. When proteins are extracted in a
1138 context of biorefinery, mild conditions of extractions must be used such as alkali
1139 or enzymatic extractions [82]. Regarding economical costs, it was demonstrated
1140 that protein production using enzyme extraction was less expensive than when
1141 obtained after an alkali extraction. Higher revenue can be generated when the
1142 residue after protein extraction can be sold as fuel or feed for animal.

1143 Nowadays scientists are convinced that the biorefinery concept (i.e., a sequence
1144 of unit operations to achieve the whole fractionation and/or transformation of
1145 biomass to produce multiple products) applied to microalgae would render this
1146 sector profitable. The biorefinery scheme is the key for the utilization of
1147 microalgal biomass, but the main constraint is that in most cases final recoveries
1148 are low due to the number of steps required to achieve the purity levels specified
1149 for each compound and improving these yields represents a real challenge for
1150 the future. Moreover, conditions of cultivation of strains that produce high value
1151 compounds have been well studied, however recovery of intracellular metabolites
1152 at large scale is still a challenge since not all cell disruption, extraction or
1153 purification methods are scalable. Nevertheless, some technologies such as high-
1154 pressure homogenization can be viable for scale up [129]. Another key point to
1155 challenge is the set-up of more environmentally friendly process regarding
1156 extraction of high value compounds and finding new way to avoid toxic solvent
1157 and the use of too much energy (replacing high temperature processes for
1158 example).

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1169 **4.4 Methods for polysaccharides determination in** 1170 **microalgae**

1171 [Guillaume Pierre, Clément Gaignard, Cédric Delattre, Céline Laroche, Philippe
1172 Michaud]

1173

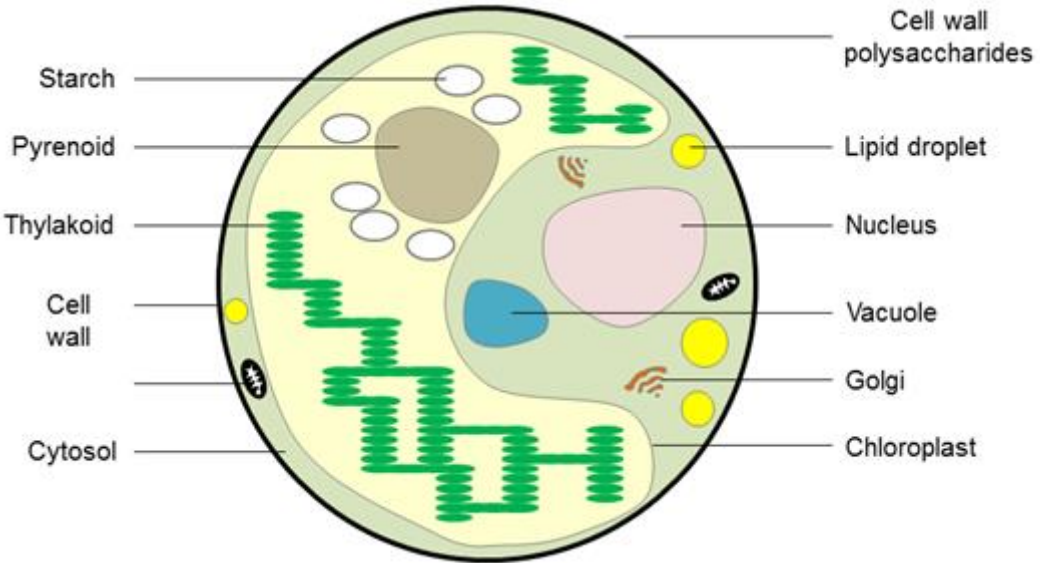
1174

1175 **4.4.1 Introduction**

1176 Polysaccharides are highly complex macromolecules exhibiting a great variety of
1177 biochemical structures based on glycosidic linkages of up to 50 different
1178 monosaccharides including numerous isomers (hexoses and pentoses), some of
1179 them being complex sugars. Various non-sugar substituents such as sulphates,
1180 acyl or methyl groups but also amino acids may be attached on these linear or
1181 ramified backbones. Understanding this complexity needs to consider that two
1182 monosaccharides such as hexoses may be theoretically polymerized by
1183 glycosyltransferases or transglycosylases by 5 different glycosidic bonds. This
1184 wide range of polysaccharidic structures dominates their conformation, flexibility
1185 and interactions as they adopt different architectures in solution [130]. In their
1186 meeting report, published in 2007 in the famous review, Nature Chemical
1187 Biology, Jeremy E. Turnbull and Robert A. Field, wrote “the key bottlenecks in
1188 the development of glycobiology has been analysis of glycan structures, from
1189 natural sources” [131]. Ten years later, this fact is definitely true in the field of
1190 polysaccharides from animals, terrestrial plants, macroalgae and, above all,
1191 microalgae. For a long time, the structure identification of carbohydrate polymers
1192 was limited by suitable and sensitive approaches and despite the recent
1193 development of powerful analytical tools, more and more efficient, the analysis of
1194 polysaccharidic sequences is still a challenge compared to those of nucleic acids
1195 and proteins. Moreover, the sole overview of methodologies currently used in
1196 polysaccharide analysis resumes well its complexity [132]. These methodologies
1197 include monosaccharide analysis, linkage methylation analysis,
1198 chemical/enzymatical degradation procedures, fractionation and purification of
1199 degradation products, infrared spectroscopy, mass spectrometry using different
1200 ionization techniques and 1-D/2-D NMR spectroscopy using $^1\text{H}/^{13}\text{C}$ dual probe.

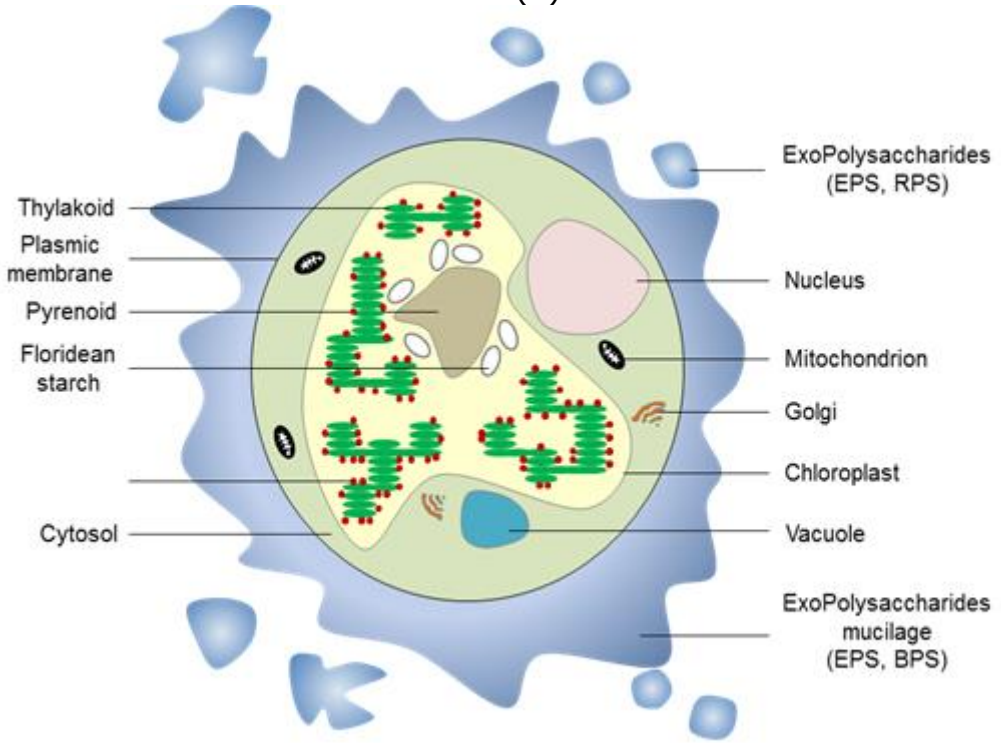
1201 Numerous microalgae are known to produce polysaccharides with several cellular
1202 locations (**Fig. 4.4.1**). They are sometimes excreted as extracellular
1203 polysaccharides [133] or are cell wall polysaccharides, intracellular starch or
1204 chrysolaminarin. Excepting starch, and chrysolaminarin, their structures are
1205 highly complex including often up to 10 different monosaccharides and non-
1206 sugars groups such as methyl, pyruvyl, acetyl, sulphate and others. Note also
1207 the lack of units of repetition for the major part of them limiting significantly the
1208 structural investigations despite the potential of these biopolymers as texturing
1209 or biological agents [133-134-135]. Exopolysaccharides from microalgae can
1210 remain associated to the cell surface (cell-bound polymers) and/or liberated into
1211 the surrounding environment as released polysaccharides [136-137-138]. The
1212 status of cell-bound exopolysaccharides is often unclear. Exopolysaccharides
1213 (EPS) are sometimes called ExoPolymeric Substances (EPS), Extracellular
1214 Polysaccharides (ECPS), Released polysaccharides (RPS), Extracellular
1215 Proteoglycan (EPG), capsular polysaccharides (CPS), Polysaccharide (PS) and
1216 sulphated Polysaccharides (sPS) [133-135-137-139-140]. Exopolysaccharides
1217 are the most studied compounds among polysaccharides from microalgae. This
1218 popularity is explained by their structural diversity, extractability and solubility,
1219 as these biopolymers have not covalent linkages with cell walls. Their

1220 characterization may be impacted by culture conditions for their production, but
 1221 also by the extraction and purification used to collect them [141,142]. The
 1222 identification by some authors of structures differences depending on culture
 1223 conditions could be in some cases artefacts related to different extraction,
 1224 purification and/or structural characterization protocols. From today and at our
 1225 knowledge, only few full structures from microalgae polysaccharides have been
 1226 described in the literature and a significant part of them are just oligosaccharides
 1227 extracted and purified from complex polysaccharides [133]. These partial
 1228 characterizations are not totally representative of the native macromolecular
 1229 structures.
 1230
 1231



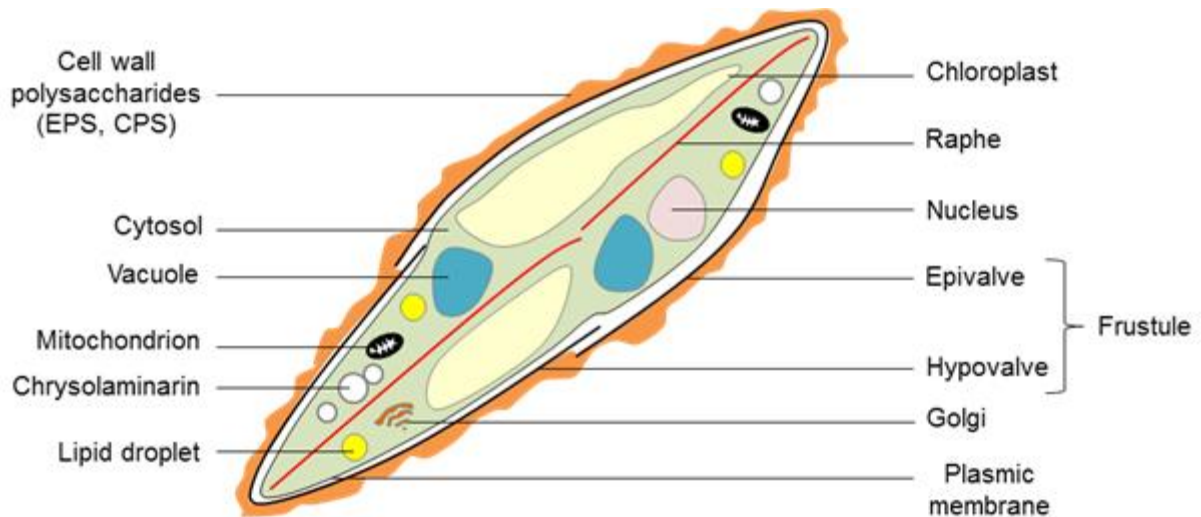
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(A)



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(B)



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(C)

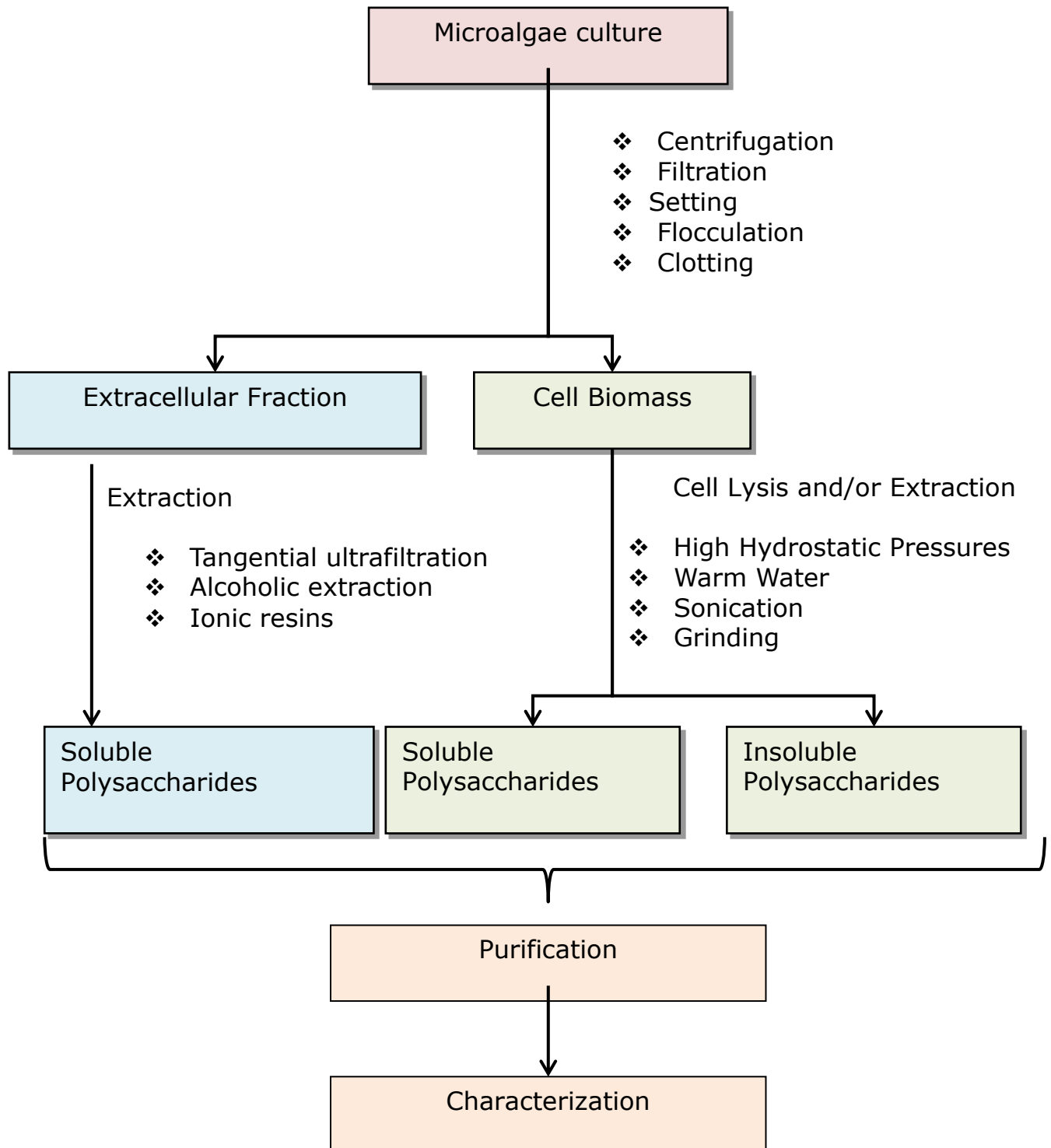
1238 **Fig. 4.4.1:** Schematic representation of microalgae (A and B) and Diatoms (C).

1239

1240 **4.4.2 Polysaccharides sampling and extraction strategies**

1241 Mostly, the largest part of microalgae polysaccharides and notably EPS stay
 1242 poorly understood because of the low extraction yields and the difficulty to
 1243 properly separate them. In the following sections are presented the most
 1244 important strategies described and developed for the extraction and purification
 1245 of microalgae polysaccharides (**Fig. 4.4.2**).

1246



1247

1248

Fig. 4.4.2: Overview of polysaccharide extraction from microalgae.

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4.4.2.1 Alcoholic precipitation of polysaccharides as conventional extraction processes

1254 Production yields of microalgae polysaccharides are closely dependent to growth
1255 and cultures conditions [143]. Then, many cost-effective and environmental
1256 friendly parameters have to be investigated to selectively extract and purify EPS
1257 and cell-bound or intracellular polysaccharides (IPS) such as chrysolaminarin,
1258 starch, capsular or sheath polysaccharides [144,147]. Works of Ramus were the
1259 first to recommend an experimental approach to separate and isolate the
1260 different categories of polysaccharides from *Porphyridium*. In his strategy, after a
1261 preliminary depigmentation of microalgae biomass using ethanol/acetone
1262 treatment, cell bound exopolysaccharides were extracted using hot water. A
1263 second step with cetyl pyridinium chloride selectively isolated EPS by
1264 precipitation in calcium salt prior to a final ethanol precipitation. Focusing only on
1265 EPS, Delattre et al. [133] have proposed a general process to selectively extract
1266 and purify these hydrocolloids from culture media of several microalgae. Briefly,
1267 after microalgae cultivation, biomass is extracted from culture medium using
1268 centrifugation or microfiltration [148,149]. Afterwards, soluble EPS (in
1269 supernatant or in permeate) are concentrated under vacuum (depending on the
1270 viscosity) and finally precipitated with 2-3 volumes of cold alcohol (-20 °C) such
1271 as ethanol, isopropanol, or methanol [133,150-152]. The main advantage of this
1272 alcoholic precipitation in industrial point of view is the possibility to work with
1273 highly viscous solutions and to recycle the solvent (water, alcohol) by distillation
1274 to reduce the production cost. Nevertheless, the main drawback of this method is
1275 the co-precipitation of a part of salts coming from the culture medium, notably
1276 when the cultivated microorganisms are marine microalgae (up to 30-40 g L⁻¹ of
1277 NaCl depending on culture media). This phenomenon can be partially avoided
1278 repeating the alcoholic precipitation 2 or 3 times and or using several selective
1279 alcohol concentrations. Then, tangential ultrafiltration (TUF) in diafiltration
1280 operating mode is commonly applied to purify polysaccharide and remove salts.
1281 Patel et al. clearly showed that the desalting of EPS from *Porphyridium cruentum*
1282 was optimal using diafiltration of medium onto 300 kDa membranes
1283 comparatively to the conventional alcoholic precipitation which gave EPS fraction
1284 polluted by high salt contents [151]. Finally, note to mention that additional
1285 combined purification steps such as trichloroacetic acid (TCA) treatment,
1286 selective precipitation (alcoholic gradient) and TUF lead also to the removing of
1287 all low molecular weight impurities (phenolic compounds, pigments, proteins,
1288 salt, mineral,...) [133,151]. Lastly, the high purity EPS is dried (freeze-drying or
1289 in ventilated oven at 40-50 °C). When polysaccharides are dried in a ventilated
1290 oven at 40-50 °C, this step is sometimes preceded by a precipitation of
1291 polysaccharide and its washing by acetone. It is important to notice that as
1292 mentioned by Delattre et al. [133] during the purification step of
1293 polysaccharides, other high-value molecules such as proteins, pigments, and
1294 lipids could be valorized for industrial purposes [153]. The next section gives an
1295 overview of the extraction/purification optimization of polysaccharides using TUF.
1296

1297 **4.4.2.2 Tangential ultrafiltration process for EPS purification**

1298 Recently, many researchers have proposed TUF processes as an alternative to
1299 the conventional extraction of EPS using alcoholic precipitation. This
1300 methodology was largely investigated to extract biomolecules with high yields
1301 from complex medium without using additives (chemical, enzymes, solvents...)
1302 [154,155]. Therefore, many proteins and polysaccharides purification processes
1303 were investigated on several ultrafiltration modules such as rotating, hollow
1304 fiber, tubular, spiral wound or flate plate system [154]. Generally, the
1305 clarification/desalting/concentration steps were carried out using nominal

1306 molecular weight cut-off (NMWCO) from 1 to 500 kDa [155]. To be efficient for
1307 the purification of polysaccharides, the membrane system has to be studied
1308 depending on the concentration and viscosity of media but also the
1309 transmembrane pressure and flow rate velocity. In the works of Li et al. [148] a
1310 combined microfiltration (polypropylene membrane, 0.2 μm) and ultrafiltration
1311 (polyethersulfone membrane with NMWCO of 5 kDa) pilot-scale extraction
1312 processes was proposed to concentrate EPS from varied microalgae and
1313 Cyanobacteria including: *Nostoc sphaeroide*, *Nostoc commune*, *Chlorella*
1314 *pyrenoidosa*, *Chaetoceros muelleri*, *Haematococcus pluvialis* and *Spirulina*
1315 *platensis*. Recently, the same approach was used to efficiently isolate and purify
1316 polysaccharides from *Porphyridium* sp. using two ultrafiltration steps with two
1317 polyethersulfone membranes (NMWCOs of 10 and 300 kDa) [156]. Generally, as
1318 demonstrated by the works of Zhang and Santschi [157] on the purification of
1319 *Amphora* sp. EPS, the ultrafiltration technology was well-recommended to purify
1320 microalgae EPS from sea water culture media. However it should be noticed that
1321 to be generalized to all microalgae EPS extraction/purification processes, TUF
1322 technology system must be optimized for industrial scale-up potential. There are
1323 some drawbacks limiting TUF process such as (i) the great amount of water used
1324 for desalting/purification/concentration of EPS and, (ii) the clogging of
1325 membranes due to the high viscosity of microalgae EPS solution
1326 [148,149,158,159] which reduce considerably the EPS extraction yield and
1327 increase the operating prices compared to the alcoholic precipitation.
1328 Consequently, it is essential for the development of new efficient membrane
1329 systems to study rigorously the combination of both numerical and theoretical
1330 analyses in order to upgrade both mass-transfer and hydrodynamics setting
1331 [154,160]. For this purpose, the works of Jhaveri and Murthy [161] gave a very
1332 attractive overview on anti-fouling nanocomposite membranes development for
1333 industrial application. Indeed, authors clearly highlighted the importance for
1334 innovation in synthetic material fields in particular to enhance the membrane
1335 filtrations system performance by improving the hydrophilicity, the anti-fouling
1336 and the self-cleaning properties. In this context, the use of organic
1337 (polyacrylonitrile) and inorganic (AZT: Aluminum/Zirconium/Titanium Oxide)
1338 membranes has been efficiently performed with the cyanobacterium *Arthrospira*
1339 *platensis* [162,163].

1340 1341 **4.4.2.3 Specific treatments for the extraction of cell-bound** 1342 **exopolysaccharides**

1343 As well-established, around 50 % of the total amount of EPS were cell-bounded
1344 to the microalgae cell as slime layer [136]. Numerous procedures have been
1345 described in literature to purify and isolate cell-bound exopolysaccharides.
1346 Among them, treatment of microalgae with ethylene diamine tetracetic acid
1347 (EDTA), formaldehyde (FA), hydroxide sodium (NaOH), water, sonication,
1348 heating and ionic resins have been proposed [147,152,164-167]. Treatment with
1349 cationic resins is known to break specific interactions between microorganisms
1350 and EPS without any cellular damage [146,147,152]. By this way, Pierre et al.
1351 [152,168,169] isolated several microalgae EPS from a diatom-dominated
1352 intertidal mudflat using the cationic exchange resin Dowex Marathon C. In other
1353 studies, researchers proposed during the extraction of EPS, the preliminary
1354 combined treatment of microalgae cell by FA and glutaraldehyde (GTA) as
1355 fixative agents in order to reduce cell lysis [144,146,147,165]. Nevertheless,
1356 these chemical treatments caused the structural modifications of EPS disturbing
1357 extraction [144,165,170]. That is why the general alternative to extract cell-

1358 bounded EPS is to use washing treatment with water at different temperature
1359 (30 to 95 °C) and pH (basic of acid). According to the treatment duration (1-4 h)
1360 and biomass/water ratio, microalgae cell lysis was observed. Thus, the
1361 contamination of EPS by intracellular compounds such as starch, chrysolaminarin
1362 and proteins was described for the extraction of EPS from *Craspedostauros*
1363 *australis*, *Nitzschia epithemioides*, *Thalassiosira pseudonana*, *Anacystis nidulans*,
1364 *Navicula jeffreyi*, *Cylindrotheca fusiformis*, *Anabaena cylindrical*, *Cyanospira*
1365 *capsulate*, *Navicula phyllepta* and *Phaeodactylum tricornutum*
1366 [145,147,171,172,173].
1367

1368 **4.4.2.4 Specific treatments for the extraction and quantification of** 1369 **starch**

1370 Despite growing interest for the use of starch from microalgae as feedstock for
1371 bioethanol production within a biorefinery point of view, only few studies describe
1372 a protocol for extraction and purification of starch. Extraction of starch is only
1373 performed when structural characterization is needed, especially for the
1374 understanding of mechanisms ruling the metabolic patterns depending on culture
1375 conditions. As starch is an intracellular polysaccharide, first step involves a
1376 cellular lysis. Several methods exist, that can be classified as physical,
1377 enzymatical, or chemical methods, but physical methods are generally preferred.
1378 Delrue et al. [174] and Deschamps et al. [175], have purified starch from
1379 microalgae using similar protocols. The cellular lysis was achieved by sonication,
1380 of a cellular suspension in phosphate buffer (pH 7.5-8.0) containing 5 mM EDTA.
1381 For Deschamps et al. [175] the buffer was specifically supplemented by 1 mM of
1382 dithiotreitol. After centrifugation, the pellets (starch and cell fragments) were
1383 resuspended in 90 % Percoll and centrifuged (10,000 g, 30 min) to separate
1384 high-density starch granules from cell debris of lower density. The Percoll
1385 gradient step is repeated to ensure complete removal of cell debris from the
1386 starch pellet. The starch is then washed in sterile water and freeze-dried after its
1387 recovery by centrifugation. Kobayashi et al. [176] used a 10 % toluol solution in
1388 order to separate starch and cell debris. After mixing vigorously during 10
1389 minutes, an emulsion layer, containing cells debris, was formed at the top of the
1390 tube. After discarding this layer, the starch was recovered and toluol step
1391 repeated, until no coloration appeared. Starch was then washed with water and
1392 ethanol before drying. Other alternative can be, after cellular lysis and the first
1393 centrifugation step, to resuspend pellets in water and to boil (30 min), in order to
1394 solubilize starch. After a new centrifugation (10 000 g, 15 min), the supernatant
1395 containing starch was precipitated with 3 volume of ethanol, or more specifically
1396 with iodine [177]. Nevertheless, starch from microalgae is structurally different
1397 from that of higher plants regarding the ratio amylose: amylopectin. Whereas
1398 starch from higher plants contains generally around 30 % of amylose, amounts
1399 of 5-25 % have been reported for chlorophyceae [176] and is rather absent in
1400 starch from rhodophyceae except in few unicellular strains [178]. Consequently
1401 the efficiency of iodine precipitation can differ from one genus to another [179].
1402 When starch has been extracted and purified, classical methods can be used for
1403 its quantification and characterization. Nevertheless, most authors are interested
1404 in quantifying starch without time consuming extraction. Several methods are
1405 found in literature for quantification of starch content in microalgae. Most of
1406 them are derived from the characterization and quantification of carbohydrates
1407 from terrestrial plants. For example, starch content can be estimated after starch
1408 solubilization from ethanol-treated biomass (defatted and depigmented) by
1409 boiling. After centrifugation, supernatant containing starch is mixed with iodine

1410 solution and color is compared with standards of known starch content [180].
1411 Drawback of this method is that as previously mentioned, structural differences
1412 of starch from microalgae induce differences in color when mixed with iodine and
1413 the coloration is then not directly proportional to amount of starch. As an
1414 example, floridean starch gives a pink color due to low amylose content [181],
1415 whereas starch from Chlorophyceae gives a blue-dark coloration [182]. One
1416 consequence is that maximum of absorbance is shifted depending of microalgae
1417 class: 597 nm for Chlorophyceae [176], 530-550 nm for Rhodophyceae [181-
1418 183]. For accurate quantification, the standard should then be of the same type
1419 than the analyzed sample. Before using this method as routine procedure, it will
1420 then require an extraction of the starch that will be used as standard (as the
1421 starch type could be not commercially available), and a more accurate method of
1422 quantification to calibrate it.

1423 Chemical treatments such as 45–52 % perchloric acid, 3-15 % sulphuric acid, or
1424 90% dimethyl sulfoxide, are often used to degrade starch before quantification of
1425 released sugars by enzymatical or colorimetric methods [176,180,184]. Even if
1426 this method is frequently used by authors, the efficiency can be discussed as
1427 several drawbacks may be highlighted. First, an incomplete hydrolysis can lead
1428 to underestimation. Second, celluloses and hemicelluloses found in cell walls can
1429 be degraded together with the starch, and result will be in that case
1430 overestimated, especially if colorimetric assay is used to quantify sugars. If
1431 enzymatic assay is used (generally based on glucose oxidase activity), only
1432 cellulose will interfere as hemicelluloses from microalgae are often xylan-type.

1433 In order to circumvent the problem of non-selective degradation, enzymes can
1434 be used. A mix of α -amylase and amyloglucosidase, in appropriate amounts and
1435 reaction conditions, will provide efficient degradation of starch, without
1436 contamination from cellulose degradation as β -links are not attained by these
1437 enzymes. The released sugars are further quantified as previously described.
1438 This protocol is at the origin of starch assay kits from Megazyme, Bohringer, or
1439 Sigma-Aldrich.

1440 Recently, Ji et al. [185] have proposed a fast method to quantify starch by direct
1441 *in-situ* measurement in individual cells by Raman spectrometry. Method requires
1442 expensive equipment, as Raman spectrometer coupled to microscope is needed.
1443 Nevertheless, results revealed a nearly linear correlation between the signal
1444 intensity at 478 cm^{-1} and the starch content of the cells. This method has been
1445 tested both on *Chlamydomonas* and *Chlorella* strains, and the specificity of the
1446 band at 478 cm^{-1} has been validated on a *Chlamydomonas* mutant (non-
1447 producing starch strain).

1448 **4.4.2.5 Specific treatments for the extraction of fibrillar polysaccharides.**

1450 Some microalgae such as green microalgae have a rigid cell wall composed of
1451 fibrillar polysaccharides including chitin-like glycan or glucose-mannose polymers
1452 [186]. For fibrillar polysaccharides extraction, the method of Mc Cleary (2007)
1453 [187] can be used. This method has led to the AOAC 2009.01 method for
1454 extraction and quantification of soluble and insoluble fibers in food samples.
1455 Briefly, microalgae biomass is subjected to action of α -amylase and
1456 amyloglucosidase (16 h, 37 °C, pH 6) in order to hydrolyse starch. For better
1457 results, a mechanical cellular lysis is recommended in order to improve access of
1458 enzymes to the starch. After heating à 95 °C (for inactivation of enzymes) and
1459 pH shift to 8.2, protease is added (30 min, 60 °C) in order to degrade proteins.
1460 After filtration and washing of residues, they are dried and correspond to
1461 insoluble fibers (fibrillar polysaccharides in microalgae case). It is to notice that

1462 the filtrate obtained at the last step could be precipitated by ethanol and should
1463 correspond to soluble fibers (matricial polysaccharides in microalgae case).

1464

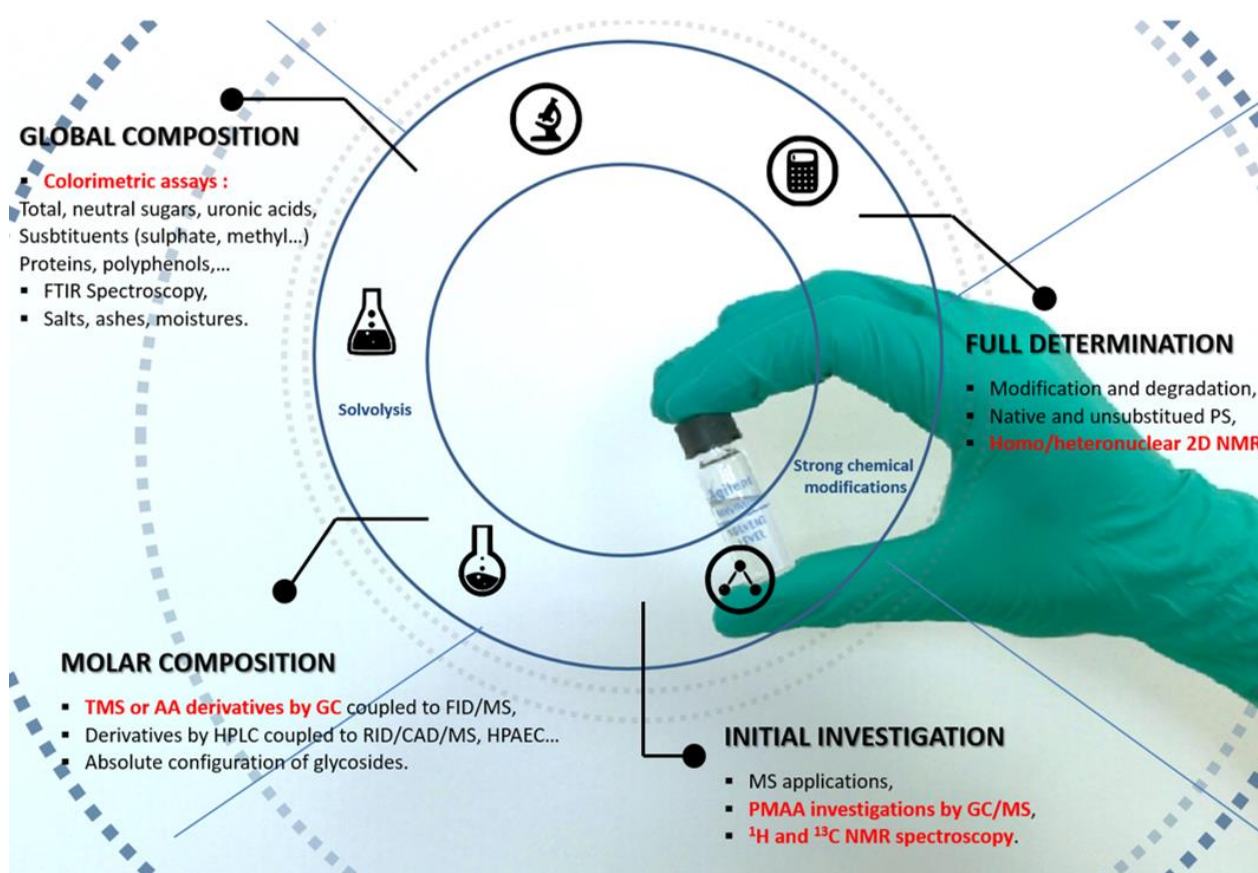
1465 **4.4.3 How to determine the global composition of polysaccharides?**

1466 Identification and quantification of carbohydrates are of primary importance
1467 when it comes to the characterization of microalgal biomass. Owing to their low
1468 absorption in UV-Visible regions and their structural complexity, the analysis of
1469 polysaccharides can be a very challenging work. Overall, a deep investigation is
1470 needed to accurately determine the structure of carbohydrates and strategy for
1471 their analyses can change depending on their sources, extractions and
1472 compositions.

1473 **Fig. 4.4.3** gives an overview of the main ways for the structural analysis of
1474 carbohydrates, from the determination of the global composition to the fine
1475 analysis of the branching patterns.

1476

1477



1478

1479 **Figure 4.4.3:** Overview of the strategies needed for the structural analysis of
1480 polysaccharides from microalgae. HPLC: High-Performance Liquid
1481 Chromatography, RID: Refractive Index Detection, CAD: Charged Aerosol
1482 Detection, MS: Mass Spectrometry, GC: Gas Chromatography, FID: Flame-
1483 Ionization Detector, TMS: TriMethylSilyl, AA: Alditol Acetates, HPAEC: High-
1484 Performance Anion-Exchange Chromatography, NMR: Nuclear Magnetic
1485 Resonance spectroscopy, EI: Electron Impact Ionization, PMAA: PerMethylated
1486 Alditol Acetates, FTIR: Fourier Transformed InfraRed spectroscopy, PS:
1487 Polysaccharides.

1488

1489 **4.4.3.1 Total carbohydrates**

1490 The phenol [188] or orcinol- [189] sulfuric acid methods, as well as the recently
1491 updated anthrone assay [190,191] can be used for determining the total amount
1492 of carbohydrates. Based on the dehydration of monosaccharides to furfural
1493 compounds in acid, the phenol-sulfuric acid is still the most used method with
1494 few alternatives (miniaturization, heating, acid concentrations) described in the
1495 literature [192,193]. Briefly, the absorbance is measured at $\lambda = 485$ nm and d-
1496 glucose is used as standard. Attention should be given to other compounds, e.g.
1497 pigments, proteins, lipids, polyphenols and salts to limit interferences.

1498
1499 **4.4.3.2 Uronic acids and neutral sugars**

1500 Based on the formation of furfural derivatives in presence of resorcinol
1501 solubilized in acid, the method of Monsigny et al. [194] can be used to determine
1502 the neutral sugars content. The absorbance is classically measured at $\lambda = 510$
1503 nm and d-galactose is used as standard. The *meta*-hydroxydiphenyl (*m*-HDP)
1504 assay [195] modified by Filisetti-Cozzi and Carpita [196] can be used to measure
1505 the uronic acids content. In presence of potassium sulfamate and *m*-HDP, uronic
1506 acids form chromogens which absorb at $\lambda = 525$ nm. In general, d-galacturonic
1507 acid is used as standard. It is noteworthy that the corrective formula from
1508 Montreuil and Spick [197] should be used to accurately quantify both neutral
1509 sugars and uronic acids in the same sample since the latter can be detected by
1510 the resorcinol-sulfuric acid assay.

1511
1512 **4.4.3.3 Substituents and non-carbohydrate content**

1513 Sulphate, pyruvate, methyl and acetyl groups have also to be quantified. The
1514 sulphate groups can be measured by using the turbidimetric protocol of Craigie
1515 et al. [198], estimated by the gelatin/BaCl₂ assay [199] or the Azure A method
1516 of Jaques et al. [200]. Pyruvate groups can be measured through the method of
1517 Sloneker and Orentas [201]. Methoxy and acetyl groups are often observed by
1518 ¹H NMR since these groups have specific resonances. However, a saponification
1519 step can be applied to release -CH₃ and -COCH₃ groups, which can be directly or
1520 indirectly monitored by High-Performance Liquid Chromatography (HPLC)
1521 [202,203]. Even if the extraction step and more specifically alcoholic
1522 precipitation (see the previous section) decreases the presence of proteins
1523 and/or glycoproteins, total proteins (which can also be covalently linked to
1524 polysaccharides) can be quantified by the Smith et al., Bradford or Lowry et al.
1525 methods [95,99,204]. For determining the polyphenol content, the Folin-
1526 Ciocalteu assay can be used from [205]. Finally, salts, ashes and moisture
1527 should be also quantified.

1528
1529 **4.4.3.4 Composing groups by Infrared spectroscopy**

1530 Fourier Transformed InfraRed (FTIR) spectroscopy can be used for identifying
1531 and/or discriminating polysaccharides as well as observing changes in their
1532 composition (footprint) by providing details on a range of vibrationally active
1533 functional groups [206]. The structural analysis of carbohydrates by FTIR
1534 spectroscopy focuses on five specific regions from 3600 to below 700 cm⁻¹, which
1535 correspond to O-H and C-H stretching vibrations, local symmetry, C-O stretching
1536 vibration and fingerprint or anomeric region and skeletal region [207].
1537 Polysaccharides usually display a strong and broad absorption peak at 3360 cm⁻¹
1538 corresponding to (O-H) stretching vibrations as well as water adsorption
1539 [208,209]. A weak signal at 2920 cm⁻¹ can be attributed to the asymmetric
1540 vibration of (C-H₂) groups [210,211]. Overall, the region from 1500 to 500 cm⁻¹

1541 is defined by several modes concerning the type of polysaccharides and
1542 glycosidic linkages. As an example, furanose derivatives from pentoses can be
1543 identified at 1250 cm⁻¹. At 1250-1170 and around 1300-1250 cm⁻¹, aliphatic
1544 esters and aromatic esters can be respectively observed. Sulphate ester (O-SO₃⁻)
1545 groups can be identified in the region from 1260 to 1210 cm⁻¹ [212], carboxylic
1546 acid ester form (C=O) or carboxylate anion form (COO⁻) can be observed around
1547 1730 and 1600 cm⁻¹ [213] whereas acetyl groups can be found at 1250-1220
1548 cm⁻¹. The signal observed around 1040 cm⁻¹ is in general from the presence of
1549 (C-O) of polysaccharides [214]. Anomeric (α and β) configurations can be
1550 identified by three types of bands from 950 to 750 cm⁻¹ [215]. Note also that
1551 glycosidic methoxy groups give a specific symmetric C-H₃ stretching band around
1552 2880-2830 cm⁻¹. FTIR spectroscopy also benefits the possibility to detect
1553 proteins, nucleic acids (NA) and other potential "contaminants" of the
1554 carbohydrate fraction through the amide I (1709-1583 cm⁻¹), amide II (1585-
1555 1481 cm⁻¹) and NA (1356-1191 cm⁻¹) bands [216].
1556

1557 **4.4.4 How to determine the monosaccharides composition of** 1558 **polysaccharides?**

1559 **4.4.4.1 Preliminary solvolysis**

1560 Monosaccharides have to be released by cleaving the glycosidic bonds before
1561 their analyses by Chromatography. Two preliminary solvolysis, *i.e.* (i)
1562 methanolysis and (ii) hydrolysis, are well described in the literature for this
1563 purpose. The first one involves the cleavage of the linkages by methanolic HCl
1564 (around 2 M) in milder conditions than for classical hydrolysis. Less degradation
1565 of monosaccharides to furfural derivatives (or more degraded products) is often
1566 reported and it is possible to analyze both neutral sugars, uronic acids, sialic
1567 acids and acetamido sugars. Moreover, methyl groups substitute the hydroxyl
1568 groups previously engaged in the glycosidic bonds [217]. Briefly, the protonation
1569 of the glycosidic-O-atom results to the cleavage of the glycosidic linkage from a
1570 specific ring form. An anomeric carbocation will be formed and be able to react
1571 with water and give α/β -anomers. Thus, ring form conversions can occur through
1572 protonation of the ring-O-atom and/or mutarotation [132]. On the other hand,
1573 hydrolysis by mineral acids (H₂SO₄, HCl, CF₃COOH) can be used to release the
1574 monosaccharides. Numerous conditions are reported in the literature and the use
1575 of TriFluoroacetic Acid (TFA) is often preferred (2-4 M, 90-240 min, 100-120 °C).
1576 Indeed, some authors reported the degradation of monosaccharides in particular
1577 when strong conditions are used to ensure the cleavage of resistant glycosidic
1578 linkages [218]. The effect of these conditions should not be neglected since it
1579 may cause underestimation of specific monosaccharides and more specifically
1580 ketoses. Here again, released monosaccharides from the polysaccharide
1581 structure give a monosaccharide-specific anomeric distribution. Finally, methyl
1582 glycosides or released monosaccharides can be analyzed by Gas Chromatography
1583 (GC) or HPLC including High-Performance Anion-Exchange Chromatography
1584 (HPAEC).
1585

1586 **4.4.4.2 Chromatography**

1587 First, monosaccharides composition can be determined by HPLC combined with
1588 various detection apparatus such as Refractive Index Detection (RID), Charged
1589 Aerosol Detection (CAD) or Low-Wavelength Ultraviolet (UV). HPLC coupled to
1590 ElectroSpray Ionization (ESI) or High-Resolution Mass Spectrometry (HRMS)
1591 should be also considered as decent methods reported in the literature. One
1592 main advantage of LC methods is probably from the sample preparation which is

1593 strongly reduced compared to classical derivatizations of monosaccharides
1594 needed for GC analysis. On the contrary, one main disadvantage could be the
1595 low resolution especially for complex mixtures [219]. Overall, the separation is
1596 based on polarity allowing to work with normal and Reversed-Phase
1597 Chromatography (RPC). Ions-exchange, polymer-, silica- or metal-based and
1598 amino-bonded can be used with heating (30-80 °C) under various mobile phases
1599 (diluted H₂SO₄, water, acetonitrile) for the quantification of the released
1600 monosaccharides. Few papers also reported derivatization of monosaccharides
1601 prior the analysis [220]. HPAEC methodology should be also considered as a
1602 first-rate alternative for quantifying monosaccharides (using CarboPac PA-1), as
1603 reported by Templeton et al. [221]. Coupled with Pulsed Amperometric Detection
1604 (PAD), the method is resolute and based on the pK_a of monosaccharides and
1605 changes in temperature or alkalinity. Schäeffler et al. [222] reported tuning
1606 procedures to improve HPAEC analyses for carbohydrates even if Fu and O'Neill
1607 [220] highlighted the drop of electrochemical response in presence of proteins.
1608 Secondly, GC is still used in many papers for quantifying monosaccharides after
1609 solvolysis due to higher sensitivity and resolution of spectra [223]. On the
1610 contrary to HPLC, released monosaccharides must be converted into volatile
1611 compounds *via* derivatization procedures including silylation and acylation. Silyl
1612 derivatives, which are the most used for GC applications, are generated by
1613 displacement of active hydrogen on -NH, -OH or -SH groups and various
1614 derivatizing agents can be used for this purpose, *e.g.* *N*-methyl-*N*-trimethylsilyl
1615 trifluoroacetamide (MSTFA), *N*-trimethylsilylimidazole (TMSI) or *N,O*-bis-
1616 (trimethylsilyl)trifluoroacetamide (BSTFA). The latter is often combined to
1617 trimethylchlorosilane (TMCS) to increase the silyl donor strength. Thus,
1618 TriMethylSilyl (TMS) alditols, aldonitrile acetates, TMS methyl ester glycosides or
1619 oxime derivatives can be prepared for GC analysis. In general, Flame-Ionization
1620 Detector (FID) is preferred to MS for the quantification since the results are more
1621 reproducible. As reported by Peña et al. [224], modifying the mass tuning and
1622 the possibility to use ion suppression strongly impact MS quantification and can
1623 result in inaccurate quantification. Note that MS benefits mass spectra
1624 (associated to specific fragmentation patterns) which are essential for
1625 discriminating carbohydrates to non-carbohydrate contaminants. Finally, GC/MS
1626 experiments can be performed using MS-Chemical Ionization (CI) or Electron
1627 Impact Ionization (EI). Pyranose and furanose ring forms can be distinguished
1628 comparing the intensities of the specific ions *m/z* 204 and *m/z* 217 for TMS
1629 (methyl) glycosides [217] as well as regarding the ion *m/z* 205. Ions such as *m/z*
1630 379, 319, 305, 217, 205, 204, 117 are characteristics of the fragmentation of
1631 TMS (methyl) glycosides by GC/MS-EI [152]. Beside to TMS derivatives, the
1632 conversion of released monosaccharides to *per-O*-acetylated alditol (AA)
1633 derivatives is another way to quantify monosaccharides composition [224]. Each
1634 monosaccharide gives only one peak with specific fragmentation patterns and
1635 main ions such as *m/z* 289, 217, 187, 157, 115, 145, 103, 43. Overall, a wide
1636 range of columns can be used for GC/MS-EI analysis, *e.g.* AT-1 [132], HP-1, CP-
1637 Sil 5 CB, DB-1701 [225], DB-225MS [221], Optima-1MS [226], OV-17 [227] or
1638 SP 2330 [224].

1639 **4.4.5 How to elucidate the branching patterns of polysaccharides?**

1640 **4.4.5.1 Absolute configuration analysis**

1641 Usually, GC and chiral stationary phase are used for determining the absolute *d*
1642 or *l* configuration of monosaccharides. Glycosidation reactions can also be used
1643 with a chiral alcohol ((-)-2-butanol or (+)-2-octanol) to generate an extra chiral
1644

1645 center, allowing the use of a nonchiral stationary phase for analyzing TMS (-)-2-
1646 butyl or (+)-2-octyl glycosides [132].

1647

1648

1649

1650

1651 **4.4.5.2 NMR analysis**

1652 NMR is probably one of the best tools for establishing native glycan structures of
1653 polysaccharides [228,229]. Two decades ago, Duus et al. [230] published an
1654 extensive review giving a deep overview of how to determine the structure of
1655 carbohydrates by NMR. Basically, 1D NMR (^1H and ^{13}C) spectroscopies give
1656 primary information about the structure and conformation, *e.g.* the type of
1657 monosaccharides, anomeric configurations, branching types, presence of
1658 substituents such as sulphate, methyl, acetyl or carboxyl groups [132]. Indeed,
1659 signals at specific resonance frequencies (shifts) are from carbon and non-
1660 equivalent hydrogen and the shape of each signal corresponds to the chemical
1661 environment of the corresponding carbon and hydrogen [231]. Considering the
1662 wide literature and numerous fingerprints spectra (^1H and ^{13}C) of polysaccharides
1663 described, authors usually compare chemical shift values for attribution. Today
1664 and especially for new and/or original structures, homo- and heteronuclear 2D
1665 techniques should be performed to assess and clarify ambiguous assignments
1666 and give accurate and deep understanding of polysaccharides linkages [232]. For
1667 NMR users, partial depolymerization of the analyzed polysaccharide (combined to
1668 a long accumulation at 60 °C), *e.g.* by using hydrolysis, ozonolysis, chemical
1669 degradation or hydrogenation, should be done since it greatly helps solubilizing
1670 the polymer (molecular weight under 100 kDa), increasing the quality and
1671 accuracy of NMR spectra and thus facilitating the structural elucidation. Note that
1672 various conditions should be performed since hydrolysis for example is well-
1673 known for removing some branching residues, side chains and/or terminal units
1674 [233]. Mass Spectrometry (MS) should also be used to analyze kind of
1675 derivatives.

1676

1677 **4.4.5.3 Mass spectrometry analysis**

1678 MS applications can be combined with a broad range of chromatographic
1679 technologies such as MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization
1680 with Time of Flight mass spectrometry), GC/MS-CI, HPLC-ES-MS, HPAEC-
1681 PAD/MS, HPLC-ESI-MS-MS, etc. [132]. MALDI-TOF MS, electrospray ionization
1682 mass spectrometry as well as GC/MS-EI are often preferred for the structural
1683 studies of glycans. The latter involves the preparation of PerMethylated Alditol
1684 Acetates (PMAA) which can be used to determine terminal units, glycosidic
1685 linkages, branching points and the ring size of monosaccharides. Before
1686 hydrolysis, permethylation of the polysaccharide is done by using CH_3I in alkaline
1687 medium, *e.g.* solid NaOH in $\text{C}_2\text{H}_6\text{OS}$ [224]. As previously described, various
1688 conditions of hydrolysis can be used but 2-4 M TFA give good depolymerization
1689 results. Reduction using (NaBH_4 or NaBD_4 for isotopic labeling in $\text{NH}_4\text{OH}/$
1690 $\text{C}_2\text{H}_6\text{OS}$) then peracetylation $(\text{CH}_3\text{CO})_2\text{O}$ in presence of co-catalyst $\text{C}_4\text{H}_6\text{N}_2$) are
1691 performed to yield PMAA. Interpreting the fragmentation patterns from mass
1692 spectra (mostly without molecular ion peaks) leads up to determine the position
1693 of glycosidic bonds initially composing the polysaccharide. A comprehensive
1694 study of these fragmentations patterns and tables can be found in Kamerling and
1695 Gerwig [132]. Further analyses are needed to complete the initial
1696 monosaccharides linkages analysis such as (i) uronic acid reduction due to their

1697 non-esterified carboxyl function and low volatility, (ii) partial hydrolysis to
1698 determine repeating unit patterns, (iii) acetolysis, (iv) uronic acid, smith and
1699 periodate degradations, (v) partial alkaline hydrolysis, (vi) removing of
1700 substituent(s) and/or (vii) enzymatic depolymerizations for determining anomeric
1701 configuration.

1702

1703 **4.4.6 Conclusion**

1704 In the emerging field of glycosciences the specific detection and quantification of
1705 polysaccharides from microalgae is in progress even if the cracking of
1706 polysaccharidic structures is always a real challenge. However the recent interest
1707 of scientific and industrial communities for the culture of microalgae (mainly for
1708 production of high value molecules) led to a better understanding of the
1709 physiology of these microorganisms and to the development of technologies for
1710 their culture. At this time the ability of scientific community to cultivate in
1711 controlled conditions numerous strains of microalgae isolated from diverse
1712 environments open the way to identification of new polysaccharides with original
1713 structures and physico-chemical properties. Many exciting applications after
1714 analysis of polysaccharides from microalgae will probably become evident with
1715 the development of essential tools for their structure analysis and modeling.

1716

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1722

4.5 Methods for lipids determination in microalgae

1723

[Junko Ito, Makoto M. Watanabe]

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Sources of lipids include soybean, corn, palm, canola, and jatropha as oleaginous plants and animal fat and waste cooking oil [234-236]. It is also widely anticipated that microalgae can be used as a new lipid source [235, 237,238]. Microalgae grow rapidly, and many species are exceedingly rich in oil. Microalgae typically double their biomass within 24 to 72 h, and the doubling times of the fastest-growing algae are as short as 3.5 h during the exponential growth phase. The oil content of microalgae is 20–50 %, and some microalgae can produce oil content of 80% per weight of dry biomass [233,237-241]. Therefore, the oil productivity of microalgae (the mass of oil produced per unit volume of broth per day) can reach tens to hundreds of times higher than that of oil crops.

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Lipids accumulate in microalgae in the forms of acylglycerols (triacylglycerol, diacylglycerol, and monoacylglycerol), phospholipids, glycolipids, lipoprotein, free fatty acids (FFA), sterols, hydrocarbons, and pigments [242]. **Fig. 4.5.1** shows the chemical structures of major representatives from microalgal lipids. This section explains qualitative and quantitative analyses of these lipids (the summary shown in **Fig. 4.5.2**).

1738

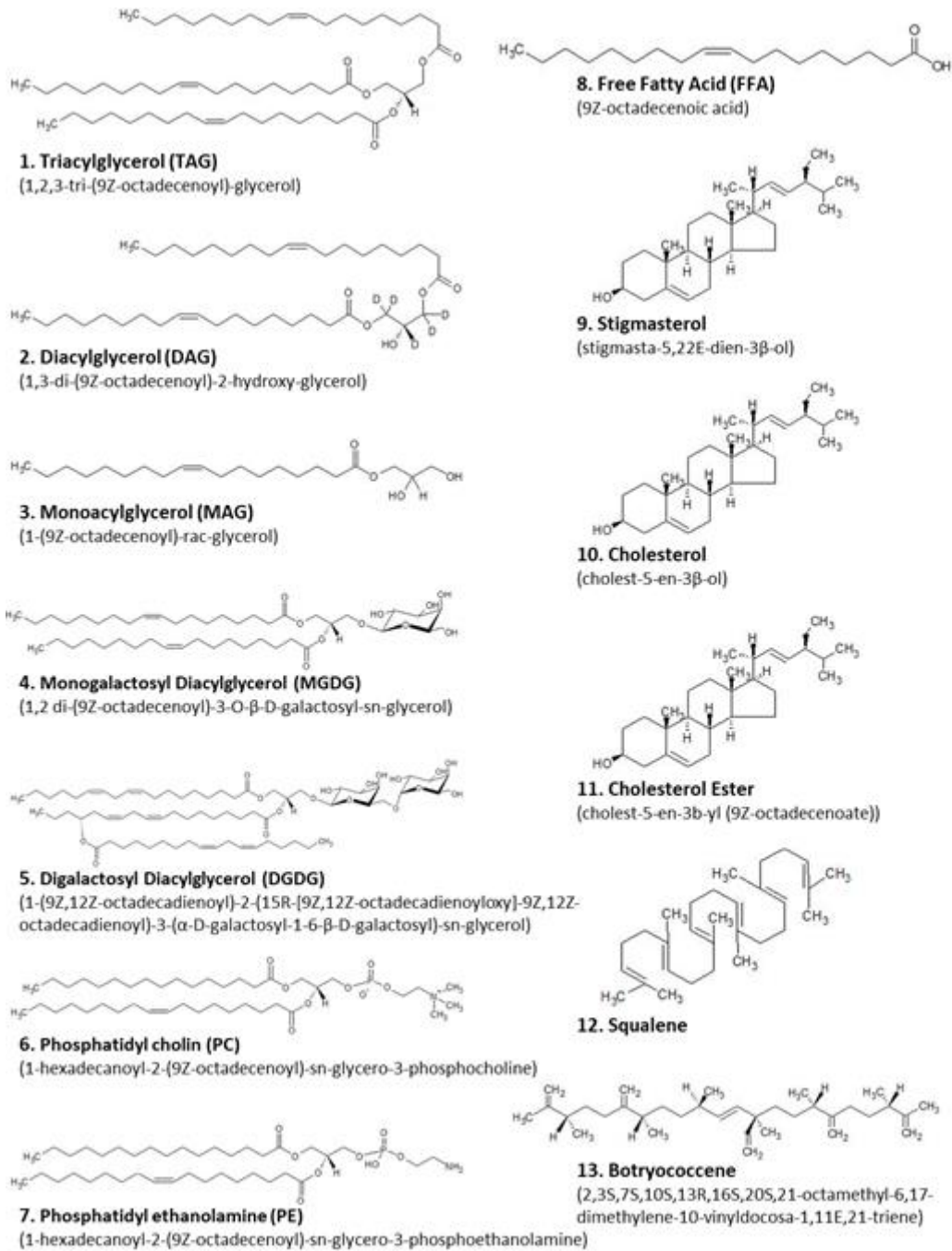
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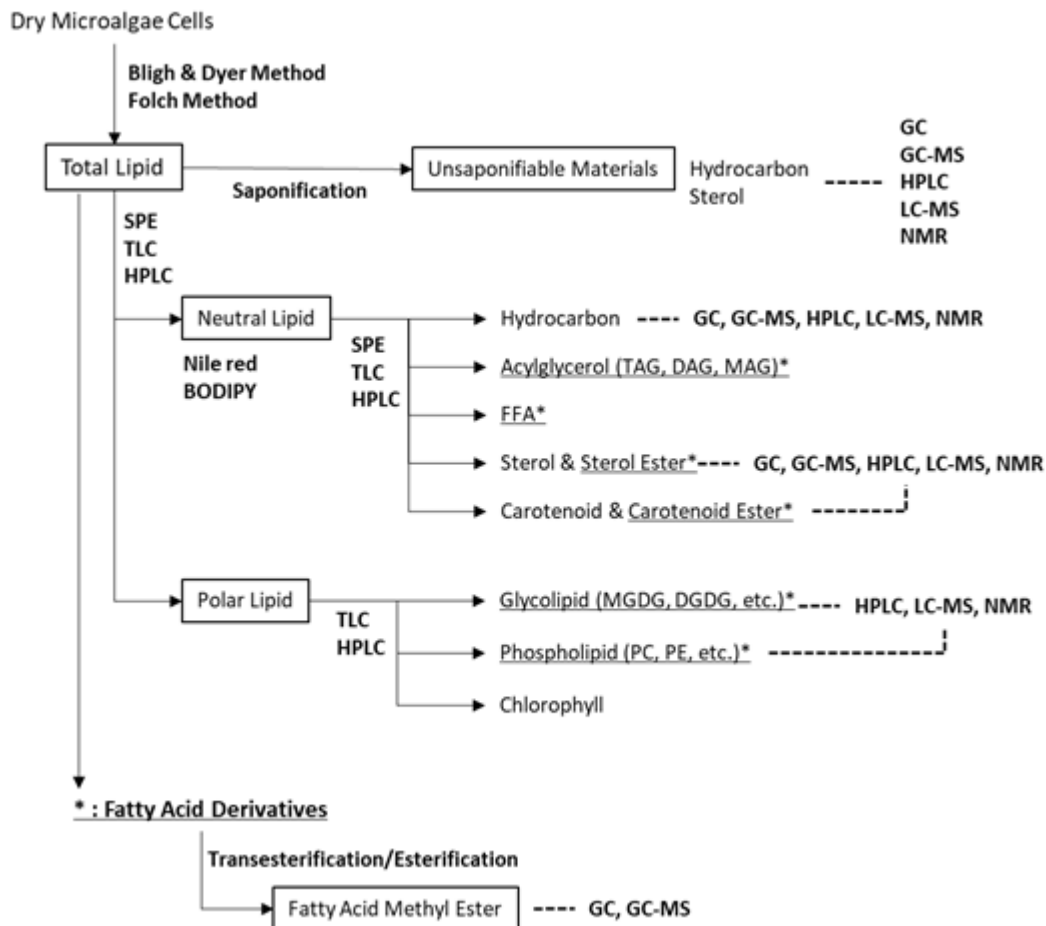
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Fig. 4.5.1: Chemical structures of the major representatives from microalgal lipid. Structures derived from www.lipidmaps.org.



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Fig. 4.5.2 The summary of lipid analysis steps and available analysis technics. SPE: Solid Phase Extraction, TLC: Thin-Layer Chromatography HPLC: High-Performance Liquid Chromatography, GC: Gas-Chromatography, MS: Mass Spectrometry, NMR: Nuclear Magnetic Resonance, TAG: Triacylglycerol, DAG; Diacylglycerol, MAG: Monoacylglycerol, FFA: Free Fatty Acid MGDG: Monogalactosyl Diacylglycerol, DGDG: Digalactosyl Diacylglycerol, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine

4.5.1 Preparation of microalgal samples

Microalgae are a highly diverse group of organisms, having a wide range of different cell walls, and various molecular components, intramolecular and intermolecular linkages, and overall structures [243,244]. The cell wall characteristics and chemical compositions vary not only according to the microalgal species but also depending on the growth conditions and growth phase [243,245]. These differences affect the barrier of intracellular lipid recovery and the ease of cell disruption.

The basic microalgal samples for lipid analysis are dried cells. To obtain such samples, microalgae are cultivated in medium and harvested by centrifugation. The obtained pellet is then washed with sterile water or medium, and the wet cells are obtained after centrifugation. Although the wet cells can be dried by either heating [246-247] or lyophilization, the optimal approach is lyophilization

1776 [248]. After washing, the wet pellets are stored at $-80\text{ }^{\circ}\text{C}$. These frozen pellets
1777 are then lyophilized in a lyophilizer. This removes water effectively without
1778 reducing the quality of the molecular components, compared with drying using
1779 heat. Lyophilization also increases the surface area of the sample, leading to
1780 better lipid extraction and easy rehydration. Lyophilized cells are also easily
1781 weighed, stored, and handled [248,249]. Furthermore, lyophilized cells contain
1782 very little water, and there is no need to add lipase deactivators during lipid
1783 extraction.

1784
1785 Oil extraction from wet microalgae has been a subject of intense investigations
1786 [237-242,250-256]. These techniques are very important for practical and
1787 scalable processes for extracting lipids from microalgae because the drying of
1788 microalgae involves high energy costs. Some lipid analyses can use wet cells
1789 without any dehydrating, but there is a need to pay close attention to the cell
1790 disruption and the efficiency of oil extraction [237, 242].

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1792

1793 **4.5.2 Analysis of total lipid content**

1794 Lipids are defined as being molecules that are not soluble in water but in
1795 nonpolar solvent instead, although the definition is relatively vague [19]. In this
1796 section, the entire oil content of microalgae is defined as total lipids. The total
1797 lipid content in microalgae is a very important value for considering the
1798 characteristics of microalgae and the possibility of practically using them as a
1799 lipid source.

1800
1801 The standard method of analyzing total lipid content is the method of Folch et al.
1802 [255], or that of Bligh and Dyer [256], involving the use of chloroform-methanol
1803 mixture as an extracting solvent [247,248]. The co-solvent containing methanol
1804 as a polar solvent can extract not only neutral lipids like hydrocarbon and
1805 acylglycerols but also polar lipids like glycolipids and phospholipids. Furthermore,
1806 the polar solvent releases the lipids from their protein-lipid complexes, and the
1807 lipids subsequently dissolve in the nonpolar solvent [248,254]. Modified versions
1808 of the methods of Bligh and Dyer [256] or Folch et al. [255] have also been
1809 proposed in many studies [19, 243,247,248,250,251,257-262].

1810 Ryckebosch et al. [248] explained that the method of Folch et al. [255] is the
1811 most widely used for the extraction of total lipids from microalgae. This method
1812 was originally optimized for the isolation and purification of total lipids from
1813 animal tissues. It uses chloroform/methanol at a 2:1 ratio for the extraction of
1814 lipids and water along with a small amount of NaCl to remove non-lipid
1815 substances from the extract. The method of Bligh and Dyer has also often been
1816 used for the extraction of total lipids from microalgae and was originally
1817 optimized for the extraction of phospholipids from fish muscle. It uses
1818 chloroform/methanol at a 1:2 ratio and followed by extraction with chloroform.
1819 Iverson et al. [256] compared the methods of Bligh and Dyer [256] to Folch et
1820 al. [255] for the determination of total lipids in marine fish tissue. They
1821 concluded that the unmodified method of Bligh and Dyer resulted in lower
1822 estimation of lipid content in samples containing $>2\%$ lipid, and this
1823 underestimation increased significantly with increasing lipid content of the
1824 sample.

1825
1826 Ryckebosch et al. [248] showed that the total lipids could be extracted from
1827 lyophilized microalgae through them being immersed in a chloroform/methanol

1828 mixture, without any cell disruption. However, some samples need to undergo
1829 cell disruption before the oil extraction process. This is largely influenced by the
1830 cell wall characteristics, which depend on the microalgal species, the growth
1831 condition and phase, and the existence and intensity of stress factors [243].
1832 When the level of oil recovery is low, cell disruption such as bead beating,
1833 microwave irradiation, sonication, and exposure to a homogenizer should be
1834 performed as pretreatment for oil extraction. The presence of lipids in microalgal
1835 cells can be evaluated by the fluorescence measurement of Nile red- or BODIPY-
1836 stained cells [246,263,264]. These dyes can stain the lipids inside cells,
1837 especially neutral lipid droplets. After cells have been stained with Nile red or
1838 BODIPY, the fluorescence measurement or microscopic observation of stained
1839 microalgae is performed. In this context, one option is first to immerse the
1840 lyophilized microalgae in co-solvent and extracted oil and then to evaluate the
1841 oil-extracted cells by fluorescence analysis. When there is residual oil inside the
1842 cells, the microalgal samples should be disrupted as pretreatment for oil
1843 extraction.

1844
1845 Chloroform and methanol can be used to extract oil from microalgae, but these
1846 organic solvents are associated with serious risks. For example, chloroform has
1847 been classified into Group B2 in risk assessment for carcinogenic effects, that is,
1848 probably carcinogenic to humans, by the US EPA [259]. Moreover, methanol is a
1849 flammable solvent and damages the visual system of animals [265]. These
1850 solvents should be carefully handled to avoid damaging the environment and
1851 human health. For this reason, other solvents have been investigated as less
1852 toxic substitutes for lipid extraction [251,259,263,265-268]. However, it has
1853 been difficult to determine the best solvents because of different results being
1854 obtained depending on the microalgal species, growth conditions, and growth
1855 phase. The current best way of determining the total lipid amount is to follow the
1856 methods of Folch et al. [255,] or Bligh and Dyer [256]. Finally, the following
1857 should be kept in mind: all extraction processes may not recover all lipids and
1858 may extract non-lipid components, such as chlorophyll, pigments, protein, and
1859 soluble carbohydrates, among the total lipids [19].

1860 **4.5.3 Separation and analysis of lipid classes**

1861 Lipids are generally separated into neutral lipids (hydrocarbons, acylglycerols,
1862 and sterol esters) and polar lipids (glycolipids and phospholipids). Neutral lipids
1863 such as triacylglycerol (TAG) are present in the cytoplasm as a form of energy
1864 storage, and polar lipids such as glycolipids and phospholipids are present in
1865 chloroplast membranes and in plasma membranes [269]. It is very useful to
1866 determine which lipid classes and how much of each class are contained among
1867 lipids extracted from microalgae, in order to characterize the extracted lipids,
1868 and to evaluate a method for applying and purifying these lipids. Solid-phase
1869 extraction (SPE) [248,269-271], thin-layer chromatography (TLC)
1870 [248,259,270,272], high-performance liquid chromatography (HPLC) [273-275],
1871 and nuclear magnetic resonance (NMR) spectroscopy [275] are well-known
1872 methods for isolating lipid classes, such as neutral lipids, glycolipids, and
1873 phospholipids, from among extracted lipids.

1874
1875 SPE is a simple and effective method for determining lipid class composition
1876 [248,269-271]. This method has the advantage that the amount of each lipid
1877 class can be determined gravimetrically and yielded as a fraction for further
1878 detailed analysis. Guckert et al. [269] reported an isolation method using simple
1879 silica column chromatography for lipid class separation. In this case, the total

1880 lipids were applied to a silica column, and elution with chloroform yielded the
1881 neutral lipids, acetone yielded the glycolipids, and methanol yielded the
1882 phospholipids. An easier SPE method involves the use of a commercial cartridge.
1883 The major SPE cartridge is Waters Sep-Pak™ of silica gel or Alltech Extract-Clean
1884 column for the separation of lipid classes [248,270-272].
1885

1886 TLC is also a typical method for lipid class separation [248,259,272]. Yao et al.
1887 [272] reported that a spot of total lipids was applied on a silica TLC plate and
1888 developed by a mixed solvent of hexane/diethyl ether/acetic acid. This was
1889 separated into TAG and FFA as neutral lipids, and polar lipids including, sterols,
1890 and chlorophyll derivatives. The separation could be visualized by spraying with
1891 staining reagents or by using ultraviolet light. The separated spots were scraped
1892 off the plate and extracted in solvent.
1893

1894 The major usage of TLC for lipid separation is to analyze the profile of neutral- or
1895 polar-lipid fractions [248,259,272]. After separation of lipid classes by the SPE
1896 method, each fraction is applied to TLC for profiling of the lipid composition in
1897 more detail. TLC for neutral lipids can separate TAG, diacylglycerol (DAG),
1898 monoacylglycerol (MAG), FFA, carotenoids, and sterols. TLC for polar lipids can
1899 separate monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol
1900 (DGDG), among others, as glycolipids, and phosphatidylcholine (PC) and
1901 phosphatidylethanolamine (PE), among others, as phospholipids.
1902

1903 HPLC with evaporative light-scattering detectors (ELSD) can identify major lipid
1904 classes in total lipids [273-275]. The HPLC method can be fully automated and
1905 has higher separation capacity. Furthermore, each fraction of lipid classes can be
1906 easily collected for further identification and analysis of molecular species. Jones
1907 et al. [273] showed that normal-phase HPLC with ELSD can identify and quantify
1908 all major lipid classes in total lipid extracts. The used column was polyvinyl
1909 alcohol bonded on a silica stationary phase, such as YMC Pack PVA-Sil-NP. This
1910 method covers the lipid classes of nonpolar groups (hydrocarbons, sterol esters,
1911 and wax esters), sterols, chlorophyll, acylglycerols (TAG, DAG, and MAG), FFA,
1912 glycolipids (MGDG and DGDG), and phospholipids (PC, PE, etc.) in a single
1913 chromatographic run.
1914

1915 NMR spectroscopy measures the interaction of nuclear spins under a powerful
1916 magnetic field and can identify the molecular structure of materials. Nuzzo et al.
1917 [276] showed that proton NMR (¹H NMR) spectroscopy using the electronic
1918 reference to access *in vivo* concentration (ERETIC) method could identify and
1919 quantify all lipid classes (TAG, PL, GL, and FFA). Total lipids from microalgae
1920 were measured by ¹H NMR spectroscopy, and these spectra were compared with
1921 those of standard compounds under the same conditions. Some peaks showed
1922 pronounced overlaps, such as aliphatic and olefinic protons, but each class of
1923 molecules showed diagnostic signals that could be selected as NMR markers of
1924 each lipid class. This method allows assessment of the total lipid content and also
1925 of the degree of saturation.
1926

1927 **4.5.4 Analysis of fatty acid composition and content**

1928 For the commercial application of microalgae, a wide range of products are
1929 available, such as biofuels, feeds, fertilizer, chemical feedstock, nutrient
1930 ingredients, and raw materials for cosmetics and drugs [240,241]. It is very

1931 important to determine the fatty acid composition and content of microalgae
1932 when considering their applications.

1933

1934 The analysis of fatty acids can be conducted on the lipids extracted from
1935 microalgae and lipids separated into lipid classes. It is very difficult to analyze
1936 fatty acids in crude extracted lipids without any pretreatment using analytical
1937 equipment directly. The standard method is first to convert lipids to fatty acid
1938 methyl esters (FAME) through transesterification and then to analyze FAME by
1939 gas-chromatography (GC) [84,248,259,261,262,264,266,270,272,277,278].

1940 Carrapiso et al. [278] reported the principles of transesterification by acidic or
1941 basic catalysts. In an acidic or basic methanol solution, the fatty acid derivatives
1942 (TAG and phospholipids) are changed to FAME. Major catalysts are hydrochloric
1943 acid (HCl) [259,262], sulfuric acid (H₂SO₄) [248,261,262,271,272], and boron
1944 trifluoride (BF₃) [262,276] as acids, and sodium hydroxide (NaOH), potassium
1945 hydroxide (KOH), and sodium methoxide (NaOCH₃) as bases.

1946

1947 Acidic catalysts are most widely used for microalgal fatty acid analysis. They
1948 have strong esterification power, reacting with not only TAG and phospholipids
1949 but also FFA and N-acyl lipids (sphingolipids). Furthermore, there is less water
1950 interference than for basic catalysts. The method of Christie [270] referenced by
1951 many papers [248,261,262,271,272,277] uses H₂SO₄ as a catalyst. H₂SO₄ has a
1952 longer life of catalytic activity and involves safer preparation than HCl. In the
1953 case of BF₃, it has higher esterifying power than H₂SO₄ and can be used to
1954 analyze a small amount of sample [264]. However, BF₃ cannot be used for
1955 water-containing samples because it is rapidly destroyed by water [278,279].

1956

1957 Basic catalysts have the advantage of having a high reaction speed and relatively
1958 mild heating conditions, compared with acidic catalysts [278,280,281].
1959 Furthermore, for lipids containing acid-labile fatty acids (cyclopropane rings,
1960 epoxy groups, or conjugated unsaturated molecules), it is better for the analysis
1961 to be performed under basic than acidic conditions. However, basic catalysts
1962 cannot react with FFA and sphingolipids, and the saponification reaction occurs in
1963 the presence of water.

1964 A method combining the use of both basic and acidic catalysts is the American
1965 Oil Chemists' Society method [282]. This is now recognized as a standard
1966 method for oil and lipids, which means that it is not specific to microalgal oil. This
1967 method involves first transesterification (or saponification) by NaOH in methanol,
1968 followed by esterification by BF₃ in methanol [264,282,283]. Yi et al. [284]
1969 applied KOH in methanol to fish oil, which contains high levels of
1970 docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), prior to BF₃ in
1971 methanol. *Schizochytrium* sp. can produce higher levels of multiple
1972 polyunsaturated fatty acids such as DHA, EPA, and docosapentaenoic acid. For
1973 the analysis of fatty acids produced by *Schizochytrium* sp., KOH and BF₃ were
1974 used in methanol [285,286].

1975

1976 The FAME obtained by transesterification/esterification can be analyzed by GC-
1977 flame ionization detector (FID) or GC-MS
1978 [84,248,259,261,262,264,266,270,272,277,278]. Before sample analysis, each
1979 fatty acid should be identified by GC or GC-MS by comparing the retention times
1980 with those of FAME standards. In the case of the quantification of fatty acids in
1981 lipids, an internal standard should be added in samples before the
1982 transesterification/esterification reaction. The FFA, methyl ester or TAG of

1983 pentadecanoic acid (C15:0), heptadecanoic acid (C17:0), and tricosanoic acid
1984 (C23:0) were used as internal standards [261,262,264,271,277,282,283]. The
1985 internal standard should be a saturated fatty acid that is not included among the
1986 sample lipids.

1987
1988 *In situ* (direct) transesterification is a method of applying methylation agent
1989 directly to the biomass and reducing the extraction steps [262,264,287-292]. To
1990 dry microalgae, catalyst solution with methanol is added, followed by incubation
1991 with heating. Excluding the lipid extraction process leads to less organic solvent
1992 and reduces the process time. Acidic catalysts such as BF₃ [262,264,290], HCl
1993 [262,288,290], and H₂SO₄ [289,290,292] are commonly used. Cavonius et al.
1994 [262] compared 11 methods to analyze fatty acids in three different microalgae.
1995 These were four different conventional methods of extraction prior to
1996 transesterification, two types of two-step transesterification (saponification
1997 followed by esterification), and five different *in situ* transesterifications. Their
1998 results showed that the conventional method resulted in underestimation of the
1999 fatty acid content compared with two-step and *in situ* transesterification.
2000 Regarding the catalyst, HCl and H₂SO₄ could recover the most fatty acids,
2001 whereas BF₃ recovered slightly less. Velasquez-Orta et al. [291] performed a
2002 comparison between a basic catalyst (NaOH) and an acidic catalyst (H₂SO₄) for *in*
2003 *situ* transesterification of *Chlorella vulgaris* and concluded that the basic catalyst
2004 outperformed the acidic one, obtaining higher conversion at shorter reaction
2005 times. At any rate, *in situ* transesterification is very useful to obtain FAME for
2006 analyses of fatty acid composition and content. The obtained FAME can be
2007 analyzed by GC-FID or GC-MS.

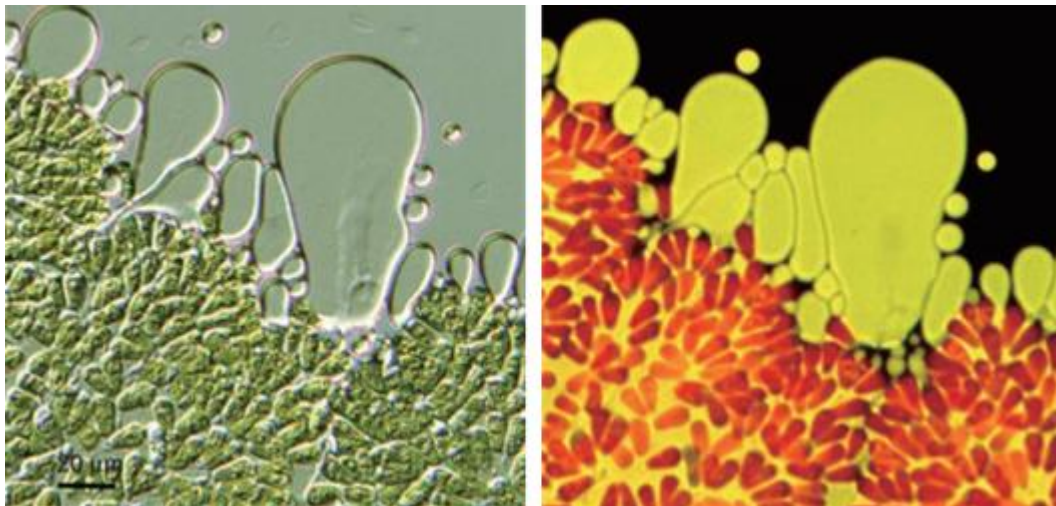
2008
2009 Compared with GC methods, HPLC has the advantage in fatty acid analysis that
2010 higher-molecular-weight and non-volatile materials can be analyzed without
2011 raising the temperature to a high level. LC methods have been employed for
2012 fatty acid analysis of FFA, TAG, phospholipids, and others [293-295]. The fatty
2013 acid analysis of FFA using GC requires the derivatization of fatty acids (FAME) by
2014 thermal and chemical reactions and high-temperature operation for volatile
2015 FAME. The high temperature is associated with a risk of isomerization of
2016 saturated fatty acids [293]. On the other hand, LC can be applied to FFA analysis
2017 directly without any derivatization [295] or with a derivatization reaction at a
2018 lower temperature [293,294]. LC-MS and LC-MS/MS have been employed for
2019 lipid analysis without additional separation and derivatization steps [296-298].
2020 Samburova et al. [297] analyzed the quantification of algal TAG, and the
2021 characterization and identification of TAG structures using direct LC-MS and
2022 MS/MS analysis. The advantage of this method is that the lipids are injected into
2023 the LC instrument without any derivatization, and that the structure of individual
2024 TAG can be estimated. These techniques have also been used in lipidomics
2025 research.

2026
2027 **4.5.5 Hydrocarbon analysis**
2028 Some microalgal species can produce hydrocarbon oil. The green alga
2029 *Botryococcus braunii* is characterized by its ability to produce and accumulate a
2030 large amount of hydrocarbons, known as botryococcene [299-302]. This alga is
2031 subclassified into four chemical races (A, B, L, and S), according to the
2032 hydrocarbon structures [302,303]. *B. braunii* Race A produces normal alkenes of
2033 an odd number of carbons in the range of C₂₅-C₃₁ with two to three carbon
2034 double bonds. Race B produces triterpenic hydrocarbons of C_nH_{2n-10} (n = 30-37).

2035 Race L produces tetraterpenic hydrocarbons of lycopadiene (C₄₀H₇₈) [304]. Race
2036 S comprises epoxy-n-alkane and saturated n-alkane chains with carbon numbers
2037 of 18 and 20 [303]. Another well-known alga producing hydrocarbons is
2038 *Aurantiochytrium* sp., which produces squalene [305-308]. Squalene is also a
2039 polyunsaturated triterpenic hydrocarbon (C₃₀H₆₀). These hydrocarbons can be
2040 used in the cosmetic industry as moisturizing agents [305] and are expected to
2041 be an alternative petroleum fuel and chemicals, among others.

2042
2043 Hydrocarbons are classified as neutral lipids. The hydrocarbons inside microalgal
2044 cells can be stained using Nile red or BODIPY. In the case of *B. braunii*,
2045 hydrocarbons are mainly accumulated in the extracellular space and stained by
2046 Nile red [299,309]. This fluorescence measurement or microscopic observation of
2047 stained microalgae can be used to estimate the presence of hydrocarbons (**Fig.**
2048 **4.5.3**).

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Fig. 4.5.3: *Botryococcus braunii*

Left: photomicrograph of colony

Right: fluorescence micrograph of Nile Red stained cell (yellow fluorescence indicates oil)

2058 In general, the level of hydrocarbons in lipid-extracted microalgae is low because
2059 major components in lipids are those with fatty acids, such as TAG, GL, and PL.
2060 Hydrocarbons should be analyzed using lipids after pretreatment to remove the
2061 fatty acids. There are three major methods for purifying hydrocarbons: (1) TLC,
2062 (2) column chromatography, and (3) saponification.

2063
2064 TLC is a typical method for the separation of lipid classes. TLC for neutral lipids
2065 can separate TAG, DAG), MAG, FFA, carotenoids, and sterols. Hydrocarbons can
2066 also be separated by TLC [310-312]. Nakazawa et al. [310] used TLC for the
2067 screening of thraustochytrid strains that produce squalene. The lipids extracted
2068 from lyophilized thraustochytrid cells by Folch's method [255] were subjected to
2069 TLC and hydrocarbon was separated. The existence of hydrocarbons could be
2070 estimated by the clarity and strength of the hydrocarbon spot in TLC. The
2071 hydrocarbon spot on TLC can also be picked up and used for further detailed
2072 analysis. Cavanagh et al. [312] used TLC-FID for the analysis of biodegradation
2073 hydrocarbons. The lipids were developed by TLC, and separated hydrocarbons

2074 were detected by a FID, which is well-known as a GC detector. This method
2075 involves not only qualitative analysis but also rough quantitative determination.
2076 TLC is a simple method and can be completed in a short time. When the analyses
2077 of many samples are needed in screening and there is a need to obtain rough
2078 results in a short time, TLC is a very good option.
2079

2080 SPE is also a simple and effective method for analyzing lipid class composition. In
2081 this approach, neutral lipids or lipids extracted using hexane are applied to a
2082 silica column, and elution with hexane yields hydrocarbons. This method can be
2083 performed gravimetrically and yields a fraction for further detailed analysis. Many
2084 studies of hydrocarbons produced by *Botryococcus* sp. and *Aurantiochytrium* sp.
2085 have been conducted using the SPE method, with analyses of the quantity,
2086 identity, and structure of separated hydrocarbons by GC, GC-MS, HPLC, LC-MS,
2087 and NMR [302,305-308,313-318].
2088

2089 Saponification can be used to make soap through the reaction of fatty acids with
2090 a base. Fatty acids in lipid extracted from microalgae are changed to soap by
2091 saponification and can be removed by washing because soap more easily
2092 dissolves in water. Meanwhile, hydrocarbons are not changed by saponification
2093 and can be recovered in organic solvents like hexane. Many studies
2094 of hydrocarbons produced by *Botryococcus* sp. and *Aurantiochytrium* sp. have
2095 been conducted using the saponification method [301,308,319,320].
2096 Saponification is useful for removing fatty acid materials and recovering
2097 unsaponifiable matter such as hydrocarbons and sterols.
2098

2099 The purified hydrocarbons were subjected to quantification, identification, and
2100 structural determination by GC, GC-MS, HPLC, LC-MS, and NMR. When the
2101 hydrocarbons extracted from microalgae were unknown materials, GC-MS or LC-
2102 MS was useful for the identification of each hydrocarbon. More information on
2103 unknown materials such as their structure can be obtained by NMR spectroscopy
2104 after further purification of hydrocarbon. In the case of botryococcene, there are
2105 no standard reagents on the market. Furthermore, hydrocarbons produced by
2106 *Botryococcus* sp. have many types of molecular weight and structure.
2107 Identification of these hydrocarbons has been conducted not only by analysis of
2108 purified hydrocarbons using GC-MS and NMR spectroscopy but also by GC-MS or
2109 NMR spectroscopy after ozonation of hydrocarbon [318,321] or hydrogenation
2110 [316,320]. Quantification of hydrocarbons can be performed using an external or
2111 internal standard method. The added internal standards were reported to be
2112 triacontane [301] or squalene [313] for botryococcene by *Botryococcus* sp., and
2113 squalene [322-324], heptadecanyl stearate [325], and 5 α -cholestane [272,326]
2114 for squalene, by GC or GC-MS analysis. The internal standard for HPLC or LC-MS
2115 analysis [327] was octadecylbenzene for squalene [319].
2116
2117

2118 **4.5.6 Phytosterol analysis**

2119 Phytosterols are mainly found in the cellular membranes of microalgae and one
2120 of the most interesting compounds for plant-based ingredients [328].
2121 Phytosterols have many health-promoting effects in humans, due to their ability
2122 to lower cholesterol levels, their antioxidant activity, and their reduction of
2123 inflammation and cancer risk [329]. As the phytosterol content in microalgae is
2124 equal to or higher than those in all plant oils extracted, microalgae have high
2125 potential to be a source of phytosterols.

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There are more than 100 different types of phytosterols. Phytosterols are present in lipid-extracted microalgae. The analysis of phytosterols can be performed by the same methods as for hydrocarbons. Such analyses should be applied to lipids after their pretreatment to remove fatty acid materials. Purification of phytosterols is conducted by TLC, column chromatography, and saponification. The purified phytosterols are subjected to quantification, identification, and structural determination by GC, GC-MS, HPLC, LC-MS, and NMR spectroscopy. The GC analysis requires chemical derivatization, with trimethylsilyl ether (TMS) or acetate derivatives, prior to analysis [328,330,331]. Quantification of phytosterols can be performed using an external or internal standard method. Internal standards were reported to include 5 α -cholestane [330], cholesterol [332], and 5 β -cholestan-3 α -ol [333] for GC analysis and 6-ketcholestanol [334], 1-hexacosanol [335], and d6-cholesterol [336] for LC analysis.

2141

2142 **4.6 Methods for pigments determination in microalgae**

2143 [Benoît Serive]

2144
2145 Organic pigments are widespread molecules in the whole biodiversity and
2146 particularly in microalgae diversity [337] since they play crucial roles in
2147 photosynthesis and photoprotection. Pigments from microalgae belong mostly to
2148 carotenoids (carotenes and xanthophylls), chlorophylls, and phycobiliproteins.
2149 However, the full definition of pigments include various molecule scaffolds such
2150 as anthocyanosides, betalains, some alkaloids (e.g. scytonemin), amino-acids
2151 (e.g. mycosporines-like, eumelanin), azaphilones, polyphenols (e.g. floridorubin,
2152 quercetin), anthraquinones (e.g. hypericin), or chlorines (e.g. purpurin-18).
2153 Since phycobiliproteins have already been presented previously in this chapter,
2154 we will focus essentially on carotenoids and chlorophylls.

2155 Since ancient times, civilizations have used pigment like Tyrian purple from
2156 gastropod molluscs or other organisms for clothes dyeing. Then, the link between
2157 food and health has been documented since Antiquity (*i.e.* Hippocrates
2158 associated the food to health and diseases) [338]. Moreover, Aztecs were one of
2159 the civilizations to use spirulina as food source. Apart from the fact this
2160 cyanobacteria is protein-rich source, it contains pigments known nowadays with
2161 health benefits. Indeed, these last decades, numerous scientific studies have
2162 shown interesting properties from pigments through notably antioxidant, anti-
2163 inflammatory, anti-AMD, anti-tumoral, anti-angiogenic, anti-diabetic, anti-
2164 obesity, anti-photoaging, and neuroprotective activities. Chlorophylls are more
2165 recommended notably to stimulate liver function recovery and increase bile
2166 secretion [339]. Thus, today, pigments found applications in chemical,
2167 pharmaceutical, poultry, cosmetics, cosmeceutical, functional food, nutraceutical
2168 and aquaculture industries [342-340]. Today, the chemical synthesis market
2169 covers about 90 % of the main carotenoid needs (e.g. asthaxanthin, β -caroten).
2170 However, natural sourcing benefits from a positive image since Western
2171 countries consumers are more and more sensitive to the impact of chemicals
2172 onto their health. The all-trans isomer of β -caroten is still chemically synthesized
2173 but the 9-cis form is produced only from natural sources, the natural form
2174 displaying better antioxidant capacity than the synthesized form. Although the
2175 natural pigment sources still compete with synthetic dyes, with the high
2176 development of agri-food industry, natural pigments are more and more used for
2177 coloring of food preparations and for their antioxidant properties. Beta-carotene
2178 market value has been estimated at US\$ 261 million in 2014. With an annual
2179 growth rate of 3 %, it is expected to reach US\$ 334 million by 2018 [343]. This
2180 is favored by stringent government regulations regarding environmental and
2181 human health hazards (Global Market Insights Inc.[344]).

2182
2183 Although yeast, fungi, or bacteria allows the production of carotenoids [347-345]
2184 microalgae biomass remains one of the best way to produce industrial amounts
2185 of natural pigments [348]. Some phytoplankton strains are easily cultivable in
2186 large open ponds. Countries like Israel, Australia, USA, China, or India became
2187 pioneers/leaders on this market [349]. Pigment-rich microalgae can be also
2188 produced in other ways like photobioreactors systems (PBRs), chemostat,
2189 turbidostat, flat-panel (*i.e.* green wall panel), disposable polymer bags, open
2190 tank or raceway, tubular glass PBR etc (batch, continuous, or semi-continuous
2191 mode).

2192 Whatever the mode of production, microalgae biomass collected has to be
2193 extracted and more or less purified according to the customer needs (as B2B or
2194 B2C). Pigments of interest, targeted applications, and production scale drive
2195 strongly the strategy to choose in term of extraction and purification. Meanwhile,
2196 characterization of targeted pigments has to be harnessed properly while
2197 acknowledging the limits of available detection and quantification techniques.
2198 Adapted extraction technique to the biomass is crucial since there is a strong
2199 relation between the amount detected and the capacity to extract targeted
2200 analytes. One of the first questions for choosing a technique is: Is my metabolite
2201 of interest sensitive to dessication, heat, oxidation, or light? For pigments like
2202 carotenoids, chlorophylls and phycobiliproteins, the answer is yes most of the
2203 time. Various techniques are available from laboratory scale to industrial scale
2204 but they are more or less efficient for large biomass and to preserve sensitive
2205 pigments. Moreover, at industrial scale, economic dimension has to be taken into
2206 account to make the whole process viable.

2207

2208 **4.6.1 Extraction**

2209

2210 **4.6.1.1 Centrifugal Partition Extraction**

2211 Also called Centrifugal Partition Chromatography (CPC) is one of the various
2212 techniques constituent of Countercurrent Chromatography (CCC). This is a liquid-
2213 liquid partitioning chromatography that uses no solid phase as the liquid
2214 stationary phase is retained in a series of channels connected by ducts, engraved
2215 on disks, which rotate around a single axis. According to the centrifugational or
2216 centripetal direction of the mobile phase, there is a descending or ascending
2217 mode to work with this equipment. Co-current method can be applied as well.
2218 This technology has been used for the continuous extraction of numerous natural
2219 products including pigments [350]. CPC is particularly adapted to extraction and
2220 purification with level up to 99.9 % (Kromaton data). Solvent choice, flow rate
2221 and rotational speed can be tuned to optimize the extraction of valuable
2222 metabolites.

2223

2224 **4.6.1.2 Supercritical CO₂ extraction**

2225 This technique (SC-CO₂) can be considered as a green strategy to reach
2226 microalgae metabolites since CO₂ gas stream is recyclable. However, prior drying
2227 or lyophilisation step is required to implement this kind of extraction. There is a
2228 large panel of equipment available from laboratory scale (*e.g.* lyophilisators,
2229 centrifugal evaporators) to industrial scale (*e.g.* vacuum drying, spray drying,
2230 extrusion porosification technology). However, dewatering pre-treatment add a
2231 substantial cost to the entire process, which is not suitable for low valuable
2232 products. In case of pigments with high added value for nutraceutical,
2233 cosmeticeutical, or pharmaceutical application, the additional cost can be
2234 acceptable. In this particular case, SC-CO₂ stays promising in term of carotenoid
2235 extraction. Thus, numerous studies have demonstrated these last years that SC-
2236 CO₂ is quite selective to extract carotenoids like β -carotene, lutein, astaxanthin,
2237 or zeaxanthin over chlorophylls from various microalgae species [351-354]. The
2238 use of ethanol as entrainer (co-solvent) is particularly efficient. However
2239 vegetable oils (*e.g.* soybean or olive oil) can be used as co-solvent in order to
2240 avoid the subsequent separation step of the co-solvent, *i.e.* the oil enriched with
2241 pigments can be sold straight away [355].

2242

2243 **4.6.1.3 Milking**

2244 This technique has been developed to extract β -carotene from *Dunaliella salina*
2245 living cells in a two phase bioreactor [356,357]. One phase is aqueous, where
2246 biomass is growing. The second phase is an organic solvent with value of Log
2247 P_{octanol} , which denotes hydrophobicity of the compound of interest. Log P_{octanol}
2248 must be >6 in order to be compatible with the species. Dodecane, tetradecane,
2249 or hexadecane are generally used for their potential to extract selectively
2250 carotenoids. This technique has the disadvantages to be not environmentally
2251 friendly and difficult to scale up.

2252

2253

4.6.1.4 Accelerated Solvent Extraction

2254 Also known as pressurized liquid extraction (PLE), this technique is based on high
2255 pressure that forces the solvent into the matrix, whereas high temperature
2256 promotes high analyte solubility, decreases the viscosity and decreases the
2257 surface tension of the solvents. This technique has the advantage to use less
2258 solvent and being less time-consuming over the simple maceration. This
2259 technique can be coupled with the use of GRAS solvent (Generally Recognized As
2260 Safe) such as ethanol and limonene. A pre-treatment prior extraction like
2261 freezing-thawing can be performed to improve the extraction yield [358].
2262 Extraction temperature, solvent composition and extraction time are parameters
2263 to watch to optimize the extraction process. However, due to the relative high
2264 temperature of this technique, it is not recommended to use for sensitive
2265 pigments.

2266

2267

4.6.1.5 High-speed homogenization

2268 This technique causes cavitation and shear stress. Our own experience showed
2269 that this technique is not applicable for all types of microalgae since the
2270 microalgae cell wall, frustule or theca can wear blades prematurely (unpublished
2271 data). Moreover, we doubt the real efficiency of this technique to treat large
2272 biomass and the energy cost would be high to treat microalgae at large scale. If
2273 for niche application this technique has to be explored, blade design and
2274 material, speed, time, microalgae amount (or flow), growth phase and
2275 conditions, and microalgae type have to be studied. One other disadvantage of
2276 this technique is the thermal heating, which occurs quickly, and makes it not
2277 compatible with extraction of sensitive pigments.

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4.6.1.6 Microwave Assisted Extraction (MAE)

2280 This extraction technology is based on microwave irradiation and can be declined
2281 in vacuum microwaves-assisted extraction (VMAE). While the first one is simple
2282 and economical, the second one can be onerous at industrial scale. This
2283 technique has the advantage to reduce solvent consumption and extraction
2284 times. Over the fact that the heating by irradiation is homogenous, temperature
2285 of the extraction medium can be regulated. That makes this methodology
2286 interesting for pigment protection against chemical transformations. This
2287 technique has been applied successfully on the unfrustulated chlorophyte
2288 *Dunaliella tertiolecta* and on the frustulated diatom *Cylindrotheca closterium*
2289 [359]. MAE seems particularly efficient when a mechanical resistance such as
2290 frustule of diatoms limits the solvent access into the cell. The optimization of this
2291 technique requires the control of agitation, dry weight, growth phase and
2292 conditions, microalgae type, power of microwave, solvent volume and time
2293 (intermittency ratio).

2294

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4.6.1.7 Sonication

2296 This technique so-called ultrasound-assisted extraction (UAE) based on acoustic
2297 cavitation leading to cell rupture is not suitable for microalgae with thick wall
2298 and/or surrounded by polysaccharides like *Porphyridium purpureum* [360]. One
2299 disadvantage of this technique is that a hot spot at the top of the probe can
2300 reach a temperature estimated about 5000 °C due to the rapid adiabatic
2301 compression of gases and vapors within bubbles or cavities. Another one is that
2302 free radical reactions leading to metabolite oxidation can occur if a small amount
2303 of water is present in the extraction batch [361]. In biorefinery, this technique
2304 can be used as pretreatment to reach lipids notably but we do not consider this
2305 technique for pigment extraction regarding the whole diversity of microalgae.
2306 However, in case of a particular interest in this methodology, in order to improve
2307 its efficacy, cycle number and time, dry weight, growth phase and conditions,
2308 microalgae type, and power of ultrasound have to be tested.

2309

2310 **4.6.1.8 Pulsed Electric Field**

2311 The goal is to apply a medium or high current field on the treatment chamber
2312 (0.3 to 1.5 kV/cm), which cause an electroporation allowing a selective
2313 extraction or an electroplasmolysis for an intense extraction. Formation and
2314 propagation of streamer and arc happens followed by the formation of the vapor
2315 cavities and a shock wave [362]. Treatment time is around 1 second per batch.
2316 Thus, there is no issue in term of energy consumption. Some essays have shown
2317 that this technique is better efficient on big cells rather than little particles like
2318 bacteria and viruses. In conclusion, this technology is not universal (species
2319 dependent). It has to be tested for the species and pigments targeted. In the
2320 framework of optimization of this process for microalgae biomass, conductivity
2321 (electrolyte concentration), current, dry weight, growth phase and conditions,
2322 microalgae type, oscillation, and time should be studied. With this technique,
2323 there is a risk to heat quickly the treated biomass. In some conditions, this
2324 technology can be used as pretreatment of another extraction technique.

2325

2326

2327 **4.6.2.9 Enzyme Assisted Extraction**

2328 Phycobiliproteins are hydrosoluble molecules and can be extracted from
2329 microalgae by bead milling without a preliminary drying step. Due to the
2330 presence of large amounts of anionic cell-wall polysaccharides, this extraction
2331 can even be improved by enzymatic hydrolysis. While proteases are generally
2332 quite efficient to extract bioactive components from algae biomass, in the case of
2333 phycobiliprotein extraction, cellulase, xylanase or β -glucanase are preferable to
2334 improve extraction yield [363]. Inactivation of enzymes at the end of the process
2335 is crucial. Most of the time, temperature and pH are two parameters allowing
2336 inactivation of enzymes. For instance, in the case of alcalase, 30 minutes at 50
2337 °C, pH 4, or 10 minutes at 85 °C, pH 8 are generally recommended (Univar
2338 data). If immobilized enzymes are used (e.g. lipases), the advantage is the
2339 possibility to re-use the enzymes up to 100 times in theory. Except for
2340 phycobiliprotein pigments, there is a lack in literature for carotenoids and
2341 chlorophylls regarding the use of enzymes as pre-treatment to improve their
2342 extraction yields from microalgae. Due to its biological specificity, mild operating
2343 conditions, low energy requirements, low capital investment, there is a great
2344 potential for biomass valorization. Moreover, this is a way to explore further
2345 since lysozyme has shown interesting results in extracting chlorophyll-*a*,
2346 chlorophyll-*b* and total carotenoids [364]. In a context of exploration of effective
2347 parameters for such a process, agitation, dry weight, enzyme concentration,

2348 enzyme type (*i.e.* currently, no enzyme is particularly dedicated to microalgae
2349 biomass), growth phase and conditions, microalgae type, oxygen level, type and
2350 amount of buffer, temperature, pressure, and time have to be taken into account
2351 in the design of experiment (DOE). Today, the main limitation in using enzymes
2352 in microalgae extraction may be the high cost to treat large volumes.
2353

2354 **4.6.1.10 Bead Milling**

2355 This is one of the most promising techniques in term of pigment extraction yield
2356 both at laboratory scale and industry scale. It generates mechanical compaction
2357 and shear stress for cells. This technique has shown excellent results with a
2358 diatom and also especially with *Porphyridium purpureum* which is known to be
2359 quite resistant to disruption [360]. Laboratory scale equipment is based on
2360 agitation of a chamber or tubes filled with beads (glass, steel, ceramic, or
2361 zirconium material), while industrial scale equipment is based on a mixing
2362 chamber filled with beads and biomass to extract.

2363 For this process, agitation disk design, speed, bead filling, size, material,
2364 microalgae amount (or concentration), feed rate, growth phase and conditions,
2365 microalgae type, time, and cooling are parameters to optimize. Our background
2366 experience has shown that it is preferable to work on a wet biomass rather than
2367 a lyophilized biomass to allow a good extraction yield. Associated with the proper
2368 solvent or buffer targeting the pigments of interest, this technique is quite
2369 suitable for most pigment projects. For microalgae biomass, a couple of studies
2370 support the idea that mechanical grinding is often the most effective technique to
2371 recover metabolites efficiently, including pigments [47,360]. However, energy
2372 requirement is a very important parameter when large biomass is treated. It
2373 seems that mechanical disruption methods are considered highly energy
2374 inefficient [365]. Thus, bead milling should be preferred for high value
2375 compounds.
2376

2377 **4.6.1.11 Soaking**

2378 Solvents are still widely used to extract pigments as additives for coloring food
2379 and beverages from other source than microalgae (carrot, turmeric,
2380 spinach/nettle, alphantha) [366]. Polar solvents (*e.g.* acetone, ethanol, ethyl
2381 acetate) are appropriate for extraction of polar carotenoids while hexane is
2382 frequently selected to extract carotenes. Moreover, solvents like DMSO or a
2383 solvent mix (hexane/acetone/ethyl alcohol) have been used to extract
2384 chlorophylls and carotenoids like astaxanthin in order to evaluate the influence of
2385 media and environmental factor allowing their production [367-369]. Soaking in
2386 solvent can be used for microalgae with a thin wall (*e.g.* most of Chlorophyceae,
2387 Trebouxiophyceae, Prasinophyceae, Mamiellophyceae, and
2388 Chlorodendrophyceae). However, generally speaking, applied alone, soaking is
2389 not suitable to extract pigments efficiently since pigments can be deeply buried
2390 and bound within the ultrastructure, which is it-self protected sometimes by
2391 strong walls. Most of solvents are produced from petrochemistry. Since it is not
2392 economically viable to use high purity grade solvents (*e.g.* HPLC/MS grades) to
2393 extract large biomass, the risk is to concentrate heavy metals with compounds of
2394 interest. One of the rules in eco-extraction is to favor the use of alternate
2395 solvents from sustainable agro-resources (biobased), which are renewable and
2396 biodegradable. Ethanol and ethyl acetate are ones of the most green and
2397 sustainable solvents. It still used to extract active components from biomass but
2398 it is more and more avoided. Moreover, in the current framework of the
2399 European regulation (novel food), no more than 10 ppm of solvent is allowed in

2400 the extracts. In brief, solvent removal is crucial. Instant Controlled Pressure Drop
2401 technique is one of the strategies available in that case.

2402

2403 **4.6.1.12 Instant controlled pressure drop**

2404 This green technology allows both drying (swell-drying) in order to obtain powder
2405 with good functional properties, and the extraction of metabolites like pigments.
2406 This process can be applied after mechanical dewatering (e.g. pressing,
2407 centrifugation, filtration). There is no particular need to reach a high level of
2408 drying. According to the targeted metabolites, organic solvent or water can be
2409 used [370]. One of the advantages of this technology is the preservation of
2410 sensitive molecules. Currently there is few literature about pigment extraction
2411 from microalgae [342]. However, this process has been applied successfully on
2412 microalgae biomass for cosmetics industry. In order to make this process
2413 efficient, cycle number, biomass, time of treatment, number of cycles, growth
2414 phase and conditions, microalgae type, and pressure have to be watched. More
2415 information is available at www.abcar-dic.com.

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2418 **4.6.1.13 Chemical treatment**

2419 This strategy is more used as pretreatment rather than a stand-alone extraction
2420 technique. For instance, to improve extraction of astaxanthin from thick-walled
2421 cysts of *Haematococcus* cells, prior extraction with acetone, cells can be treated
2422 with organic and mineral acids (e.g. hydrochloric acid) at 70 °C (1 hour).
2423 Treatment time, temperature, and concentration of the acid are critical factors
2424 for an optimized extractability [371]. However, acidic conditions are not suitable
2425 if chlorophylls are targeted since they are instable at pH under 5. Alkaline
2426 hydrolysis (0.1 M NaOH) have been also tested previously [358].

2427

2428 **4.6.1.14 Others**

2429 At laboratory scale, other techniques may be used for valuable compound
2430 extraction. Some are not desirable and/or very used anymore for carotenoids
2431 and chlorophylls. This is the case of heat reflux extraction (too degradative),
2432 liquid-liquid extraction in separating funnel. Solid phase extraction (SPE) is not
2433 very used as a stand-alone extraction for pigments. However SPE can be used as
2434 purification technique or as pretreatment before analysis. QuEChERS method
2435 (Quick, Easy, Cheap, Efficient, Rugged and Safe) is derived from SPE originally
2436 dedicated to the detection of pesticides [372]. An adaptation of this technique
2437 may be an alternative at laboratory scale in the future. Then, some industrials
2438 claim that spray drying (originally dedicated for drying) may be used as a
2439 disruption method applicable to microalgae biomass. This technique may be used
2440 to extract broadly main pigments. Some authors have reported a pairing of
2441 several techniques. For instance, astaxanthin extraction from *Chlorococcum* sp.
2442 has been performed successfully (7.09 mg.g⁻¹ DW) coupling solvent system with
2443 methanol/dichloromethane (75:25), French pressure cell (110 MPa), and a
2444 saponification [373]. A two-stage extraction of free astaxanthin with dodecane
2445 and subsequent NaOH-added methanol (0.02M) has been developed. This
2446 consists of two extraction units (i) a dodecane unit for astaxanthin mixture and
2447 (ii) a methanol unit for free astaxanthin [374]. This methodology closely related
2448 to milking has the disadvantage to exclude reuse of red cyst cells for continuous
2449 astaxanthin production and not be a green extraction process.

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4.6.1.15 Biorefinery

Techniques presented above give some hints to overcome the extraction of valuable pigments from microalgae biomass (**Fig. 4.6.1**). However, extraction scale-up stays not trivial. A good review regarding cell disruption for microalgae biorefineries has been published by Günerken et al. [375] including a classification of the cell disruption methods. Although the techniques are not focused on pigments, this may be useful for projects seeking to valorize pigments from microalgae. Outsourcing of compounds of interest extraction can be cost-effective, especially for early stage of projects, since a couple of companies are dedicated to assist and co-develop flexible and qualitative solutions for drying, extraction, and separation/purification steps. The technical and economical challenges in co-extracting marine active ingredients are still ongoing. Carotenoids represent only 0.1 to 0.2 % of dry weight on average, and up to 14% of dry weight for β -carotene in some species like *Dunaliella salina* under stress conditions. Chlorophylls represent around 0.5 to 1 % of dry weight, and phycobiliproteins up to 8 % of dry weight [376]. These data have to be considered in the targeted applications and for the business model. Cracking strategy aims to valorize a maximum from the produced biomass, and not only a short part of it. Lipids can represent up to the half of the dry weight of microalgae [233] and their polarity is similar to those of organo-soluble pigments. The specificity to reach pigments without lipids is low. For applications, which do not need a high purity grade, development of products benefiting from both, some particular lipids (e.g. DHA - Docosahexaenoic acid), and pigments is a good and sustainable strategy. In the framework of a full exploitation of microalgae components, starch and proteins can be interesting beside oil extraction for bioethanol and biodiesel production [377]. Algae are chest of other various valuable metabolites like phenolic compounds, phytoene/terpenoids, phytols, sterols, photoprotective compounds, phytohormones, halogenated compounds, cyanotoxines, phycocolloids, polyhydroxyalkanoates, which may find applications in medicine, in nutraceuticals, in cosmeceuticals, in functional food industry, aquaculture, or agriculture [378,379].

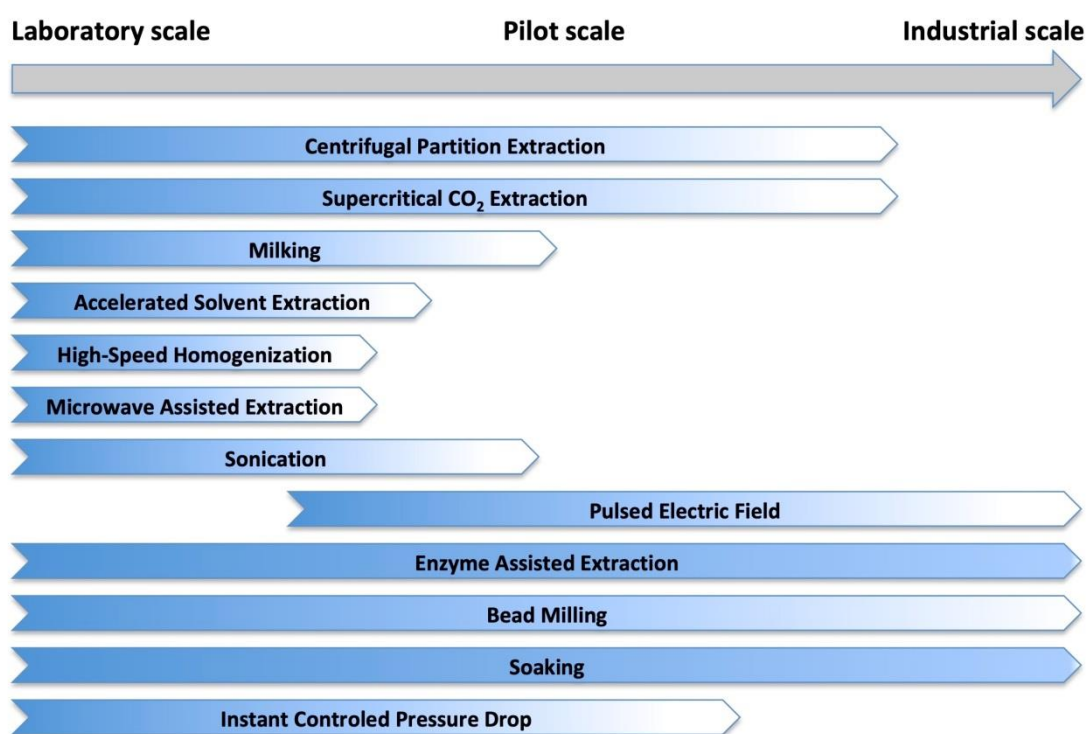


Fig. 4.6.1: Extraction techniques from laboratory to industrial scale.

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4.6.2 Pigment analysis

Disruption of a cellular system can lead to rapid degradation of carotenoids and chlorophylls, even at low temperature. Indeed, free pigments may be exposed to organic acids from the extracted cells, which can cause isomerization and rearrangement of 5,6-epoxy- to 5,8-epoxycarotenoids (e.g. in violaxanthin and neoxanthin). Free pigments are more sensitive to oxidations and UV light as well. In order to minimize pigment degradation, it can be advised to add a neutralizer to the sample (calcium carbonate, sodium bicarbonate, or magnesium bicarbonate during the extraction. An antioxidant such as tert-butylhydroquinone, butylhydroxytoluene, pyrogallol, or ascorbyl palmitate can be added to the sample (around 0.1 % w/v). Azote can be flushed as well during the process, from extraction to the sample storage to limit oxidations. Then, samples should be protected against light and particularly UV since they can cause trans-cis-photoisomerization and photodestruction [380]. Microalgae enzymes have to be mentioned as well since some of them can degrade pigments. It is the case with chlorophyllases, which are particularly present in diatoms [381]. They degrade chlorophyll-*a* to chlorophyllide-*a*, which modify the perception of the culture/biomass growth phase (chlorophyll-*a* ratio). A serious issue is that chlorophyllase can be activated by harvesting techniques before the extraction step. Moreover, the latter can amplify the phenomenon except if acetone is used, which inhibits the enzymes. Thus, pigment analyzes should be performed as early as possible or samples should be stored at -80 °C for a short period before analyzes.

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4.6.2.1 Spectrophotometry analysis

Pigment analysis science benefits from oceanography research, which aims to study phytoplankton pigments for the understanding of population dynamic and biogeography. Several techniques have been developed these last decades. With the improvement of the equipment accuracy, high performance liquid chromatography (HPLC) methods hyphenated with UV-VIS detector are the gold standard to detect and quantify known carotenoids and chlorophylls. Amongst all the existing methods, some are kind of reference in oceanography. Eleven international laboratories specialized in the determination of marine pigment concentrations using HPLC (*e.g.* LOV, CSIRO, DHI, NASA Goddard Space Flight Center) are regularly intercompared using *in situ* samples within the SeaHARRE studies (SeaWiFS HPLC Analysis Round-Robin Experiment) [380]. These laboratories use the following validated methods:

- Gieskes and Kraay, 1989 [383]
- Wright et al., 1991 [384]
- Egeland et al., 1995 [385]
- Pinckney et al., 1996 [386]
- Vidussi et al., 1996 [387]
- Barlow et al., 1997 [388]
- Zapata et al., 2000 [389]
- Van Heukelem and Thomas, 2001 [390]

Each laboratory derived their analyzes method from one of the methods mentioned above. The most up to date and used method is those of Van Heukelem and Thomas [390]. This accurate method allows separating most of pigments properly using a liquid chromatography system hyphenated with a diode-array detector. It is nowadays widely used in oceanography for determining the phytoplankton composition and for estimating the biomass of the different algal groups. However, uncertainties may arise because of the partial separation of some derivative pigments (*e.g.* monovinyl and divinyl forms of chlorophyll *b*). Numerous guidelines regarding sample handling, storage, data reporting, procedures, quality assurance have been settled along the scientific community experience [391,392]. About detection, in a nutshell, 450 nm is often dedicated to detect specifically carotenoids, 665 nm for chlorophyll related pigments, 405 nm for chlorophyll derivatives (degradation forms), while 436 nm is commonly used because this wavelength provides good detection for most pigments. Regarding quantification, pigments are often reported to the sample dry weight. However, this measurement is strongly dependent of a proper drying which is not so reliable with microalgae, especially for short samples. Oceanographers are used to report pigments as a chlorophyll-*a* ratio. Even if chlorophyll-*a* allomers can be taken into account for the calculation, a bias can occur according to the growth phase of the species within the sample to study *i.e* chlorophyll-*a* and its derivatives can be degraded in various forms like pheophorbides and pheophytins, which escape to the ratio, especially during senescence. Pigment amounts can be reported to carbon unit. An elemental analyzer is required to study the samples. This method is very accurate and reproducible. However, since microalgae have a carbon composition, which is quite different according to the species, interspecies studies should be avoided using a pigment ratio related to carbon.

2567 Regarding quantitation, saponification has to be mentioned since this step can be
2568 added just before HPLC analyzes in order to make free carotenoid esters and
2569 thus, improve carotenoid content quantitation. Moreover, it is sometimes useful
2570 to remove chlorophylls and unwanted lipids which may interfere with the
2571 chromatographic carotenoid separation and shorten the column's life [393].
2572 However, some carotenoids (especially xanthophylls) and most chlorophylls are
2573 known to be sensitive to oxygen, light, acids, alkaline bases and high
2574 temperature. It is crucial to choose a gentle protocol to avoid pigment
2575 degradation. For instance, Yuan and Chen developed a method promoting the
2576 hydrolysis of astaxanthin esters, minimizing the degradation of astaxanthin, and
2577 avoiding loss of lutein, β -carotene and canthaxanthin during saponification [394].
2578 A special care with saponification is important to keep in mind if biological
2579 activity assays have to be performed afterwards, since this method can produce
2580 non-naturally occurring pigments. Chlorophyllins are semisynthetic derivatives
2581 obtained by saponification of chlorophylls and display various potent activities
2582 like antigenotoxic, antioxidant, inhibition of cancer initiation, and progression by
2583 targeting multiple molecules and pathways involved in the metabolism of
2584 carcinogens, cell progression, resistance to apoptosis, metastasis, and
2585 angiogenesis [393]. A study with Japanese patients with trimethylaminuria found
2586 that a chlorophyllin dietary supplement significantly decreased urinary
2587 trimethylamine concentrations [394].

2588 This is potentially true with some other molecules like lipids and carotenoids
2589 even if literature is not hearty yet.

2590

2591 **4.6.2.2 Other techniques for structural characterization and** 2592 **identification**

2593 While spectrophotometry is the gold standard to identify major known pigments
2594 using standards as reference, pigment diversity/complexity is hidden in the
2595 detection baseline and it is not always easy to identify them [337]. If main
2596 organo-soluble pigments in microalgae are about 40, minor/traces pigments are
2597 probably far beyond 150. For instance, Carotenoids Database [395] provides
2598 information on 1174 natural carotenoids from 699 source organisms (not only
2599 microalgae but also plants, animals, bacteria). Identification based on UV-VIS
2600 spectrum can be tricky. In that case, a large panel of physical methods is
2601 available. For an acceptable identification, ideally, a complete spectroscopic
2602 characterization (UV-VIS, masse spectrometry, ^1H and ^{13}C -NMR, circular
2603 dichroism for chiral compounds) is required. Total synthesis would confirm the
2604 full structure elucidation as well preferably [396]. However, it is not always
2605 possible to obtain all of these identification criteria since the purified amount can
2606 be low (between few μg to 1 mg at laboratory scale) and the native molecule
2607 sensitive to various factors mentioned previously. For subtle details of
2608 conformation, some other techniques can be cited like calorimetry,
2609 roentgenography, electronography, neutronography, spin electric resonance,
2610 molecular polarization, light diffusion or refraction [397]. Most of the time, these
2611 techniques are more accessible in academia than in industry. Fluorimetric
2612 methods can be considered anecdotal in carotenoid and chlorophyll identification.

2613

2614 Metabolomics approaches are increasingly becoming a powerful strategy in
2615 identification of metabolites with the recent advances of high sensitive
2616 spectroscopy methods. Amongst the various aspects this field, untargeted
2617 metabolomics is challenging. Although identification remains a bottleneck
2618 particularly for NMR-based dereplication strategies, MS and MS/MS based

2619 metabolomics made interesting progress these last years [398-400]. Some
2620 authors developed whole online workflow to analyze metabolomics datasets
2621 [401]. Some others developed the Global Natural Products Social Molecular
2622 Networking platform so-called GNPS [402]. Based on the use of MS public
2623 database and/or customized database, and the creation of molecular networks
2624 (ion fragments) using the software Cytoscape, this powerful open-source tool
2625 allows making identification hypothesis [403]. However, confidence in
2626 identification requires a special care to reach an acceptable level [404]. GNPS
2627 authors recommend notably a minimum of 6 MS/MS fragment ions to match in
2628 addition to the parent mass to decrease false discovery rates. They advise as
2629 well a cross validation of results with additional methods (*e.g.* retention time
2630 analysis, co-migration with standards, or subsequent isolation and NMR analysis)
2631 to validate the results. This very promising methodology can be applied to
2632 pigments using various MS technologies (MALDI-TOF, Q-TOF) and various
2633 ionization sources (APCI, APPI, +/-ESI) [405-410]. Coupled for instance with
2634 Carotenoids Database mentioned previously, results may be very interesting in a
2635 close future.

2636
2637 In conclusion, the panel of extraction techniques available is large. The selection
2638 of the most appropriate process depends of the production scale, the targeted
2639 pigments, the final application and the characteristics of the biomass to extract
2640 (humidity, rigid cell wall, polysaccharides). For instance, water miscible
2641 properties of acetone and ethanol helps in the efficient extraction of wet
2642 biomass. Some techniques like those using ionic liquids seem very promising but
2643 further advancements are needed for full safety applications. When pigments are
2644 extracted, analyses can be performed keeping in mind the bias related to
2645 identification and quantitation (possible degradation of most sensitive pigments,
2646 free and esterified forms, pigment-protein complexes, carbohydrates derivatives
2647 like glycosides and glycosyl esters). Cis isomers are also common isolation
2648 artefacts although some may be natural.

2649
2650
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2652
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2655 Program.
2656

2657

2658

2659 **4.7 Methods for secondary metabolites determination in**
2660 **microalgae**

2661 [Hugh D. Burrows, Maria G. Campos, Telma Encarnação, Alberto A.C.C. Pais]

2662
2663
2664

2665 **4.7.1 Introduction**

2666 Secondary metabolites, often referred to as natural products, are low molecular
2667 weight organic molecules produced by living cells [411] that, unlike primary
2668 metabolites, are not directly required for their growth, development and
2669 reproduction. They are produced by bacteria, fungi, algae and plants, often for
2670 biochemical interactions between organisms [412] and represent a wide variety
2671 of chemicals with diverse molecular structures. Many of these chemicals have
2672 been found to possess a variety of important pharmacological properties,
2673 including anticancer, antiviral, antibiotic, anti-inflammatory and other activities
2674 [413,414]. Frequently, these molecules have contributed to the cure of deadly
2675 diseases [413], such that biologically active secondary metabolites are a rich
2676 resource as potential new drug candidates.

2677 Around 70 000 microbial metabolites have been described to date. About 33 000
2678 of these exhibit some kind of biological activity; 1290 cyanobacterial bioactive
2679 metabolites have been reported, predominantly linked to a group of enzymes
2680 referred to as nonribosomal peptide synthases (NRPS), such as cyclic
2681 depsipeptides, polyketides derivatives [415] or a hybrid of the two. Other
2682 structural classes include alkaloids, shikimate-derivatives, aminoglycosides and
2683 more rare, terpenoids [416].

2684 Secondary metabolites have considerable commercial importance. In addition to
2685 their use in pharmaceuticals, these compounds find applications in many other
2686 areas, including cosmetics, agricultural, animal feed and food products, and
2687 chemicals.

2688

2689 **4.7.2 Sampling and Preconditioning**

2690 In the screening for new metabolites or in their production, cultures from
2691 microalgae collections, commercial suppliers or the isolation of indigenous wild
2692 type microalgae may be considered. Some of the databases for culture
2693 collections worldwide include the CCALA [415] in Trebon, Czech Republic, the
2694 SAG [418] in Göttingen, Germany, the ACOI [419] in Coimbra, Portugal, the
2695 CCAP [420] in Windermere, U.K., the Chlamydomonas Resource Center [421] in
2696 Minneapolis, U.S.A. and the UTEX [422] in Austin TX, U.S.A.

2697

2698 Isolation of microalgae strains into pure cultures include traditional isolation
2699 techniques, such as single-cell isolation by a micropipette, isolation of cells on
2700 agar plates, dilution method, gravity separation, phototaxis, and automated
2701 isolation techniques, such as flow cytometry cell sorting and optical trapping.
2702 Single-cell isolation by micropipetting is the most common method for isolation
2703 of microalgae strains. Basically, the isolation is performed using a Pasteur pipette
2704 or a glass capillary to collect a single cell from a sample and transfer it to a
2705 sterile droplet. A detailed description can be found in [423]. Flow cytometry cell
2706 sorting is an automated technique that can be used for isolation of small cells,
2707 especially those less than about 5 μ m, which are much more difficult to isolate
2708 by traditional techniques. Single cells can be identified and separated from

2709 contaminants and other cells, and sorted into multiwell plates for establishing
2710 new microalgae cultures [423]. Another automated technique is optical trapping,
2711 which is not a common technique, since the reaction centers of photosystems I
2712 and II are susceptible to photodamage. However, in a recent report [424], the
2713 non-invasive optical manipulation of living cells of the microalga *Trachydiscus*
2714 *minutus* was performed, using laser wavelengths longer than 935 nm causing no
2715 observable photodamage.
2716 For additional information on microalga isolation the reader is referred to the
2717 references [423].

2718
2719

2720 **4.7.3 Extraction Techniques**

2721 The solvent extraction process is a crucial step in isolation and purification of
2722 individual distinct types of metabolites. The method chosen can directly affect
2723 the biological activity of the extracted compounds [425] as well as their
2724 reproducibility, efficiency and effectiveness. Therefore, when choosing the most
2725 appropriate method from amongst the several available options, certain
2726 important considerations must be addressed. First, microalgae and cyanobacteria
2727 present a remarkable biodiversity, which is reflected in the chemistry of their
2728 unique secondary metabolites, and associated cell wall characteristics and
2729 structure. Thick-walled, silicified membranes, multilayered walls, wall-bound
2730 exopolysaccharides and armored walls present barriers to permeation by the
2731 solvents used in extraction, and must be disrupted prior to this to allow the
2732 release of compounds of interest [360].

2733 Labile compounds may be subject to degradation upon solvent extraction under
2734 elevated temperatures, and light, air and pH are among the factors that should
2735 be controlled to prevent the decomposition of these compounds.

2736
2737

2737 *4.7.3.1 Cell Disruption*

2738 Cell disruption techniques include soaking, pestle and mortar (tissue grinders),
2739 maceration, cryogrinding, bead-beating, homogenization, planetary micro
2740 milling, sonification [426], mixer milling [360], high-pressure homogenization
2741 [427], microwave, autoclaving, and addition of hydrochloric acid, sodium
2742 hydroxide or alkaline lysis. Maceration is a cost-effective method and is
2743 commonly used in algal cell disruption. Bead beating has been demonstrated to
2744 be very effective in disrupting the cell of *Botryococcus braunii* [428] and can be
2745 suitable for industrial scale up [426]. Sonification is one of the most commonly
2746 used laboratory method to disrupt the cell wall in microalgae, and has been
2747 shown to give reliable results in several reports [426,429,430]. However,
2748 thermolabile compounds may be degraded due to the heat generated by this
2749 method, therefore, when low temperatures are required, cells should be placed in
2750 an ice bath during the entire sonification process to prevent the formation of
2751 artifacts or degradation products and disruption of the cells may be confirmed by
2752 optical microscopy.

2753
2754

2755 *4.7.3.2 Secondary Metabolite Extraction*

2756 The microalgal cell is a complex biological system, with many cellular and sub-
2757 cellular structures where several different processes occur and a panoply of
2758 chemically distinct metabolites are produced. Because different classes of
2759 secondary metabolites have different chemical characteristics, there is currently
2760 no standard established extraction procedure. Procedures described in the

2761 literature include conventional extraction techniques, supercritical fluid
2762 extraction, enzyme extraction, etc.

2763 The presence of thermolabile compounds requires the use of techniques working
2764 at low temperature, to prevent thermal degradation, hydrolysis and
2765 hydrosolubilization. The cell wall characteristics, the nature of molecules to be
2766 extracted from the matrix, extraction times and coextraction of undesirable
2767 compounds, as for example, sometimes lipids and pigments, are some of the
2768 factors that have to be considered in this.

2769

2770

4.7.3.2.1 Conventional Solvent Extraction

2771 *Solvent extraction:* in conventional solvent extraction, the appropriate solvent or
2772 mixture of solvents is added to the microalgal biomass from which the desired
2773 metabolite is to be extracted. Once the crude extract is separated from the cell
2774 residue and filtered, the solvents, if volatile, can be evaporated.

2775 *Soxhlet extraction:* the procedure described by Soxhlet [431] has commonly
2776 been used for the extraction of primary compounds as lipids from biological
2777 samples. Nevertheless, secondary metabolites are also extracted with this
2778 methodology from a known quantity of the microalgal material, which is placed in
2779 a thimble, by repeatedly washing and leaching (percloration) with the
2780 appropriate organic solvent under reflux in the Soxhlet apparatus. The solvent in
2781 the flask is heated to boiling. The solvent vapor is condensed in the condenser,
2782 flows through the sample and moves back down into the distillation flask. The
2783 resulting crude extract should be filtered, to remove any remaining unwanted
2784 matter, and can then be concentrated on a rotary evaporator or dried under a
2785 nitrogen steam. In the Soxhlet procedure, heat is required to drive the
2786 extraction, and this may cause decomposition of thermolabile compounds. In
2787 these cases, high temperatures must be avoided by choice of appropriate
2788 solvents in order to prevent the development of decomposition products.

2789 The extraction process using organic solvents has come under increasing
2790 criticism due to its reliance on solvents that are known to be toxic, carcinogenic
2791 or environmental pollutants. Chloroform, dimethyl acetamide, dimethyl
2792 formamide, dimethyl sulfoxide and methanol are some of the solvents that are
2793 becoming considered unsuitable for such extractions, and there is increasing
2794 interest in the development of green solvents or other techniques for this [360].

2795 Regardless of the choice of solvent, the selection of a suitable solvent or solvent
2796 system should address some aspects, such as grade and purity, solubility of the
2797 compounds of interest in it, safety, and environmental issues.

2798

2799

4.7.3.2.2 Non-Conventional Extraction Technique

2800 Several novel extraction techniques have been developed in recent years as
2801 alternatives to the traditional extraction procedures, to overcome the limitations
2802 and inconveniences presented by the conventional methodologies. Methods such
2803 as microwave extraction (MAE), ultrasonic extraction (UAE), subcritical water
2804 extraction (SWE), supercritical fluid extraction (SFE), microbial-aided extraction
2805 (MbAE), and enzyme-aided extraction (EAE), have all been applied for the
2806 extraction of secondary metabolites from microalgae [425].

2807 Green extraction methods, such as *Supercritical fluid extraction (SFE)*, present
2808 advantages over the traditional ones using organic solvents. This eco-friendly
2809 process does not use large amounts of organic toxic solvents, is rapid,
2810 inexpensive, selective and it is suitable when dealing with thermolabile
2811 compounds, since it avoids long extraction times and high temperatures, which
2812 may result in degradation of compounds. The most commonly used critical fluid

2813 has been supercritical carbon dioxide (SC-CO₂), which is non-flammable, has low
2814 critical conditions and is suitable for industrial applications [425].

2815

2816 **4.7.4 Chemical characterization**

2817 The separation and detailed identification of secondary metabolites has only
2818 really become possible by the development of advanced instrumental methods of
2819 analysis, particularly those involving hyphenated techniques (e.g. HPLC-MS). The
2820 traditional methods of screening for secondary metabolites lead to the successful
2821 identification and development of many drug candidates [432]. For the structural
2822 elucidation of metabolites in algae and in other natural products, a repertoire of
2823 analytical techniques is currently available including, chromatographic,
2824 spectroscopic, hyphenated and genome mining techniques. However, due to the
2825 diversity and complexity of microalgal and cyanobacterial metabolites, it is
2826 unlikely that one single analytical technique will provide enough information
2827 about the metabolites that are present in these organisms and, generally, it is
2828 necessary to use various complementary techniques that reveal different facets
2829 which enable the chemical structure characterisation.

2830

2831

2832 **4.7.4.1 Chromatographic Techniques**

2833

2834 Chromatographic techniques, such as high performance thin layer (HPTL) and
2835 liquid chromatography (HPLC), gas chromatography (GC), liquid chromatography
2836 (LC), supercritical fluid chromatography (SFC), have been used to screen various
2837 types of secondary metabolites.

2838

2839 *4.7.4.1.1 Thin Layer Chromatography (TLC)*

2840

2841 Although being an old technique, thin layer chromatography provides a simple,
2842 rapid and sensitive method for the separation and determination of secondary
2843 metabolites in crude extracts. This technique consists of a stationary phase,
2844 which involves a layer of adsorbent coated onto a solid support, such as glass,
2845 polymeric material or aluminium, and a liquid acting as a mobile phase. The
2846 sample is applied in the form of a small spot at one end of the layer, and
2847 migrates with the solvent along the plate by capillary action. The TLC spots are
2848 visualized visible, with ultraviolet light or by treatment with a suitable spray
2849 reagent, depending of the characteristics of the compounds and can be
2850 compared by simultaneously running the unknown spot with standards to identify
2851 compounds present.

2852 TLC has been used for the determination of various bioactive compounds.
2853 Metabolites such as nostocionone [433], norharmane [434], microcystins and
2854 nodularins have, for example, been detected by this technique.

2855

2856 *4.7.4.1.2 High performance thin layer chromatography (HPTLC)*

2857 Based on thin layer chromatography separation, high performance thin layer
2858 chromatography has emerged as a sophisticated technique with major
2859 improvements in resolution and accuracy over standard TLC, and has been
2860 reported to provide good separation of several biomolecules from biological
2861 samples. HPTLC is a simple and fast separation technique, and relatively
2862 inexpensive, compared with HPLC. Multiple samples can be analysed
2863 simultaneously; the method is straightforward, and does not require complex
2864 sample pre-treatment. The main disadvantages are the interferences that can

2865 occur, sometimes giving some false positives. HPTLC has been used to determine
2866 secondary metabolites such as norharmine in various cyanobacteria [435].
2867

2868

4.7.4.1.3 Gas Chromatography (GC)

2869 Gas chromatography (GC) is the most widely used technique for the separation
2870 of volatile mixtures because of the high sensitivity and selectivity it allows. It has
2871 been particularly effective when coupled with mass (GC-MS) [436], flame
2872 ionization (GC-FID) or electron capture detectors (GC-ECD). The major limitation
2873 of GC is that nonvolatile compounds cannot be analyzed directly, and require
2874 derivatization of the sample. It is limited to samples that are easily volatilized
2875 and are thermally stable. Considering that 80 % of all known natural compounds
2876 are nonvolatile or thermolabile [437], GC is not normally the method of choice in
2877 the field of secondary metabolites analysis. Nevertheless, gas chromatography
2878 has been used to identify specific secondary metabolites [438] and may provide
2879 an effective quality control of samples [439]. One such example is the bioactive
2880 exometabolite harmine, extracted from the culture medium of the
2881 cyanobacterium *Geitlerinema* sp.. The alkaloid harmine can be detected in low
2882 volumes of culture media by GC-FID, after a derivatization step [438].

2883 Some microalgae, in particular the cyanobacteria and the dinoflagellates, are
2884 known to produce toxins such as anatoxin-a, saxitoxins and microcystins, which
2885 are hazardous to human and animal health, and have been associated with the
2886 contamination of blue-green algal food supplements [440]. The microalgal
2887 biomass production systems include open ponds, which might be susceptible to
2888 contamination by a variety of microalgae species, with some capable of
2889 producing a wide range of toxins. A toxicological control guarantees the absence
2890 of potential toxins in microalgal food supplements. Toxins that can be derivatized
2891 can be successfully detected and quantified by the technique GC-ECD, at
2892 pictograms levels, in small amounts of algal samples and in small volumes of
2893 water [441].
2894

2895

4.7.4.1.4. High Performance Liquid Chromatography (HPLC)

2896

2897 HPLC is a chromatographic technique that can separate complex mixture of
2898 compounds and can be used to identify, quantify and purify individual analytes of
2899 a mixture [440]. Equipped with ultraviolet (UV) and fluorescence (FLD)
2900 detectors, is a widely used method for the analysis of secondary metabolites
2901 because of its sensitivity and ease of use. Specific examples of application of this
2902 technique include the analysis of secondary metabolic peptides [443] and
2903 alkaloids [444].
2904

2905 HPLC with ultraviolet (UV), photodiode array (PDA), and fluorescence (FLD)
2906 detectors can be applied to determine different types of cyanotoxins; HPLC-FLD
2907 can detect saxitoxins which, although they are neurotoxic, have therapeutic
2908 potential in anaesthesia [444]. When confirmation of the molecular structure of a
2909 given toxin is required, the hyphenated technique liquid chromatography mass
2910 spectrometry (LC-MS) can be used for the identification of the compound [445].

2911 Although UV detectors are limited to molecules having suitable chromophores,
2912 HPLC-UV is suitable to the detection and quantification of several secondary
2913 metabolites. Other examples include the analysis of the closely related [7.7]
2914 paracyclophanes and structural derivatives, extracted from the cyanobacterial
2915 strains belonging to the orders Nostocales and Oscillatoriales, which were
2916 detected and quantified by HPLC-UV [447]. The same technique was also applied

2917 for the determination of fourteen phenolic compounds in the extracts from the
2918 diatom *Phaeodactylum tricornutum* [448].
2919

2920

2921 **4.7.4.2 Spectroscopic Techniques**

2922 This discussion is not exhaustive, and we have not included important
2923 spectroscopic techniques such as UV/Vis, FTIR and Raman Spectroscopy. The
2924 reader is referred to standard texts for the discussion of these.

2925

2926 *4.7.4.2.1 Fluorescence Spectroscopy*

2927 Fluorescence is the emission of light when electronically excited states relax back
2928 to the ground state. Its theoretical basis is closely related to, and involves the
2929 same electronic states, as UV-vis absorption spectroscopy. However, the nature
2930 of measurement technique means that it may have a sensitivity several orders of
2931 magnitude greater than absorption spectroscopy. This is a great advantage in
2932 analytical applications, such as the study of microalgal metabolites, which may
2933 be present at very low concentrations. Not all molecules have significant
2934 fluorescence. Analytically interesting fluorescent organic molecules typically have
2935 rigid conjugated structures, such as derivatives of aromatic hydrocarbons.
2936 Experimentally, its study requires an exciting light source, the sample,
2937 appropriate dispersive elements, such as monochromators, and a detector. It is
2938 possible to scan the emission spectrum using a constant excitation wavelength,
2939 or to observe the emission at a constant wavelength and study the effect of
2940 changing the excitation wavelength. These give the emission and excitation
2941 spectra respectively. A number of excellent descriptions of the technique are
2942 available [449,450]. Typical examples of applications include analysis of
2943 chlorophylls [451] and alkaloids, such as β -carboline [452]. It is also possible to
2944 add fluorescent probes which are sensitive to particular environments, such as
2945 hydrophobic ones, which can provide detailed information on lipid content
2946 [453,454]. The high sensitivity of fluorescence means that it is valuable for
2947 combining with chromatographic methods, such as HPLC, in hyphenated
2948 techniques. It can also be combined with microscopy in fluorescent imaging, and
2949 can furnish more detailed information through time-resolved measurements.

2950

2951

2952 *4.7.4.2.2 Nuclear Magnetic Resonance (NMR)*

2953

2954 NMR is a powerful technique that provides both qualitative and quantitative
2955 information. It allows the simultaneous detection of diverse groups of secondary
2956 metabolites (aminoglycosides, alkaloids, terpenoids and so on). NMR exploits the
2957 magnetic properties of certain nuclei (such as ^1H , ^{13}C , ^{31}P , which, when placed in
2958 a magnetic field, absorb electromagnetic radiation in the radio-frequency region
2959 of the spectrum (frequency of resonance). The resonance frequency depends on
2960 the chemical environment of the nucleus, and thus, each nucleus in a molecule
2961 gives a specific and characteristic signal (given as its chemical shift), allowing
2962 structural elucidation. In addition, the technique provides information on the
2963 nearest neighbour atoms through "coupling" of the nuclear spins. There are
2964 many excellent books on NMR spectroscopy, describing the fundamental theory,
2965 applications and the typical values for chemical shifts and coupling constants,
2966 and several online databases that provide valuable data resources [455], and
2967 useful information regarding organic structures and correspondent spectra are
2968 available [455,456].

2969
2970 Although NMR is a reliable and very robust technique that provides unambiguous
2971 information, it has some disadvantages that can limit its application. These
2972 include the expensive equipment needed, the time-consuming measurements,
2973 the high concentrations of samples required, and the overlapping signals that
2974 may be present when applied to complex bio-organic compounds, making the
2975 interpretation of the exact structure of the compound difficult.
2976 In general, the NMR technique does not require elaborate sample preparation,
2977 and liquid samples, solvent extracts and dried or live microalgal cells can directly
2978 be analysed. It is a straightforward and non-destructive technique, such that
2979 samples can be further analysed by other techniques. NMR spectroscopy has
2980 been extensively used in biological studies and significant improvements are
2981 continuously being made in the use this high-throughput technique.
2982 Since a large number of NMR experiments are possible in metabolomics, different
2983 approaches can be considered when using NMR spectroscopy. One dimensional
2984 ^1H NMR is one of the most widely used in metabolomics analysis. However, when
2985 analysing complex mixtures, ^1H NMR spectra can be very challenging due to
2986 overlapping of signals, which can hinder the identification of metabolites.
2987 A relatively new methodology in the field of Metabolomics studies is *in vivo* High
2988 Resolution Magic Angle Spinning Nuclear Magnetic Resonance (^1H HR-MAS NMR)
2989 that can be applied to live microalgal cells [457].
2990 In ^{13}C NMR spectroscopy, where chemical shifts cover 200 ppm, compared
2991 typically with 10 ppm for ^1H NMR, the signals are better resolved. However, the
2992 major limitation of ^{13}C NMR spectroscopy is the lower sensitivity arising from the
2993 low natural abundance of ^{13}C . The use of multidimensional NMR spectrum can
2994 help to overcome many of the limitations of one dimensional NMR. Two-
2995 dimensional (2D) NMR include correlated spectroscopy (^1H - ^1H COSY), total
2996 correlation spectroscopy (^1H - ^1H TOCSY) and heteronuclear single-quantum
2997 correlation (^1H - ^{13}C HSQC).
2998 A customized metabolomics NMR database [458], ^1H (^{13}C)-TOCCATA, contains a
2999 complete set of ^1H and ^{13}C chemical shift information on individual spin systems
3000 and isomeric states of common metabolites, and allows the identification of
3001 metabolites in complex mixtures [459].
3002 Because NMR spectroscopy analysis frequently produces a highly complex
3003 spectrum which is difficult to interpret, it is useful to employ chemometric
3004 analysis, such as principal components analysis (PCA), that allows the reduction
3005 of multivariate data into a smaller number of principal components representing
3006 the original variables, and Partial Least Squares Discriminant Analysis (PLS-DA),
3007 which identify categories, provide a more objective and clear information.
3008 Examples of application of NMR on microalgal secondary metabolites elucidation
3009 include the detection of glycosides in powder suspensions of the blue-green algae
3010 *Aphanizomenon flos-aquae* [460].
3011
3012

3013 **4.7.4.3 Hyphenated Techniques**

3014 The combination of individual techniques with NMR and MS has led to the
3015 development of hyphenated techniques which have greatly increased the
3016 analytical capabilities in metabolomics research. Examples include gas
3017 chromatography-mass spectrometry (GC-MS) [461], liquid chromatography-
3018 nuclear magnetic resonance spectroscopy LC-NMR [462], ultra-high performance
3019 liquid chromatography-two-dimensional mass spectrometry (UHPLC-MS/MS)
3020 [463]. GC-MS combines the good resolution of GC and high selectivity and

3021 sensitivity of MS. To be detected by GC-MS analysis, the metabolites require a
3022 derivatization step in order to create volatile organic compounds, which limits the
3023 applicability of this technique [464]. If the unknown sample has derivatizable or
3024 volatile components, this technique might be employed to identify and quantitate
3025 several secondary metabolites such as different peptides, microcystins and
3026 alkaloids [461,464,465].

3027 The UHPLC-MS/MS technique provides an ultrasensitive and selective
3028 determination of several secondary metabolites, such as cyanotoxins or
3029 isoflavones in microalgae and cyanobacteria. The presence of eight isoflavones
3030 such as daidzin, genistin and formononetin was demonstrated by UHPLC-MS/MS
3031 in concentrations of ng.g^{-1} dry biomass [463]. Another example of the
3032 application of a hyphenated technique in the analysis of secondary metabolites is
3033 the determination of extracellular diterpenoids by LC-NMR from the terrestrial
3034 cyanobacterium *Nostoc commune* [462].

3035
3036

3037 **4.7.4.4 Genome Mining**

3038 In response to environmental changes such as excessive light, changes in pH,
3039 nutrients depletion, etc., many cyanobacteria activate specific genes that, in
3040 turn, synthesize specific proteins; these are involved in the pathways for the
3041 production of these metabolites that protect cells from stress or help them to
3042 adapt to the new conditions. Genome mining is a powerful technology that has
3043 emerged as a new strategy for the discovery of novel secondary metabolites
3044 [466]. Based on genetic information, it has become possible to predict and
3045 isolate new compounds. The genome sequencing of microorganisms unveils new
3046 metabolites with potential therapeutic interest in the treatment of diseases,
3047 enhancement of organism growth and increased crop yields and other
3048 biotechnology applications are also possible. At present, thousands of microbial
3049 genome sequences are available in public repositories containing information on
3050 secondary metabolite gene clusters that encode the biosynthetic pathways of
3051 secondary metabolites. With the fast development of sequencing methods and
3052 bioinformatics, several genome mining methodologies have arisen. Currently,
3053 computational and web-based analysis platforms such as the *Atlas of*
3054 *Biosynthetic Gene Clusters* [467] and the *antiSMASH* [468-472] are used to
3055 predict the products of genes whose DNA sequences are known. With the
3056 advance of modern technologies such as UHPLC, NMR and various mass
3057 spectroscopy methodologies, the products of such genes can be identified and
3058 characterized. One such example, for the discovery of new secondary
3059 metabolites, involves an interesting combination of techniques; the combination
3060 of mass spectrometric metabolic profiling and genomic analysis has resulted in
3061 the discovery of a new class of di- and tri-chlorinated acyl amides, the
3062 columbamides [473].

3063
3064

3064 **4.7.5 Summary**

3065 A summary of the main information is given in **Table 4.7.1**.

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Table 4.7.1 Information about the biological activity, culture conditions, extraction methods and analytical techniques of the compounds obtained from several species of microalgae and cyanobacteria.

Metabolite	Family/Genus /specie	Biological activity	Culture conditions	Sample treatment	Extraction method/solvents used	Analytical techniques	References
Aeruginosin 828A	<i>Planktothrix rubescens</i>	Anti-Inflammatory	Mineral medium, 6 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 20°C	Freeze/thaw cycle after harvested by centrifugation	50% methanol Water	RP-HPLC	[474]
Anatoxin-a	<i>Anabaena flos-aquae</i>	Cytotoxic	BG-11 medium, 18-20 °C, 30-50 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 16:8 h	Freeze-dried after harvested by filtration	Dichloromethane, Derivatization with pentafluorobenzyl bromide	GC-ECD	[441]
Calothrixins A and B	<i>Calothrix</i>	Antimalarial and anticancer	Allen and Arnon medium [475], 25 °C, 10mM NaNO ₃ NiSO ₄ , 24:0 h	Lyophilized	Sohxlet/ Dimethylsulfoxide, ethyl acetate, hexane, acetone	EIMS, ¹³ C NMR, ¹ H NMR, singles-crystal X-ray	[414]
Carriebowmide	<i>Lyngbya polychroa</i>	-	Collected sample	Freeze-dried	EtOAc-MeOH, EtOH-H ₂ O	IR, HPLC, NMR	[443]
Columbamides	<i>Moorea (Lyngbya)</i>	Cannabinomimetic	Collected sample, BG-11 medium	Freeze-dried	(2:1, v/v) CH ₂ Cl ₂ /MeOH	IR, ¹ H and ¹³ C NMR, HPLC, HRMS-genomic analysis	[473]
Cyanopeptolin 1020	<i>Microcystis aeruginosa</i> UV-006	Anti-Inflammatory	Mineral medium, 26°C, 40±5 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$	Harvested by centrifugation	60% acetonitrile, Water	RP-HPLC, high-resolution mass spectrometry	[474,476]
Diterpenoids comostins	<i>Nostoc commune</i>	Antibacterial, cytotoxic, molluscicidal	Inorganic culture medium, 24 °C, 29 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 24:0 h, 2% CO ₂	Harvested by filtration	Methanol	NMR, MS, single-crystal X-ray	[462]
9-Ethyliminomet hyl-12-(morpholin - 4 - ylmethoxy) - 5, 8, 13, 16-tetraaza-hexacene - 2, 3 dicarboxylic acid	<i>Nostoc</i> sp.	Antibacterial	Collected sample, BG-11 medium, 25 °C, 95 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 14:10 h	Harvested by centrifugation and lyophilized	Methanol	TLC, HPLC, ESIMS, NMR	[477]
Harmane	<i>Geitlerinema</i> sp.	Antimicrobial	Rippka et al [478], 25 °C, 10 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 24:0 h	Harvested by centrifugation	Volk's procedure, methanol, Derivatization	TLC, LC-MS/MS, GC-FID	[438]
Isoflavones	<i>Nostoc, Spongiochloris, Scenedesmus</i>	-	-	Lyophilized	Supercritical fluid extraction	UHPLC-MS/MS	[463]
Lyngbyabellin B	<i>Lyngbya majuscula</i>	antifungal	Collected sample	-	(2:1, v/v) CH ₂ Cl ₂ /MeOH,	1D and 2D NMR, GC-MS	[436]
Mycosporine-like amino acids and glycosides	<i>Aphanizomenon flos-aquae</i>	Anticancer	-	Dry powder, sonicated for 1 h	No extraction	NMR, ESI-QTOF-MS, CID-MS/MS	[460]
Norharmane	<i>Nostoc insulare, Nodularia harveyana</i>	Algicidal	Pohl et al. medium [479], 27 °C, 25-30 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$,	Centrifugation and filtration, freeze-dried	Methanol	HPLC	[480]
Norharmane	<i>Synechocystis aquatilis</i>	Algicidal, allelopathic	BG-11 medium, 24 °C, 30 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 24:0 h	Harvested by centrifugation	Volk's procedure	TLC	[434]
Nostocarboline	<i>Nostoc 78-12A</i>	Inhibitor of butyrylcholin esterase	Mineral medium, 25°C, continuously illumination, 15 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, CO ₂ -enriched air (600 mL min ⁻¹ , 0.15 vol % CO ₂	Lyophilized	60% methanol CH ₃ CN-H ₂ O, 95:5 V/V Separated by HPLC	2D-NMR	[481,482]
[7.7] paracyclophanes	<i>Nostoc, Cylindrospermum, Anabaena, Nodularia, Pseudanabaena</i>	Antibacterial, cytotoxic	BG-11, BG-11+0.5% NaCl, MBL medium, 20-28 °C, 0.5-5% CO ₂ , 20-80 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$	Freeze-dried after harvested by centrifugation	n-heptane, EtOAc, MeOH, and H ₂ O (5:2:5:2, v/v/v/v);	UV-vis spectrophotometer, HPLC-UV, 2D NMR	[447]
Phenolic compounds	<i>Phaeodactylum tricoratum</i>	Antioxidant	f/2 medium [483], 24°C, 8000 lux, 24:0 h	Freeze-dried after harvested by filtration	solid-phase extraction/ Methanol, acetone:hexane (1:4)	RP-HPLC	[448]
Sulphated polysaccharide	<i>Nostoc calcicola</i>	Antiviral	BG-11 medium, 25 °C 35 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$, 16:8 h	Harvested by centrifugation	Mouhim et al. (1993)	IR, GC	[484]

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