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

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Outline

4.1 Introduction

[Jack Legrand]

Microalgae represent a unique source of biomass and biodiversity in the living world. There are several hundred thousand species colonizing almost the entire globe. They represent the first link in the trophic chain, the main supplier of oxygen and the first consumer of CO_2, some of whose storage is in the form of calcareous sediments. The advantage of microalgae over higher plants is their rapid growth. Their continuous cultivation makes it possible to direct their metabolism, thus opening the way to a renewable chemistry which does not encroach on the grounds reserved for agriculture and the forest.

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

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

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

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

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

4.2 Methods for biomass global characterization

[Catherine Dupré]

4.2.1 Introduction

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

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

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

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

4.2.2 Dry weight method (DW)

5.2.2.1. Centrifugation or filtration

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

4.2.2.2 Washing the biomass

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

4.2.2.3 Biomass dewatering

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

organic compounds (VOCs) to escape, but also for a shorter period to reach constant weight, indicative of the end of the drying kinetic. Fast drying is obtained with halogen moisture balances, provided that, for reliable DW measurements, the drying temperature is set to 105 °C. Indeed, too high surface temperatures (> 400 °C) should be avoided [21].
The dried filters or tubes are quickly transferred in a desiccator for 30 minutes minimum before the last weighing m_2. The biomass concentration (g L^{-1}) is deduced from the ratio of the dry biomass to the volume of the sample (m_2-m_1)/v. The biomass concentration X in attached cultures is expressed as biofilm areal density (X, g m^{-2}).

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

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

4.2.3.1 Classical gravimetric method

The classical gravimetric method corresponds to the determination of the remaining ash obtained after further combustion of the dry biomass in a furnace at high temperatures (> 550 °C). The difference gives the ash free dry weight (AFDW), corresponding to the global organic fraction of the microalga biomass. It is also named as the volatile suspended solids (VSS) fraction. The values of biochemical fractions (lipids, proteins, sugars…) are higher when calculated on an AFDW basis. Moreover the AFDW allows avoiding the biomass rinsing problems. The samples are placed within porcelain, silica or platinum crucibles to prevent loss; they are pre-weighted. Then, they are placed in a combustion furnace to burn up to 700 °C with a recommended value of 575 °C and for a minimum of four hours. The duration corresponds to the time needed to reach a constant weight, defined as that obtained when mass variation is less than 0.3 mg after one hour of re-heating the crucible. This time is a function of the initial mass and the structure of the biomass to be treated. After its total oxidation in the muffle furnace, the samples within their crucibles are placed in a desiccator to cool to ambient temperature (1 hour recommended) before the final weighing.
The ash content and composition of the biomass is important in furnace operations during combustion processes, but also during bio-refinery processes [23]. When necessary, the ash composition (Si, Ca, Mg, K, Na, P…) could be easily determined using inductively coupled plasma mass spectroscopy (ICP-MS) or X-ray fluorescence (XRF).

4.2.3.2 Thermogravimetric analysis

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

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

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

4.2.4 Cell counts methods

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

4.2.4.1 Hemocytometry

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

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

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

4.2.4.2 Flow cytometry

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

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

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

4.2.4.3 Image-based cytometry

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

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

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

4.2.5 Elemental analysis

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

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

Some of them have to be considered for characterizing biomass from some bio-mineralized microalgae, such as diatoms or calcareous species [38].

The large biodiversity and chemodiversity of microalgae is of great interest in tapping in never exploited natural molecules, thanks to recent and ongoing screening studies. However, such diversity implies that the standard analytical protocols for biomass characterization have to be critically examined for potential interference.

4.3 Methods for protein determination in microalgae

[Marilyne Fauchon, Claire Hellio]

4.3.1 Introduction

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

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

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

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

Alga	**Class**	**Protein content (%)**
Chlorella pyrenoidosa	Trebouxiophyceae	57
Chlorella vulgaris		53.3
Chlorella ellipsoidea		42.2

Chlorella ovalis		10.97
Chlorellla spaerckii		6.87
Dunaliella salina	Chlorophyceae	57
Dunaliella primolecta		12.26
Dunaliella tertiolecta		11.4
Scenesdesmus obliquus		48
Scenesdesmus almeriensis		41.8
Tetraselmis chui	Chlorodendrophyceae	46.5
Porphyridium cruentum	Porphyridiophyceae	35
Porphyridium aerugineum		31.6

4.3.1.2 Applications of algal protein

4.3.1.2.1 Human nutrition

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

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

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

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

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

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

Table 4.3.3: Essential Amino acid profile of different microalgae as compared 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
Chlorella sp.	4.4	9.2	6.1	8.9	2.2	0.4	4.7	2.4
Dunaliella sp.	4.5	9.3	6.0	6.2	2.5	4.0	5.0	2.5
Scenedesmus sp.	4.7	9.4	6.0	6.8	2.4	0.1	4.9	2.6

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

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4.3.1.2.2 Industrial application

4.3.1.2.2.1 High value metabolites: phycobiliproteins

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

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

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

4.3.1.2.2.2 Functional food-ingredients

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

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

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

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

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

4.3.1.2.2.3 Animal feed

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

4.3.1.2.2.3.1 Agriculture

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

4.3.1.2.2.3.2 Aquaculture

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

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

4.3.1.2.2.4 Recombinant proteins

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

4.3.1.2.2.5 By-product of biofuel consumption

Microalgae are considered as one of the most promising biobased feedstocks, as their productivity in converting CO_2 into lipids exceeds that of oilseed crops [81]. Applying biorefinery, not only for oil, but as well for protein, may lead to the sustainable and economical microalgae-based fuels. With this approaches, the biomass treatment for protein extraction, alongside other compounds of interests (lipids, carbohydrates), is different to when only proteins are extracted and may affect the yield [82].

4.3.2 Protein extraction and quantification

4.3.2.1 Pre-treatment prior to extraction

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

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

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

Cell disruption	**Time**	**Experimental set-up**
Acid treatment	25 min	Hot Ac2O* + H_2SO_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 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

***** acetic anhydride

4.3.2.2 Protein extraction

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

4.3.2.3 Protein content determination

For a given algal species, the intracellular concentration of inorganic and organic nitrogen depends on the growth phase [93]. Various methodologies have been developed for proteins quantification and analysis. Main methods are based on colorimetry (Coomassie, Lowry, BCA, Amido Black, Biuret), fluorescence (fluorescamin, Nano-orange, CBQCA, OPA), immunology (Elisa) and spectroscopy (amino acids analysis, UV, mass spectrometry) protocols [94].
For microalgae, commonly protein are quantified by elemental analysis, Kjeldahl, Lowry assay, Bradford assay [95] or dye binding method [91]. However, the first two analyses take into consideration total nitrogen present in the microalga, multiplying it by the standard nitrogen to protein conversion factor (NTP) 6.25 which may lead to overestimation or underestimation of the true protein quantity because microalgae contains high concentration of non-protein nitrogenous substances such as pigments (chlorophyll or phycoerythrin for example), nucleic acids, free amino acids and inorganic nitrogen (nitrate, nitrite, ammonia) [93] whose presence makes the factor 6.25 unsuitable since it overestimates the real protein content [96]. Several authors recommended to use a value of NTP lower then the standard 6.25 [48,97]. Moreover, a study by Safi et al. [89] highlighted that no universal conversion factor could be recommended due to the fact that multiple factors, such as cell wall rigidity, growth conditions, growth media and environmental uncertainty, can affect the NTP value. Gonzales Lopez et al. [48] determined the NTP using a technique that correlates protein content (Lowry assay) to total nitrogen content (Kjeldahl and elemental analysis). In addition, Servaites et al. [98] quantified proteins of microalgae by staining the protein isolate with Coomassie brilliant blue R-250 (CBB) on a paper and then eluting the remaining stained proteins in 1 % sodium dodecyl sulphate (SDS) followed by measuring the absorbance at 600 nm. On the other hand, the colorimetric method of Lowry [99] was also considered as one of the most accurate methods to quantify proteins, but with time this method showed to only quantify hydro-soluble proteins [89], which represents the major part of proteins. Lowry assay is considered as more acceptable then Bradford assay because the latter does not react with all amino acids present in the extract and thus giving lower protein concentrations [91].
General methodologies can be used but it is important to state that some protein may lead to challenges depending on its structure and mode of action. So far, no single method of quantification can be used for the determination of the true protein concentration for all proteins in all kinds of buffers to serve as a "gold standard" [94]. This can be explained by the fact that proteins can have a very large variety of structures and physicochemical properties. Thus, the choice of the right quantification method is crucial.The different assays available for protein quantification have various characteristics that must be considered before choosing the right protocol depending on the type of experimental work planned. Many different methodologies to assay proteins have been developed; the purpose of this chapter is not to review all but describe the most commonly applied and/or appropriate assays.

4.3.2.3.1 Total nitrogen content

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

is determined with a thermal conductivity detector, after removal of carbon dioxide and water aerosols. The Kjeldahl method was chosen as an example of this analytical principle as it is still recognized as the official method for food protein determination by the AOAC International [87].
For microalgae, measurements can be done using 200 mg samples of the dry biomass. The biological material is hydrolyzed with 3 mL concentrated sulfuric acid (H_2SO_4) containing one copper catalyst tablet in a heat block (for example, Kjeltec system 2020 digestor, Tecator Inc.,Herndon, VA, USA) at 420 °C for 2 h. After cooling, H_2O was added to the hydrolysates before neutralization and titration. Following the nitrogen determination, crude protein content is estimated using a conversion factor (see 4.3.2.3).

4.3.2.3.2 Colorimetric methods

Colorimetric assays rely on the appearance of a chromophore as a consequence of either the binding of a dye to a protein or the protein being involved in a redox reaction. Such methods are sensitive to interferences and their accuracy depends significantly on the methods used for pre-treating the samples. Indeed, for microbial proteins to be measured accurately, the cells must be pretreated in order to fully release the intracellular proteins [48]. For better results, Barbino and Lourenço [91] suggested to start the protein extraction from 50 mg of freeze-dried material. The most common treatments involved physical or chemical disruption of the cell wall (see 4.3.2.1 pre-treatment).
Prior to protein quantification by Bradford, BCA or Lowry methods, it is necessary to proceed to a protein precipitation step. For this a commonly used procedure is the Berges et al. [100] methodology which is based on the use of trichloroacetic acid (TCA) to allow for protein precipitation. The advantage of this method is that the pellet containing the proteins from microalgae can be stored at -20 °C until further analysis. Prior to colorimetric analysis, precipitated proteins are respectively suspended in 0.5 mL 1.0 N NaOH for the Bradford assay and 2.0 mL for the Lowry assay (when starting from 50 mg freeze dried biomass before pre-treatment).
A study by Berges et al. [100] stated that even if it remains unclear which spectrophotometric assay is the most accurate (between Lowry, BCA and Coomassie), it appears that Coomassie assay is faster and simpler, and, has the advantage of being less affected by non-protein compounds found in marine phytoplankton. The reactivity of either of the assays to a particular protein will be a function of that protein's composition as well as any other compound which might oxidize the Folin phenol reagent or bind the Coomassie dye. The bicinchoninic acid (BCA) assay, similarly to the Lowry assay, measure the conversion of Cu^{2+} to Cu^{+} under alkaline conditions, and, both assays have similar sensitivity but BCA is stable under alkaline conditions.

4.3.2.3.2.1 Lowry assay

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

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

Example of experimental protocol:
Step 1 : Pre-treatment of microalgal samples : for example, 20 mg of aliquots of microalgal freeze-dried biomass suspended for 20 min in 10 mL of lysis buffer
Step 2: An aliquot of this suspension is diluted with the lysis buffer such that the protein concentration is within the range of 0 and 1000 mg L^{-1}.
Step 3: 0.1 mL SDS and 1 mL of reagent C is added to 0.1 mL of the above solution, the solution is then vortexed.
Step 4: 10 min later 0.1 mL of Folin reagent is added and well mixed. The sample is then kept in the dark to avoid the degradation of the Folin reagent.
Step 5: after 30 min the absorbance of the sample is measured at 750 nm (a blank is prepared is prepared without the algal extract).
Step 6: The spectrophotometric absorbance is converted to protein concentration using a calibration curve established with bovine serum albumin (BSA) dissolved in lysis buffer. The protein content of the biomass is calculated using the formula:

Protein (%; w=w) = (CVD/m) x 100

with C the protein concentration (mg L1) obtained from the calibration curve, V the volume (L) of the lysis buffer used to resuspend the biomass, D is the dilution factor and m is the amount of biomass (mg).

4.3.2.3.2.2 Coomassie (Bradford) Bradford assay

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

Example of experimental protocol:
The Quick Start™ Bradford protein assay is a simple and accurate procedure for determining the concentration of protein in solution. The assay supplies ready-to-use dye reagent at 1x concentration and two protein standards at seven prediluted concentrations (0.125, 0.25, 0.5, 0.75, 1.0, 1.5, and 2.0 mg mL^{-1}). Protein concentration is determined in one step, with no need to dilute standards. Quick Start Bradford protein assay kits offer either bovine serum albumin or bovine γ-globulin standard sets.
Step 1: Prepare calibration curves using either Bovin Serum Albumin (BSA) with a range of 1,25-10 µg mL^{-1} or bovine γ-globulin in the range of 1,25-20 µg mL^{-1}

Step 2: Pour 150 µL of samples/standard in a microplate
Step 3: in each well of the microplate, add 150 µl of 1X Dye Reagent and shake manually
Step 4: incubate 5 minutes at room temperature
Step 5: Take absorbance readings at 595 nm against a blank

Note: In classical protein staining protocols using Coomassie Brilliant Blue (CBB), solutions with high contents of toxic and flammable organic solvents (Methanol, Ethanol or 2-Propanol) and acetic acid are used for fixation, staining and destaining of proteins in a gel after SDS-PAGE. To speed up the procedure, heating the staining solution in the microwave oven for a short time is frequently used. This usually results in evaporation of toxic or hazardous Methanol, Ethanol or 2-Propanol and a strong smell of acetic acid in the lab which should be avoided due to safety considerations.
In a protocol published by Lawrence and Besir [103], an alternative composition of the staining solution is described in which no organic solvent or acid is used. The CBB is dissolved in bidistilled water (60-80 mg of CBB G-250 per liter) and 35 mM HCl is added as the only other compound in the staining solution. The CBB staining of the gel is done after SDS-PAGE and thorough washing of the gel in bidistilled water. By heating the gel during the washing and staining steps, the process can be finished faster and no toxic or hazardous compounds are evaporating. The staining of proteins occurs already within 1 minute after heating the gel in staining solution and is fully developed after 15-30 minutes with a slightly blue background that is destained completely by prolonged washing of the stained gel in bidistilled water, without affecting the stained protein bands.

4.3.2.3.2.3 Bicinchoninic Acid (BCA) Microplate Assay

The bicinchoninic acid (BCA) assay measures the conversion of Cu^{2+} to Cu^{+} under alkaline conditions. The amount of Cu^{2+} reduced is a function of protein concentration that can be determined spectrophotometrically by a color change of the sample solution into purple, which absorbs at 562 nm. The absorbance is directly proportional to the amount of protein present in the solution and it can be estimated by comparison with a known protein standard, such as bovine serum albumin (BSA). This assay is also generally less affected than the Lowry assay by interfering compounds such as some detergents and denaturing agents (urea, guanidine chloride). However, the BCA assay is more sensitive to the presence of reducing sugars and high level of lipids [104]. The effects of these interferences can be eliminated or reduced through several strategies such as removing the interfering substances through dialysis, gel filtration or if the protein concentration is high enough, by diluting the sample [105]. Compared to other methods BCA assay is one of the most sensitive (it can detect proteins at concentrations as low as 5 ug mL^{-1}). It has less variability than others (i.e., Bradford assay), and it can be used to measure a wide range of protein concentration [106].
Example of experimental protocol for samples containing 0.1–1 mg protein mL^{-1}:
Step 1: Prepare calibration curves with Bovin Serum Albumin (BSA) with a range of 5-2,000 µg mL^{-1}.
Step 2: Prepare BCA working reagent (WR)
Step 3: Dilute the microalgal samples with distilled water (1:3, v/v).

Step 4: Pour 10 µL of distilled water with 10 µL of diluted samples/standard in a microplate.
Step 5: Add 200 µL of working reagent.
Step 6: Cover the microplate and incubate for 30 min at 37 °C in the dark.
Step 7: Keep all tubes at room temperature for 15 min before measurement.
Step 8: Take absorbance readings at 562 nm against a blank (distilled water).

4.3.2.3.3 Amino acids analysis

Amino acid analysis is one of the analytical principles for protein determination. The principle is that the proteins are broken down into their constituent amino acids by hydrolysis of the peptide bonds. The liberated amino acid residues are then determined, most often chromatographically, and protein content is calculated as the sum of individual amino acid residues after subtraction of the molecular mass of H_2O.
After sample preparation (hydrolysis of the protein with 1 mL of 6 N HCl in vacuum-sealed hydrolysis vials at 110 °C for 22 h), sodium or lithium buffers (depending on the amino acids present in the sample) are prepared for separation of the amino acids by Ion Exchange Chromatography. The eluate from the ion exchange column is passed through in a teflon coil placed in a boiling water bath, or other heating apparatus. Before entering, the column effluent is mixed with reduced ninhydrin reagent, which is dissolved in acetate buffer. The ninhydrin reacts with amino acids forming a dye complex. The absorption is determined in a flow photometer, and registered on the chart of a recorder or a computer. The area under the peaks corresponds to the amounts of amino acids present in the sample. The evaluation can be done manually or automatically with an integrator or a computer. The circumstances of the analysis make it possible to quantitate as little as one nanomol amino acid with a high degree of accuracy.
Example of protocol (from Mæhre [107])
From microalgal crude samples: 50 mg of extracts are dissolved in 0.7 mL distilled H_2O and 0.5 mL 20 mM norleucine (internal standard).

1. From protein extract samples: 500 µL extract is mixed with 50 µL 20 mM norleucine (internal standard).
2. Subsequently, for all samples, concentrated hydrochloric acid (HCl, 12 M) is added, to a final concentration of 6 M.
3. The sample mixture is flushed with nitrogen gas for 15 s in order to minimize oxidation, before hydrolysis at 110 °C for 24 h.
4. Following hydrolysis, 100 µL aliquots of the hydrolysates are evaporated under nitrogen gas until complete dryness and re-dissolved to a suitable concentration in lithium citrate buffer at pH 2.2.
5. All amino acids are analyzed chromatographically using an ion exchange column followed by ninhydrin post column derivatization on a Biochrom 30 amino acid analyzer (for example, Biochrom Co).
6. Amino acid residues were identified using the A_{9906} physiological amino acids standard (Sigma).
7. Protein content was calculated as the sum of individual amino acid residues (the molecular weight of each amino acid after subtraction of the molecular weight of H_2O).

4.3.2.3.4 Validation of protein quantification methodology

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

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

4.3.2.4 Extraction and purification of high value phycobiliproteins from microalgae

4.3.2.4.1 Phycoerythrin

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

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

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

- Cell disruption: several methods can be used (sonication, mechanical maceration or lysozyme treatment). From the literature, the best recovery yields are obtained with sonication [108]. Jubeau et al. [109] evaluated the extraction of B-phycoerythrin from *Porphyridium cruentum* by high-pressure cell disruption varying the parameters of pressure (25–270 MPa) and they proposed a two-step selective extraction with a first passage at 50 MPa (to eliminate the contaminating protein present in the cytoplasm) in culture medium followed by a second passage at 270 MPa (to extract the B-phycoerythrin) in distilled water, achieving a 0.79 purity ratio.

- Primary recovery: the most commonly, fast and inexpensive method used for primary recovery is selective precipitation with ammonium sulfate. Various concentration of ammonium sulfate is used depending of the microalgal strains, for example 65 % for *Porphyridium cruentum* [110] or a a two-step ammonium sulfate precipitation, at 20 % and 70 % saturation for *Phormidium spp.* [111].

- Purification: Purification is typically achieved by chromatographic methods like ion exchange chromatography, hydroxyapatite chromatography, gel filtration and expanded bed adsorption chromatography [39].
  - Parmar et al. [111] purified phycoerythrin from *Phormidium sp*. A27DM with a single-step gel permeation chromatography using a Sephadex G-150 matrix pre-equilibrated and eluted with a 10 mM Tris-HCl buffer (pH 8.1) at a flow rate of 60 mL h^{-1}. This protocol yielded a final purity ratio of 3.9.
  - Bermejo et al. [110] purified phycoerythrin from *P. cruentum* using an anionic chromatographic column of Diethylaminoethanol (DEAE) cellulose. Elution was performed as a discontinuous gradient of acetic acid-sodium acetate buffer (pH 5.5). The best results were achieved with flow rate of 100 mL h^{-1}. Later, Bermejo et al. [112]

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

4.3.2.4.2 Phycocyanin

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

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

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

4.3.3 Proteomics

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

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

PDQuest (Bio- Rad Laboratories) and DeCyder (GE Healthcare) for the analysis of 2-DE gels.
Jia et al. [121] described 2-DE as an elaborate pre-fractionation step that precedes MS analysis in a typical proteomic workflow and, as such, some researchers prefer to omit this step and apply different strategies which scale up much better, thus gel-free strategies are used more and more, since they allow higher analytical throughput and deeper proteome coverage than gel-based methods.

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

4.3.3.1 Gel-based approach

4.3.3.1.1 Sample preparation

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

4.3.3.1.2 Protein separation and quantification

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

4.3.3.1.3 Protein identification and characterization

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

4.3.3.2 Gel free approach

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

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

4.3.3.3 Obtention of peptides and identifying proteins from peptides

4.3.3.3.1 Peptides obention

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

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

4.3.3.3.2 Identifying proteins from peptides

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

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

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

4.3.4 Challenges and future perspectives

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

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

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

4.4 Methods for polysaccharides determination in microalgae

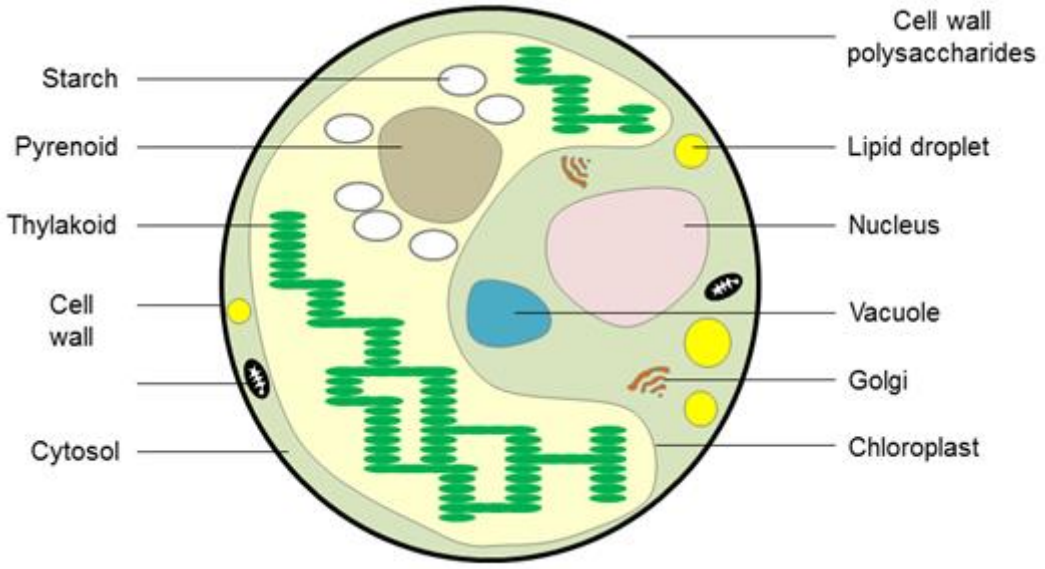
[Guillaume Pierre, Clément Gaignard, Cédric Delattre, Céline Laroche, Philippe Michaud]

4.4.1 Introduction

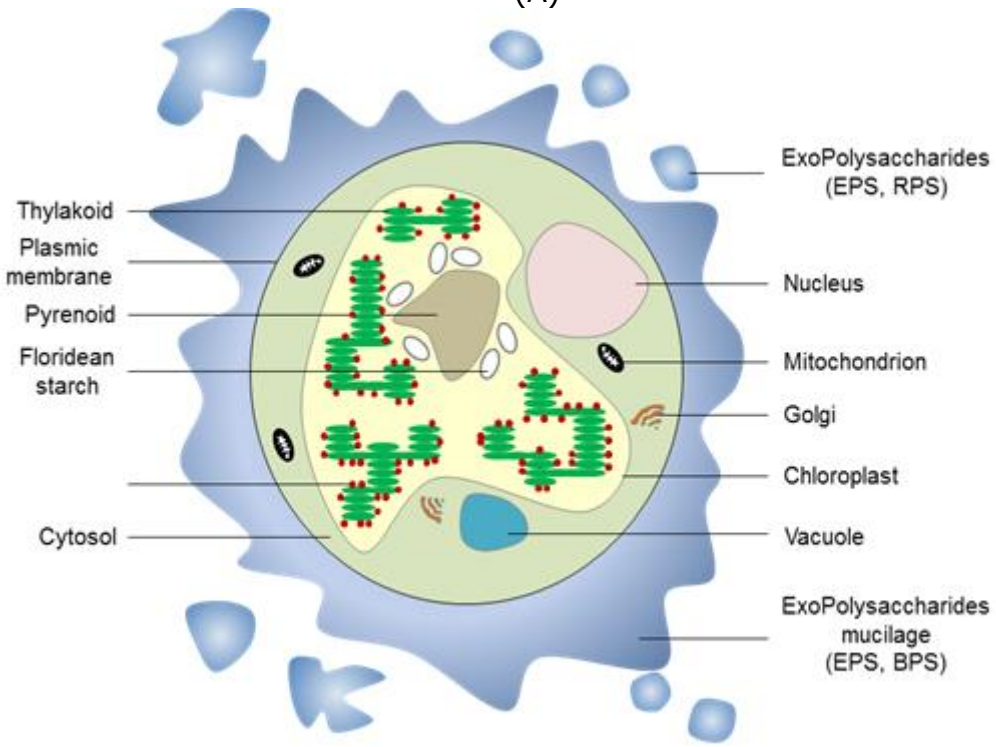
Polysaccharides are highly complex macromolecules exhibiting a great variety of biochemical structures based on glycosidic linkages of up to 50 different monosaccharides including numerous isomers (hexoses and pentoses), some of them being complex sugars. Various non-sugar substituents such as sulphates,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

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

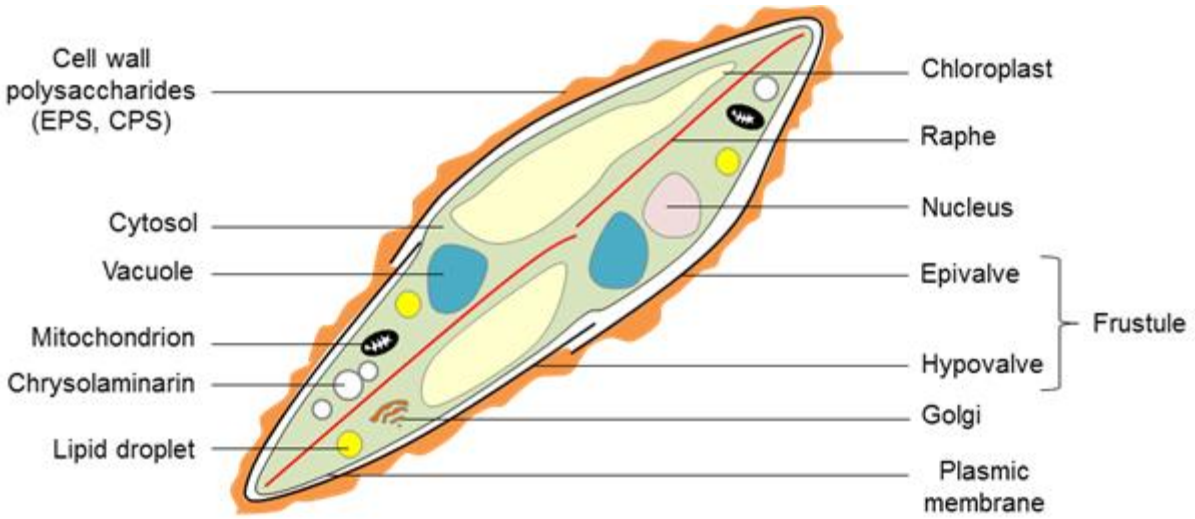
characterization may be impacted by culture conditions for their production, but also by the extraction and purification used to collect them [141,142]. The identification by some authors of structures differences depending on culture conditions could be in some cases artefacts related to different extraction,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



(A)



(B)



(C)

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

4.4.2 Polysaccharides sampling and extraction strategies

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

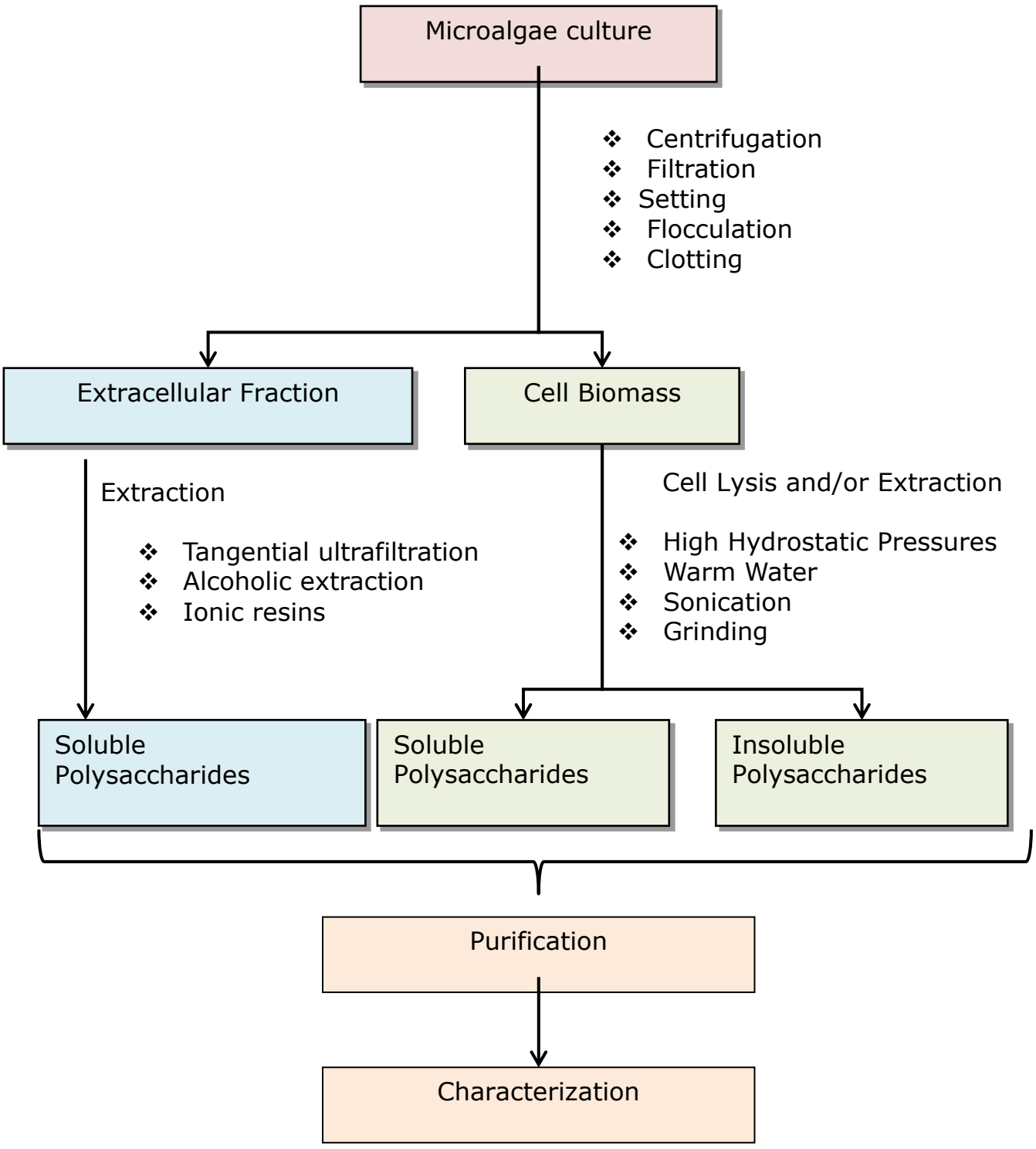


Fig. 4.4.2: Overview of polysaccharide extraction from microalgae.

4.4.2.1 Alcoholic precipitation of polysaccharides as conventional extraction processes

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

4.4.2.2 Tangential ultrafiltration process for EPS purification

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

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

4.4.2.3 Specific treatments for the extraction of cell-bound exopolysaccharides

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

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

4.4.2.4 Specific treatments for the extraction and quantification of starch

Despite growing interest for the use of starch from microalgae as feedstock for bioethanol production within a biorefinery point of vue, only few studies describe a protocol for extraction and purification of starch. Extraction of starch is only performed when structural characterization is needed, especially for the understanding of mechanisms ruling the metabolic patterns depending on culture conditions. As starch is an intracellular polysaccharide, first step involves a cellular lysis. Several methods exist, that can be classified as physical, enzymatical, or chemical methods, but physical methods are generally preferred. Delrue et al. [174] and Deschamps et al. [175], have purified starch from microalgae using similar protocols. The cellular lysis was achieved by sonication, of a cellular suspension in phosphate buffer (pH 7.5-8.0) containing 5 mM EDTA. For Deschamps et al. [175] the buffer was specifically supplemented by 1 mM of dithiotreitol. After centrifugation, the pellets (starch and cell fragments) were resuspended in 90 % Percoll and centrifuged (10,000 g, 30 min) to separate high-density starch granules from cell debris of lower density. The Percoll gradient step is repeated to ensure complete removal of cell debris from the starch pellet. The starch is then washed in sterile water and freeze-dried after its recovery by centrifugation. Kobayashi et al. [176] used a 10 % toluol solution in order to separate starch and cell debris. After mixing vigorously during 10 minutes, an emulsion layer, containing cells debris, was formed at the top of the tube. After discarding this layer, the starch was recovered and toluol step repeated, until no coloration appeared. Starch was then washed with water and ethanol before drying. Other alternative can be, after cellular lysis and the first centrifugation step, to resuspend pellets in water and to boil (30 min), in order to solubilize starch. After a new centrifugation (10 000 g, 15 min), the supernatant containing starch was precipitated with 3 volume of ethanol, or more specifically with iodine [177]. Nevertheless, starch from microalgae is structurally different from that of higher plants regarding the ratio amylose: amylopectin. Whereas starch from higher plants contains generally around 30 % of amylose, amounts of 5-25 % have been reported for chlorophyceae [176] and is rather absent in starch from rhodophyceae except in few unicellular strains [178]. Consequently the efficiency of iodine precipitation can differ from one genus to another [179].

When starch has been extracted and purified, classical methods can be used for its quantification and characterization. Nevertheless, most authors are interested in quantifying starch without time consuming extraction. Several methods are found in literature for quantification of starch content in microalgae. Most of them are derived from the characterization and quantification of carbohydrates from terrestrial plants. For example, starch content can be estimated after starch solubilization from ethanol-treated biomass (defatted and depigmented) by boiling. After centrifugation, supernatant containing starch is mixed with iodine

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

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

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

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

4.4.2.5 Specific treatments for the extraction of fibrillar polysaccharides.

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

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

4.4.3 How to determine the global composition of polysaccharides?

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

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

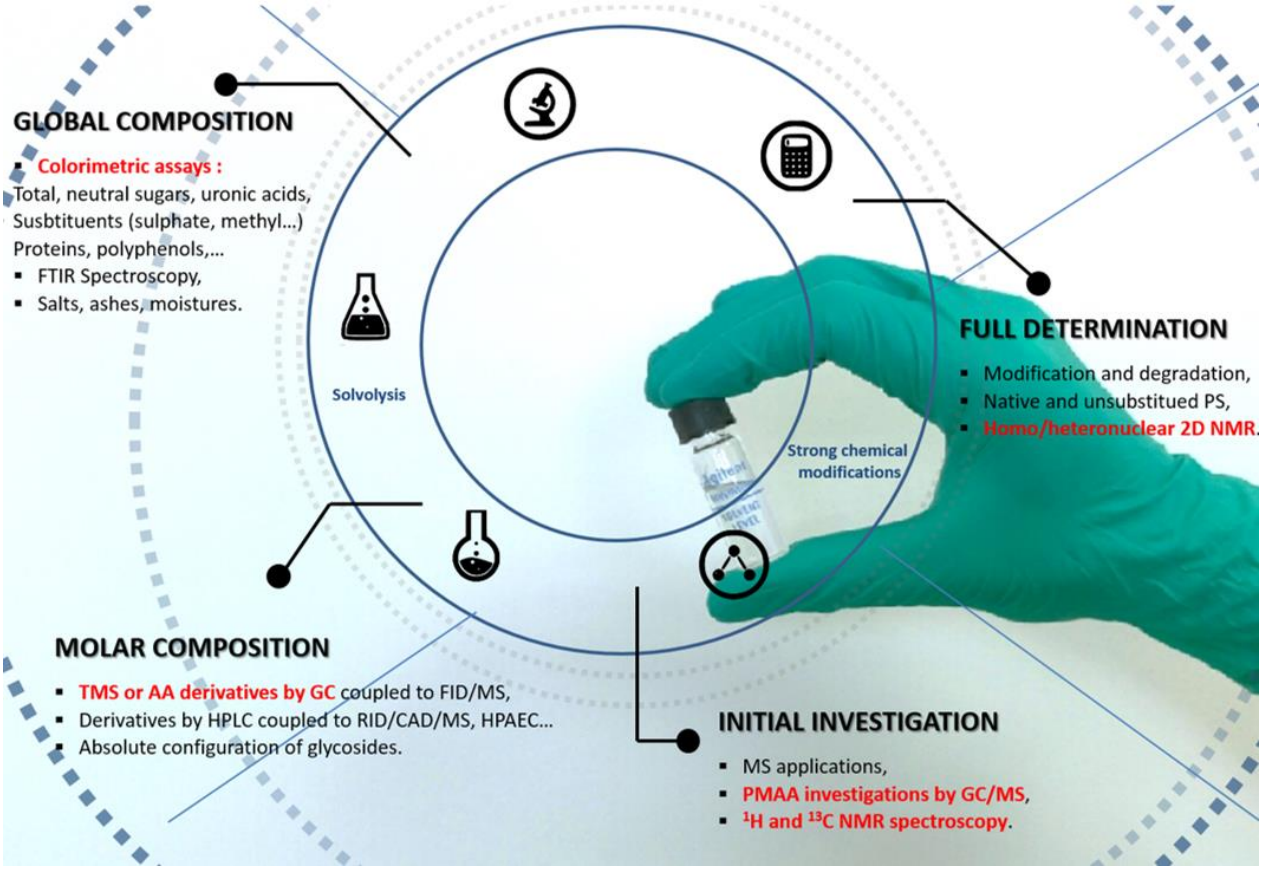


Figure 4.4.3: Overview of the strategies needed for the structural analysis of polysaccharides from microalgae. HPLC: High-Performance Liquid Chromatography, RID: Refractive Index Detection, CAD: Charged Aerosol Detection, MS: Mass Spectrometry, GC: Gas Chromatography, FID: Flame-Ionization Detector,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

4.4.3.1 Total carbohydrates

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

4.4.3.2 Uronic acids and neutral sugars

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

4.4.3.3 Substituents and non-carbohydrate content

Sulphate, pyruvate, methyl and acetyl groups have also to be quantified. The sulphate groups can be measured by using the turbidimetric protocol of Craigie et al. [198], estimated by the gelatin/$BaCl_2$ assay [199] or the Azure A method of Jaques et al. [200]. Pyruvate groups can be measured through the method of Sloneker and Orentas [201]. Methoxy and acetyl groups are often observed by ^{1}H NMR since these groups have specific resonances. However, a saponification step can be applied to release -CH_3 and -$COCH_3$ groups, which can be directly or indirectly monitored by High-Performance Liquid Chromatography (HPLC) [202,203]. Even if the extraction step and more specifically alcoholic precipitation (see the previous section) decreases the presence of proteins and/or glycoproteins, total proteins (which can also be covalently linked to polysaccharides) can be quantified by the Smith et al., Bradford or Lowry et al. methods [95,99,204]. For determining the polyphenol content, the Folin-Ciocalteu assay can be used from [205]. Finally, salts, ashes and moisture should be also quantified.

4.4.3.4 Composing groups by Infrared spectroscopy

Fourier Transformed InfraRed (FTIR) spectroscopy can be used for identifying and/or discriminating polysaccharides as well as observing changes in their composition (footprint) by providing details on a range of vibrationally active functional groups [206]. The structural analysis of carbohydrates by FTIR spectroscopy focuses on five specific regions from 3600 to below 700 cm^{-1}, which correspond to O-H and C-H stretching vibrations, local symmetry, C-O stretching vibration and fingerprint or anomeric region and skeletal region [207]. Polysaccharides usually display a strong and broad absorption peak at 3360 cm^{-1} corresponding to (O-H) stretching vibrations as well as water adsorption [208,209]. A weak signal at 2920 cm^{-1} can be attributed to the asymmetric vibration of (C-H_2) groups [210,211]. Overall, the region from 1500 to 500 cm^{-1}

is defined by several modes concerning the type of polysaccharides and glycosidic linkages. As an example, furanose derivatives from pentoses can be identified at 1250 cm^{-1}. At 1250-1170 and around 1300-1250 cm^{-1}, aliphatic esters and aromatic esters can be respectively observed. Sulphate ester ($O\text{-}SO_3^-$) groups can be identified in the region from 1260 to 1210 cm^{-1} [212], carboxylic acid ester form (C=O) or carboxylate anion form (COO-) can be observed around 1730 and 1600 cm^{-1} [213] whereas acetyl groups can be found at 1250-1220 cm^{-1}. The signal observed around 1040 cm^{-1} is in general from the presence of (C-O) of polysaccharides [214]. Anomeric (*α* and *β*) configurations can be identified by three types of bands from 950 to 750 cm^{-1} [215]. Note also that glycosidic methoxy groups give a specific symmetric $C\text{-}H_3$ stretching band around 2880-2830 cm^{-1}. FTIR spectroscopy also benefits the possibility to detect proteins, nucleic acids (NA) and other potential "contaminants" of the carbohydrate fraction through the amide I (1709-1583 cm^{-1}), amide II (1585-1481 cm^{-1}) and NA (1356-1191 cm^{-1}) bands [216].

4.4.4 How to determine the monosaccharides composition of polysaccharides?

4.4.4.1 Preliminary solvolysis

Monosaccharides have to be released by cleaving the glycosidic bonds before their analyses by Chromatography. Two preliminary solvolysis, *i.e.* (i) methanolysis and (ii) hydrolysis, are well described in the literature for this purpose. The first one involves the cleavage of the linkages by methanolic HCl (around 2 M) in milder conditions than for classical hydrolysis. Less degradation of monosaccharides to furfural derivatives (or more degraded products) is often reported and it is possible to analyze both neutral sugars, uronic acids, sialic acids and acetamido sugars. Moreover, methyl groups substitute the hydroxyl groups previously engaged in the glycosidic bonds [217]. Briefly, the protonation of the glycosidic-*O*-atom results to the cleavage of the glycosidic linkage from a specific ring form. An anomeric carbocation will be formed and be able to react with water and give α/β-anomers. Thus, ring form conversions can occur through protonation of the ring-*O*-atom and/or mutarotation [132]. On the other hand, hydrolysis by mineral acids (H_2SO_4, HCl, CF_3COOH) can be used to release the monosaccharides. Numerous conditions are reported in the literature and the use of TriFluoroacetic Acid (TFA) is often preferred (2-4 M, 90-240 min, 100-120 °C). Indeed, some authors reported the degradation of monosaccharides in particular when strong conditions are used to ensure the cleavage of resistant glycosidic linkages [218]. The effect of these conditions should not be neglected since it may cause underestimation of specific monosaccharides and more specifically ketoses. Here again, released monosaccharides from the polysaccharide structure give a monosaccharide-specific anomeric distribution. Finally, methyl glycosides or released monosaccharides can be analyzed by Gas Chromatography (GC) or HPLC including High-Performance Anion-Exchange Chromatography (HPAEC).

4.4.4.2 Chromatography

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

strongly reduced compared to classical derivatizations of monosaccharides needed for GC analysis. On the contrary, one main disadvantage could be the low resolution especially for complex mixtures [219]. Overall, the separation is based on polarity allowing to work with normal and Reversed-Phase Chromatography (RPC). Ions-exchange, polymer-, silica- or metal-based and amino-bonded can be used with heating (30-80 °C) under various mobile phases (diluted H_2SO_4, water, acetonitrile) for the quantification of the released monosaccharides. Few papers also reported derivatization of monosaccharides prior the analysis [220]. HPAEC methodology should be also considered as a first-rate alternative for quantifying monosaccharides (using CarboPac PA-1), as reported by Templeton et al. [221]. Coupled with Pulsed Amperometric Detection (PAD), the method is resolutive and based on the pKa of monosaccharides and changes in temperature or alkalinity. Schäeffler et al. [222] reported tuning procedures to improve HPAEC analyses for carbohydrates even if Fu and O'Neill [220] highlighted the drop of electrochemical response in presence of proteins.
Secondly, GC is still used in many papers for quantifying monosaccharides after solvolysis due to higher sensitivity and resolution of spectra [223]. On the contrary to HPLC, released monosaccharides must be converted into volatile compounds *via* derivatization procedures including silylation and acylation. Silyl derivatives, which are the most used for GC applications, are generated by displacement of active hydrogen on -NH, -OH or -SH groups and various derivatizating agents can be used for this purpose, *e.g. N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA), *N*-trimethylsilyimidazole (TMSI) or *N*,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA). The latter is often combined to trimethylchlorosilane (TMCS) to increase the silyl donor strength. Thus, TriMethylSilyl (TMS) alditols, aldonitrile acetates, TMS methyl ester glycosides or oxime derivatives can be prepared for GC analysis. In general, Flame-Ionization Detector (FID) is preferred to MS for the quantification since the results are more reproducible. As reported by Peña et al. [224], modifying the mass tuning and the possibility to use ion suppression strongly impact MS quantification and can result in inaccurate quantification. Note that MS benefits mass spectra (associated to specific fragmentation patterns) which are essential for discriminating carbohydrates to non-carbohydrate contaminants. Finally, GC/MS experiments can be performed using MS-Chemical Ionization (CI) or Electron Impact Ionization (EI). Pyranose and furanose ring forms can be distinguished comparing the intensities of the specific ions m/z 204 and m/z 217 for TMS (methyl) glycosides [217] as well as regarding the ion m/z 205. Ions such as m/z 379, 319, 305, 217, 205, 204, 117 are characteristics of the fragmentation of TMS (methyl) glycosides by GC/MS-EI [152]. Beside to TMS derivatives, the conversion of released monosaccharides to *per-O*-acetylated alditol (AA) derivatives is another way to quantify monosaccharides composition [224]. Each monosaccharide gives only one peak with specific fragmentation patterns and main ions such as m/z 289, 217, 187, 157, 115, 145, 103, 43. Overall, a wide range of columns can be used for GC/MS-EI analysis, *e.g.* AT-1 [132], HP-1, CP-Sil 5 CB, DB-1701 [225], DB-225MS [221], Optima-1MS [226], OV-17 [227] or SP 2330 [224].

4.4.5 How to elucidate the branching patterns of polysaccharides?

4.4.5.1 Absolute configuration analysis

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

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

4.4.5.2 NMR analysis

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

4.4.5.3 Mass spectrometry analysis

MS applications can be combined with a broad range of chromatographic technologies such as MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization with Time of Flight mass spectrometry), GC/MS-CI, HPLC-ES-MS, HPAEC-PAD/MS, HPLC-ESI-MS-MS, etc. [132]. MALDI-TOF MS, electrospray ionization mass spectrometry as well as GC/MS-EI are often preferred for the structural studies of glycans. The latter involves the preparation of PerMethylated Alditol Acetates (PMAA) which can be used to determine terminal units, glycosidic linkages, branching points and the ring size of monosaccharides. Before hydrolysis, permethylation of the polysaccharide is done by using CH_3I in alkaline medium, *e.g.* solid NaOH in C_2H_6OS [224]. As previously described, various conditions of hydrolysis can be used but 2-4 M TFA give good depolymerization results. Reduction using ($NaBH_4$ or $NaBD_4$ for isotopic labeling in NH_4OH/ C_2H_6OS) then peracetylation $(CH_3CO)_2O$ in presence of co-catalyst $C_4H_6N_2$) are performed to yield PMAA. Interpreting the fragmentation patterns from mass spectra (mostly without molecular ion peaks) leads up to determine the position of glycosidic bonds initially composing the polysaccharide. A comprehensive study of these fragmentations patterns and tables can be found in Kamerling and Gerwig [132]. Further analyses are needed to complete the initial monosaccharides linkages analysis such as (i) uronic acid reduction due to their

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

4.4.6 Conclusion

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

Acknowledgment

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4.5 Methods for lipids determination in microalgae

[Junko Ito, Makoto M. Watanabe]

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.

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**.

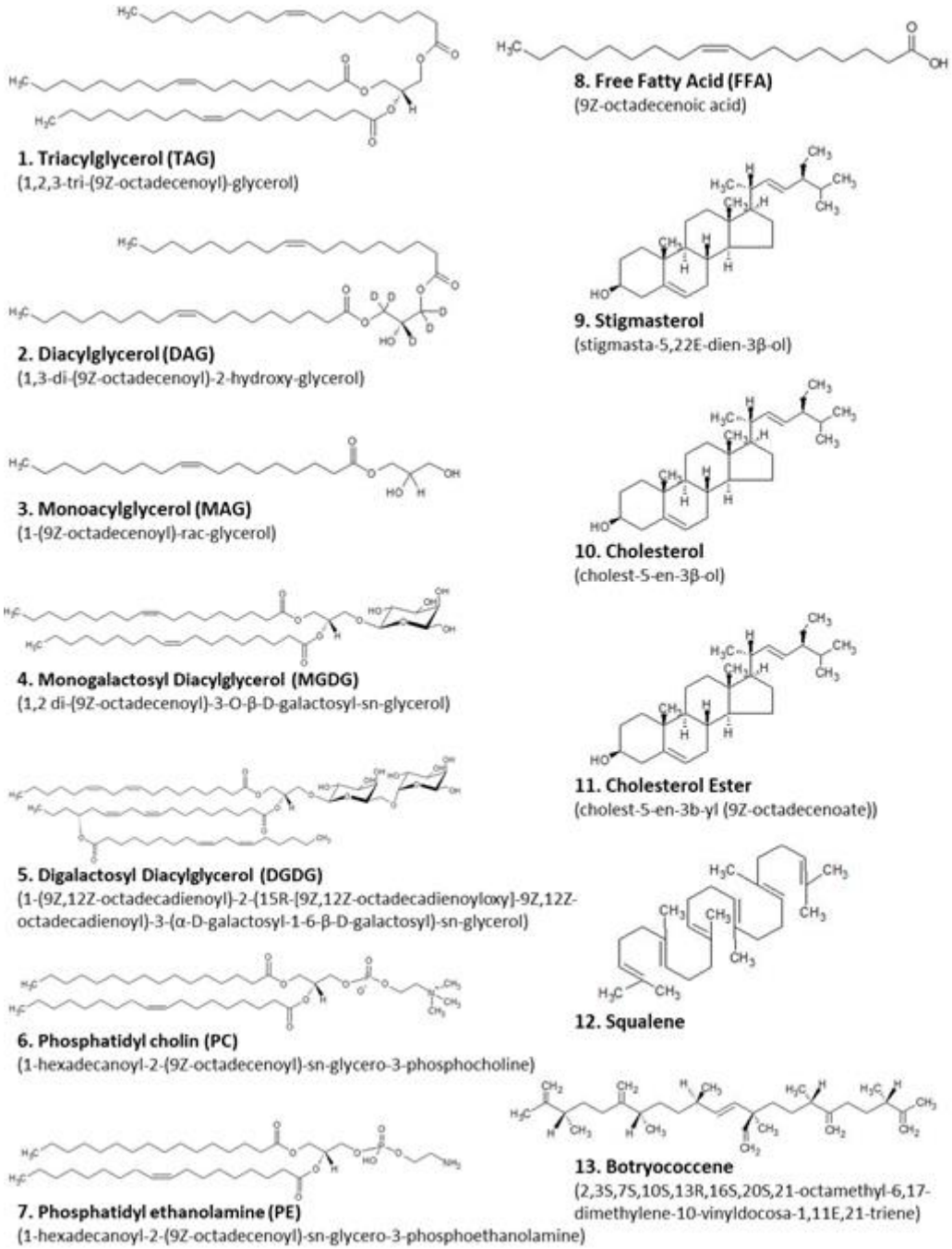

Fig. 4.5.1: Chemical structures of the major representatives from microalgae lipid. Structures derived from www.lipidsmaps.org.

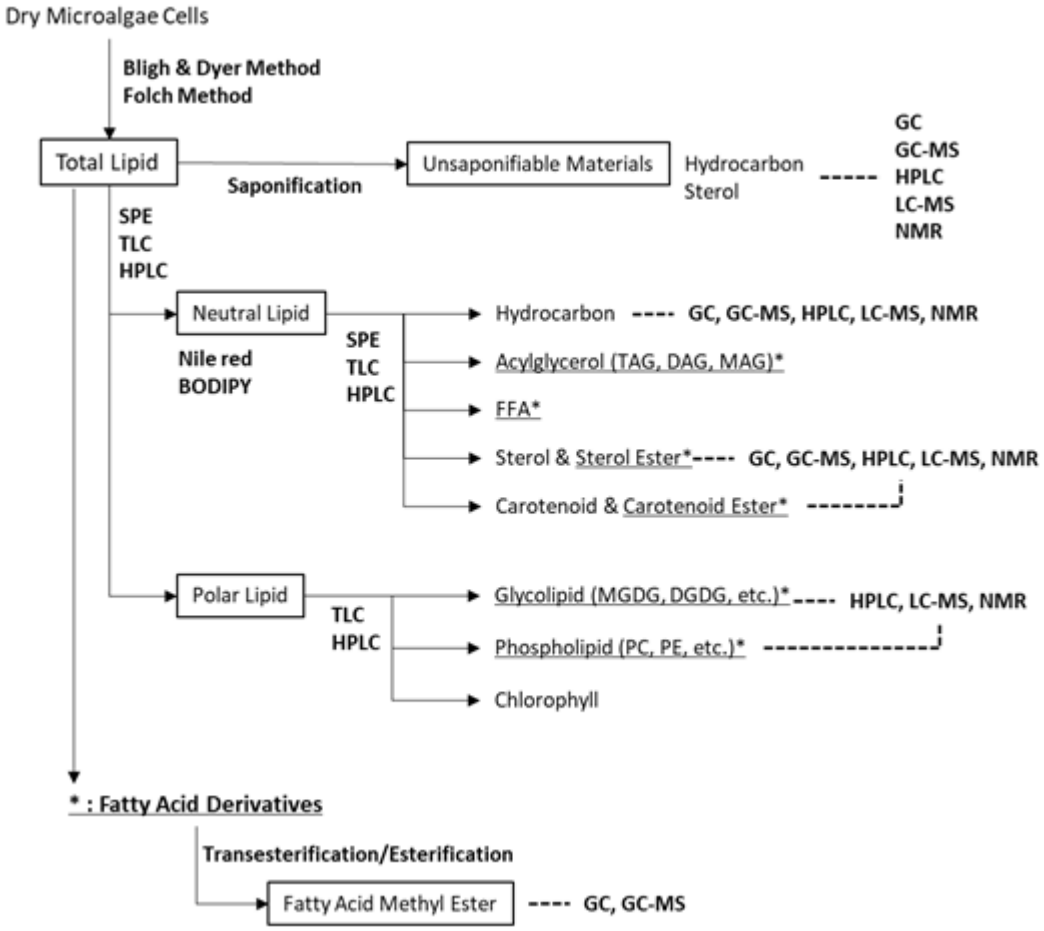

Fig. 4.5.2 The summary of lipid analysis steps and available analysis technics. SPE: Solid Phase Extraction, TLC: Thin-Layer Chromatography HPLC: High-Performance Liquid Chromatography, GC: Gas-Chromatography, MS: Mass Spectrometry, NMR: Nuclear Magnetic Resonance,
TAG: Triacylglycerol, DAG; Diacylglycerol,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

[248]. After washing, the wet pellets are stored at −80 °C. These frozen pellets are then lyophilized in a lyophilizer. This removes water effectively without reducing the quality of the molecular components, compared with drying using heat. Lyophilization also increases the surface area of the sample, leading to better lipid extraction and easy rehydration. Lyophilized cells are also easily weighed, stored, and handled [248,249]. Furthermore, lyophilized cells contain very little water, and there is no need to add lipase deactivators during lipid extraction.

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

4.5.2 Analysis of total lipid content

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

The standard method of analyzing total lipid content is the method of Folch et al. [255], or that of Bligh and Dyer [256], involving the use of chloroform-methanol mixture as an extracting solvent [247,248]. The co-solvent containing methanol as a polar solvent can extract not only neutral lipids like hydrocarbon and acylglycerols but also polar lipids like glycolipids and phospholipids. Furthermore, the polar solvent releases the lipids from their protein–lipid complexes, and the lipids subsequently dissolve in the nonpolar solvent [248,254]. Modified versions of the methods of Bligh and Dyer [256] or Folch et al. [255] have also been proposed in many studies [19, 243,247,248,250,251,257-262].
Ryckebosch et al. [248] explained that the method of Folch et al. [255] is the most widely used for the extraction of total lipids from microalgae. This method was originally optimized for the isolation and purification of total lipids from animal tissues. It uses chloroform/methanol at a 2:1 ratio for the extraction of lipids and water along with a small amount of NaCl to remove non-lipid substances from the extract. The method of Bligh and Dyer has also often been used for the extraction of total lipids from microalgae and was originally optimized for the extraction of phospholipids from fish muscle. It uses chloroform/methanol at a 1:2 ratio and followed by extraction with chloroform. Iverson et al. [256] compared the methods of Bligh and Dyer [256] to Folch et al. [255] for the determination of total lipids in marine fish tissue. They concluded that the unmodified method of Bligh and Dyer resulted in lower estimation of lipid content in samples containing >2 % lipid, and this underestimation increased significantly with increasing lipid content of the sample.

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

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

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

4.5.3 Separation and analysis of lipid classes

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

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

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

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

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

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

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

4.5.4 Analysis of fatty acid composition and content

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

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

The analysis of fatty acids can be conducted on the lipids extracted from microalgae and lipids separated into lipid classes. It is very difficult to analyze fatty acids in crude extracted lipids without any pretreatment using analytical equipment directly. The standard method is first to convert lipids to fatty acid methyl esters (FAME) through transesterification and then to analyze FAME by gas-chromatography (GC) [84,248,259,261,262,264,266,270,272,277,278].
Carrapiso et al. [278] reported the principles of transesterification by acidic or basic catalysts. In an acidic or basic methanol solution, the fatty acid derivatives (TAG and phospholipids) are changed to FAME. Major catalysts are hydrochloric acid (HCl) [259,262], sulfuric acid (H_2SO_4) [248,261,262,271,272], and boron trifluoride (BF_3) [262,276] as acids, and sodium hydroxide (NaOH), potassium hydroxide (KOH), and sodium methoxide ($NaOCH_3$) as bases.

Acidic catalysts are most widely used for microalgal fatty acid analysis. They have strong esterification power, reacting with not only TAG and phospholipids but also FFA and N-acyl lipids (sphingolipids). Furthermore, there is less water interference than for basic catalysts. The method of Christie [270] referenced by many papers [248,261,262,271,272,277] uses H_2SO_4 as a catalyst. H_2SO_4 has a longer life of catalytic activity and involves safer preparation than HCl. In the case of BF_3, it has higher esterifying power than H_2SO_4 and can be used to analyze a small amount of sample [264]. However, BF_3 cannot be used for water-containing samples because it is rapidly destroyed by water [278,279].

Basic catalysts have the advantage of having a high reaction speed and relatively mild heating conditions, compared with acidic catalysts [278,280,281]. Furthermore, for lipids containing acid-labile fatty acids (cyclopropane rings, epoxyl groups, or conjugated unsaturated molecules), it is better for the analysis to be performed under basic than acidic conditions. However, basic catalysts cannot react with FFA and sphingolipids, and the saponification reaction occurs in the presence of water.
A method combining the use of both basic and acidic catalysts is the American Oil Chemists' Society method [282]. This is now recognized as a standard method for oil and lipids, which means that it is not specific to microalgal oil. This method involves first transesterification (or saponification) by NaOH in methanol, followed by esterification by BF_3 in methanol [264,282,283]. Yi et al. [284] applied KOH in methanol to fish oil, which contains high levels of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), prior to BF_3 in methanol. *Schizochytrium* sp. can produce higher levels of multiple polyunsaturated fatty acids such as DHA, EPA, and docosapentaenoic acid. For the analysis of fatty acids produced by *Schizochytrium* sp., KOH and BF_3 were used in methanol [285,286].

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

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

In situ (direct) transesterification is a method of applying methylation agent directly to the biomass and reducing the extraction steps [262,264,287-292]. To dry microalgae, catalyst solution with methanol is added, followed by incubation with heating. Excluding the lipid extraction process leads to less organic solvent and reduces the process time. Acidic catalysts such as BF_3 [262,264,290], HCl [262,288,290], and H_2SO_4 [289,290,292] are commonly used. Cavonius et al. [262] compared 11 methods to analyze fatty acids in three different microalgae. These were four different conventional methods of extraction prior to transesterification, two types of two-step transesterification (saponification followed by esterification), and five different *in situ* transesterifications. Their results showed that the conventional method resulted in underestimation of the fatty acid content compared with two-step and *in situ* transesterification. Regarding the catalyst, HCl and H_2SO_4 could recover the most fatty acids, whereas BF_3 recovered slightly less. Velasquez-Orta et al. [291] performed a comparison between a basic catalyst (NaOH) and an acidic catalyst (H_2SO_4) for *in situ* transesterification of *Chlorella vulgaris* and concluded that the basic catalyst outperformed the acidic one, obtaining higher conversion at shorter reaction times. At any rate, *in situ* transesterification is very useful to obtain FAME for analyses of fatty acid composition and content. The obtained FAME can be analyzed by GC-FID or GC-MS.

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

4.5.5 Hydrocarbon analysis

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

Race L produces tetraterpenic hydrocarbons of lycopadiene ($C_{40}H_{78}$) [304]. Race S comprises epoxy-n-alkane and saturated n-alkane chains with carbon numbers of 18 and 20 [303]. Another well-known alga producing hydrocarbons is *Aurantiochytrium* sp., which produces squalene [305-308]. Squalene is also a polyunsaturated triterpenic hydrocarbon ($C_{30}H_{60}$). These hydrocarbons can be used in the cosmetic industry as moisturizing agents [305] and are expected to be an alternative petroleum fuel and chemicals, among others.

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

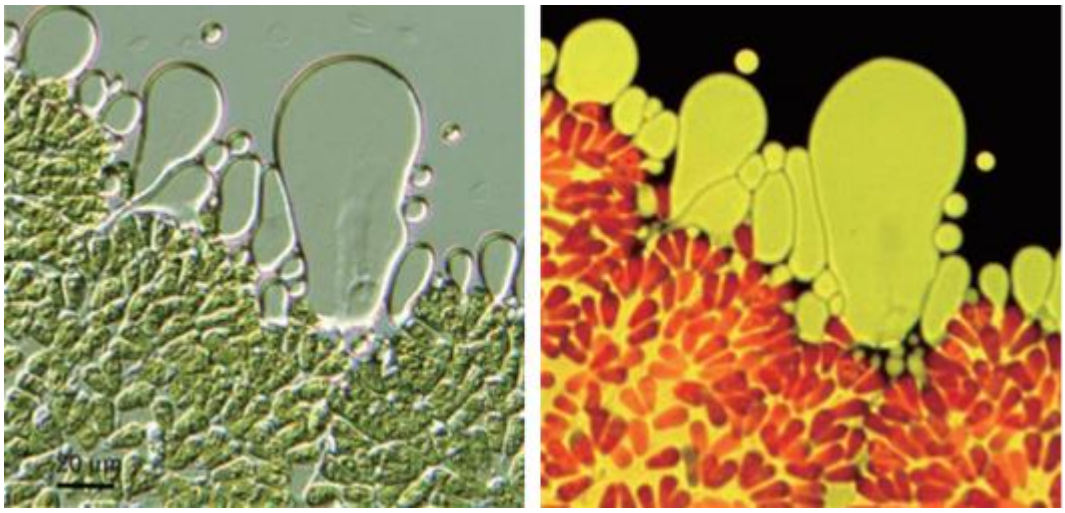

Fig. 4.5.3: *Botryococcus braunii*
Left: photomicrograph of colony
Right: fluorescence micrograph of Nile Red stained cell (yellow fluorescence indicates oil)

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

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

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

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

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

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

4.5.6 Phytosterol analysis

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

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 5α-cholestane [330], cholesterol [332], and 5β-cholestan-3α-ol [333] for GC analysis and 6-ketocholestanol [334], 1-hexacosanol [335], and d6-cholesterol [336] for LC analysis.

4.6 Methods for pigments determination in microalgae

[Benoît Serive]

Organic pigments are widespread molecules in the whole biodiversity and particularly in microalgae diversity [337] since they play crucial roles in photosynthesis and photoprotection. Pigments from microalgae belong mostly to carotenoids (carotenes and xanthophylles), chlorophylls, and phycobiliproteins. However, the full definition of pigments include various molecule scaffolds such as anthocyanosides, betalains, some alkaloids (*e.g.* scytonemin), amino-acids (*e.g.* mycosporines-like, eumelanin), azaphilones, polyphenols (*e.g.* floridorubin, quercetin), anthraquinones (*e.g.* hypericin), or chlorines (*e.g*. purpurin-18). Since phycobiliproteins have already been presented previously in this chapter, we will focus essentially on carotenoids and chlorophylls.
Since ancient times, civilizations have used pigment like Tyrian purple from gastropod molluscs or other organisms for clothes dying. Then, the link between food and health has been documented since Antiquity (*i.e.* Hippocrates associated the food to health and diseases) [338]. Moreover, Aztecs were one of the civilizations to use spirulina as food source. Apart from the fact this cyanobacteria is protein-rich source, it contains pigments known nowadays with health benefits. Indeed, these last decades, numerous scientific studies have shown interesting properties from pigments through notably antioxidant, anti-inflammatory, anti-AMD, anti-tumoral, anti-angiogenic, anti-diabetic, anti-obesity, anti-photoaging, and neuroprotective activities. Chlorophylls are more recommended notably to stimulate liver function recovery and increase bile secretion [339]. Thus, today, pigments found applications in chemical, pharmaceutical, poultry, cosmetics, cosmeceutical, functional food, nutraceutical and aquaculture industries [342-340]. Today, the chemical synthesis market covers about 90 % of the main carotenoid needs (*e.g.* asthaxanthin, β-caroten). However, natural sourcing benefits from a positive image since Western countries consumers are more and more sensitive to the impact of chemicals onto their health. The all-trans isomer of β-caroten is still chemically synthetized but the 9-cis form is produced only from natural sources, the natural form displaying better antioxidant capacity than the synthetized form. Although the natural pigment sources still compete with synthetic dyes, with the high development of agri-food industry, natural pigments are more and more used for coloring of food preparations and for their antioxidant properties. Beta-carotene market value has been estimated at US$ 261 million in 2014. With an annual growth rate of 3 %, it is expected to reach US$ 334 million by 2018 [343]. This is favored by stringent government regulations regarding environmental and human health hazards (Global Market Insights Inc.[344]).

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

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

4.6.1 Extraction

4.6.1.1 Centrifugal Partition Extraction

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

4.6.1.2 Supercritical CO_2 extraction

This technique (SC-CO_2) can be considered as a green strategy to reach microalgae metabolites since CO_2 gas stream is recyclable. However, prior drying or lyophilisation step is required to implement this kind of extraction. There is a large panel of equipment available from laboratory scale (*e.g*. lyophylisators, centrifugal evaporators) to industrial scale (*e.g.* vacuum drying, spray drying, extrusion porosification technology). However, dewatering pre-treatment add a substantial cost to the entire process, which is not suitable for low valuable products. In case of pigments with high added value for nutraceutical, cosmeticeutical, or pharmaceutical application, the additional cost can be acceptable. In this particular case, SC-CO_2 stays promising in term of carotenoid extraction. Thus, numerous studies have demonstrated these last years that SC-CO_2 is quite selective to extract carotenoids like β-carotene, lutein, astaxanthin, or zeaxanthin over chlorophylls from various microalgae species [351-354]. The use of ethanol as entrainer (co-solvent) is particularly efficient. However vegetable oils (*e.g.* soybean or olive oil) can be used as co-solvent in order to avoid the subsequent separation step of the co-solvent, *i.e.* the oil enriched with pigments can be sold straight away [355].

4.6.1.3 Milking

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

4.6.1.4 Accelerated Solvent Extraction

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

4.6.1.5 High-speed homogenization

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

4.6.1.6 Microwave Assisted Extraction (MAE)

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

4.6.1.7 Sonication

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

4.6.1.8 Pulsed Electric Field

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

4.6.2.9 Enzyme Assisted Extraction

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

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

4.6.1.10 Bead Milling

This is one of the most promising techniques in term of pigment extraction yield both at laboratory scale and industry scale. It generates mechanical compaction and shear stress for cells. This technique has shown excellent results with a diatom and also especially with *Porphyridium purpureum* which is known to be quite resistant to disruption [360]. Laboratory scale equipment is based on agitation of a chamber or tubes filled with beads (glass, steel, ceramic, or zirconium material), while industrial scale equipment is based on a mixing chamber filled with beads and biomass to extract.
For this process, agitation disk design, speed, bead filling, size, material, microalgae amount (or concentration), feed rate, growth phase and conditions, microalgae type, time, and cooling are parameters to optimize. Our background experience has shown that it is preferable to work on a wet biomass rather than a lyophilized biomass to allow a good extraction yield. Associated with the proper solvent or buffer targeting the pigments of interest, this technique is quite suitable for most pigment projects. For microalgae biomass, a couple of studies support the idea that mechanical grinding is often the most effective technique to recover metabolites efficiently, including pigments [47,360]. However, energy requirement is a very important parameter when large biomass is treated. It seems that mechanical disruption methods are considered highly energy inefficient [365]. Thus, bead milling should be preferred for high value compounds.

4.6.1.11 Soaking

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

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

4.6.1.12 Instant controlled pressure drop

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

4.6.1.13 Chemical treatment

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

4.6.1.14 Others

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

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 nutraceutics, in cosmeceuticals, in functional food industry, aquaculture, or agriculture [378,379].

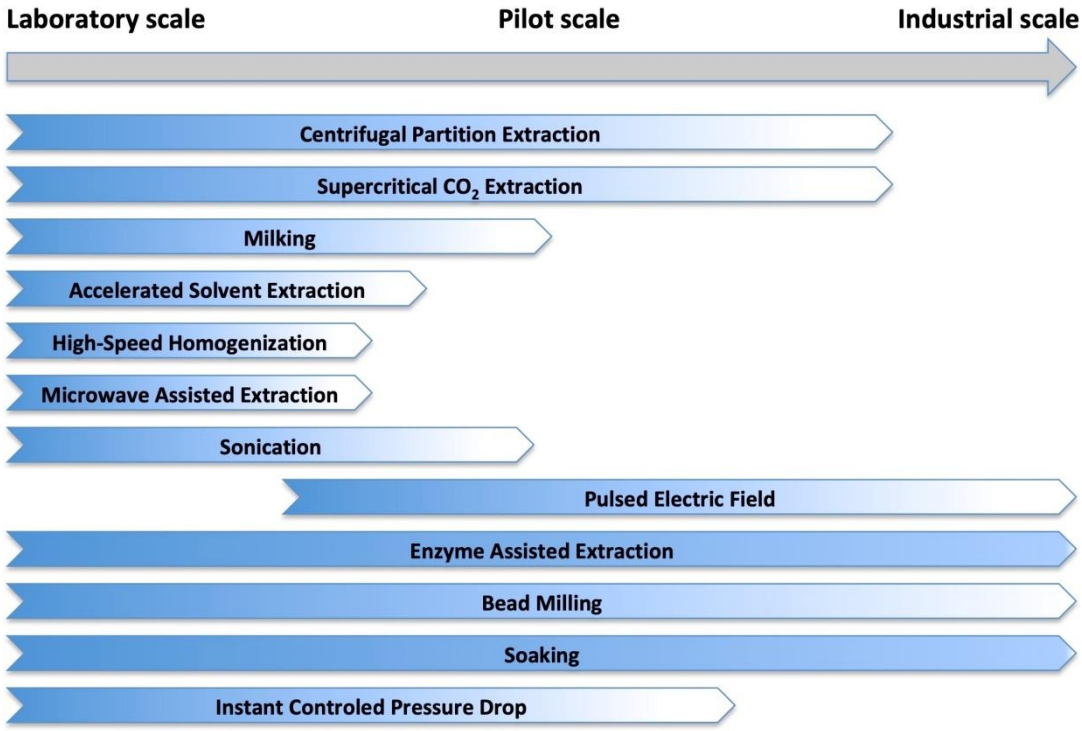

Fig. 4.6.1: Extraction techniques from laboratory to industrial scale.

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.

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.

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

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

4.6.2.2 Other techniques for structural characterization and identification

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

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

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

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

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4.7 Methods for secondary metabolites determination in microalgae

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4.7.1 Introduction

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

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

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

4.7.2 Sampling and Preconditioning

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

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

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

4.7.3 Extraction Techniques

The solvent extraction process is a crucial step in isolation and purification of individual distinct types of metabolites. The method chosen can directly affect the biological activity of the extracted compounds [425] as well as their reproducibility, efficiency and effectiveness. Therefore, when choosing the most appropriate method from amongst the several available options, certain important considerations must be addressed. First, microalgae and cyanobacteria present a remarkable biodiversity, which is reflected in the chemistry of their unique secondary metabolites, and associated cell wall characteristics and structure. Thick-walled, silicified membranes, multilayered walls, wall-bound exopolysaccharides and armored walls present barriers to permeation by the solvents used in extraction, and must be disrupted prior to this to allow the release of compounds of interest [360].
Labile compounds may be subject to degradation upon solvent extraction under elevated temperatures, and light, air and pH are among the factors that should be controlled to prevent the decomposition of these compounds.

4.7.3.1 Cell Disruption

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

4.7.3.2 Secondary Metabolite Extraction

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

literature include conventional extraction techniques, supercritical fluid extraction, enzyme extraction, etc.
The presence of thermolabile compounds requires the use of techniques working at low temperature, to prevent thermal degradation, hydrolysis and hydrosolubilization. The cell wall characteristics, the nature of molecules to be extracted from the matrix, extraction times and coextraction of undesirable compounds, as for example, sometimes lipids and pigments, are some of the factors that have to be considered in this.

4.7.3.2.1 Conventional Solvent Extraction

Solvent extraction: in conventional solvent extraction, the appropriate solvent or mixture of solvents is added to the microalgal biomass from which the desired metabolite is to be extracted. Once the crude extract is separated from the cell residue and filtered, the solvents, if volatile, can be evaporated.
Soxhlet extraction: the procedure described by Soxhlet [431] has commonly been used for the extraction of primary compounds as lipids from biological samples. Nevertheless, secondary metabolites are also extracted with this methodology from a known quantity of the microalgal material, which is placed in a thimble, by repeatedly washing and leaching (percloration) with the appropriate organic solvent under reflux in the Soxhlet apparatus. The solvent in the flask is heated to boiling. The solvent vapor is condensed in the condenser, flows through the sample and moves back down into the distillation flask. The resulting crude extract should be filtered, to remove any remaining unwanted matter, and can then be concentrated on a rotary evaporator or dried under a nitrogen steam. In the Soxhlet procedure, heat is required to drive the extraction, and this may cause decomposition of thermolabile compounds. In these cases, high temperatures must be avoided by choice of appropriate solvents in order to prevent the development of decomposition products.
The extraction process using organic solvents has come under increasing criticism due to its reliance on solvents that are known to be toxic, carcinogenic or environmental pollutants. Chloroform, dimethyl acetamide, dimethyl formamide, dimethyl sulfoxide and methanol are some of the solvents that are becoming considered unsuitable for such extractions, and there is increasing interest in the development of green solvents or other techniques for this [360].
Regardless of the choice of solvent, the selection of a suitable solvent or solvent system should address some aspects, such as grade and purity, solubility of the compounds of interest in it, safety, and environmental issues.

4.7.3.2.2 Non-Conventional Extraction Technique

Several novel extraction techniques have been developed in recent years as alternatives to the traditional extraction procedures, to overcome the limitations and inconveniences presented by the conventional methodologies. Methods such as microwave extraction (MAE), ultrasonic extraction (UAE), subcritical water extraction (SWE), supercritical fluid extraction (SFE), microbial-aided extraction (MbAE), and enzyme-aided extraction (EAE), have all been applied for the extraction of secondary metabolites from microalgae [425].
Green extraction methods, such as *Supercritical fluid extraction (SFE),* present advantages over the traditional ones using organic solvents. This eco-friendly process does not use large amounts of organic toxic solvents, is rapid, inexpensive, selective and it is suitable when dealing with thermolabile compounds, since it avoids long extraction times and high temperatures, which may result in degradation of compounds. The most commonly used critical fluid

has been supercritical carbon dioxide (SC-CO_2), which is non-flammable, has low critical conditions and is suitable for industrial applications [425].

4.7.4 Chemical characterization

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

4.7.4.1 Chromatographic Techniques

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

4.7.4.1.1 Thin Layer Chromatography (TLC)

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

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

4.7.4.1.2 High performance thin layer chromatography (HPTLC)

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

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

4.7.4.1.3 Gas Chromatography (GC)

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

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

4.7.4.1.4. High Performance Liquid Chromatography (HPLC)

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

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

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

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

4.7.4.2 Spectroscopic Techniques

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

4.7.4.2.1 Fluorescence Spectroscopy

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

4.7.4.2.2 Nuclear Magnetic Resonance (NMR)

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

Although NMR is a reliable and very robust technique that provides unambiguous information, it has some disadvantages that can limit its application. These include the expensive equipment needed, the time-consuming measurements, the high concentrations of samples required, and the overlapping signals that may be present when applied to complex bio-organic compounds, making the interpretation of the exact structure of the compound difficult.

In general, the NMR technique does not require elaborate sample preparation, and liquid samples, solvent extracts and dried or live microalgal cells can directly be analysed. It is a straightforward and non-destructive technique, such that samples can be further analysed by other techniques. NMR spectroscopy has been extensively used in biological studies and significant improvements are continuously being made in the use this high-throughput technique.

Since a large number of NMR experiments are possible in metabolomics, different approaches can be considered when using NMR spectroscopy. One dimensional ^{1}H NMR is one of the most widely used in metabolomics analysis. However, when analysing complex mixtures, ^{1}H NMR spectra can be very challenging due to overlapping of signals, which can hinder the identification of metabolites.

A relatively new methodology in the field of Metabolomics studies is *in vivo* High Resolution Magic Angle Spinning Nuclear Magnetic Resonance (^{1}H HR-MAS NMR) that can be applied to live microalgal cells [457].

In ^{13}C NMR spectroscopy, where chemical shifts cover 200 ppm, compared typically with 10 ppm for ^{1}H NMR, the signals are better resolved. However, the major limitation of ^{13}C NMR spectroscopy is the lower sensitivity arising from the low natural abundance of ^{13}C. The use of multidimensional NMR spectrum can help to overcome many of the limitations of one dimensional NMR. Two-dimensional (2D) NMR include correlated spectroscopy (^{1}H-^{1}H COSY), total correlation spectroscopy (^{1}H-^{1}H TOCSY) and heteronuclear single-quantum correlation (^{1}H-^{13}C HSQC).

A customized metabolomics NMR database [458], ^{1}H(^{13}C)-TOCCATA, contains a complete set of ^{1}H and ^{13}C chemical shift information on individual spin systems and isomeric states of common metabolites, and allows the identification of metabolites in complex mixtures [459].

Because NMR spectroscopy analysis frequently produces a highly complex spectrum which is difficult to interpret, it is useful to employ chemometric analysis, such as principal components analysis (PCA), that allows the reduction of multivariate data into a smaller number of principal components representing the original variables, and Partial Least Squares Discriminant Analysis (PLS-DA), which identify categories, provide a more objective and clear information.

Examples of application of NMR on microalgal secondary metabolites elucidation include the detection of glycosides in powder suspensions of the blue-green algae *Aphanizomenon flos-aquae* [460].

4.7.4.3 Hyphenated Techniques

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

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

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

4.7.4.4 Genome Mining

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

4.7.5 Summary

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

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	*Planktothrix rubescens*	Anti-Inflammatory	Mineral medium, 6 µmol photon m^{-2} s^{-1}, 20ºC	Freeze/thaw cycle after harvested by centrifugation	50% methanol Water	RP-HPLC	[474]
Anatoxin-a	*Anabaena flos-aquae*	Cytotoxic	BG-11 medium, 18-20 ºC, 30-50 µmol photon m^{-2} s^{-1},16:8 h	Freeze-dried after harvested by filtration	Dichloromethane, Derivatization with pentafluorobenzyl bromide	GC-ECD	[441]
Calothrixins A and B	*Calothrix*	Antimalarial and anticancer	Allen and Arnon medium [475], 25 ºC, 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	*Lyngbya polychroa*	-	Collected sample	Freeze-dried	EtOAc-MeOH, EtOH-H_2O	IR, HPLC, NMR	[443]
Columbamides	*Moorea (Lyngbya)*	Cannabinomimetic	Collected sample, BG-11 medium	Freeze-dried	(2:1, v/v) CH_2Cl_2/MeOH	IR, ^{1}H and ^{13}C NMR, HPLC, HRMS-genomic analysis	[473]
Cyanopeptolin 1020	*Microcystis aeruginosa* UV-006	Anti-Inflammatory	Mineral medium, 26ºC, 40±5 µmol photon m^{-2} s^{-1}	Harvested by centrifugation	60% acetonitrile, Water	RP-HPLC, high-resolution mass spectrometry	[474,476]
Diterpenoids commostins	*Nostoc commune*	Antibacterial, cytotoxic, molluscicidal	Inorganic culture medium, 24 ºC, 29 µmol photon m^{-2} s^{-1}, 24:0 h, 2% CO_2	Harvested by filtration	Methanol	NMR, MS, single-crystal X-ray	[462]
9-Ethyliminomethyl-12-(morpholin - 4 - ylmethoxy) - 5, 8, 13, 16–tetraaza–hexacene - 2, 3 dicarboxylic acid	*Nostoc* sp.	Antibacterial	Collected sample, BG-11 medium, 25 ºC, 95 µmol photon m^{-2} s^{-1}, 14:10 h	Harvested by centrifugation and lyophilized	Methanol	TLC, HPLC, ESIMS, NMR	[477]
Harmane	*Geitlerinema* sp.	Antimicrobial	Rippka et al [478], 25 ºC, 10 µmol photon m^{-2} s^{-1}, 24:0 h	Harvested by centrifugation	Volk´s procedure, methanol, Derivatization	TLC, LC-MS/MS, GC-FID	[438]
Isoflavones	*Nostoc, Spongiochloris, Scenedesmus*	-	-	Lyofilized	Supercritical fluid extraction	UHPLC-MS/MS	[463]
Lyngbyabellin B	*Lyngbya majuscula*	antifungal	Collected sample	-	(2:1, v/v) CH_2Cl_2/MeOH,	1D and 2D NMR, GC-MS	[436]
Mycosporine-like amino acids and glycosides	*Aphanizomenon flos-aquae*	Anticancer	-	Dry powder, sonicated for 1 h	No extraction	NMR, ESI-QTOF-MS, CID-MS/MS	[460]
Norharmane	*Nostoc insulare, Nodularia harveyana*	Algicidal	Pohl et al. medium [479], 27 ºC, 25-30 µmol photon m^{-2} s^{-1},	Centrifugation and filtration, freeze-dried	Methanol	HPLC	[480]
Norharmane	*Synechocystis aquatilis*	Algicidal, allelopathic	BG-11 medium, 24 ºC, 30 µmol photon m^{-2} s^{-1}, 24:0 h	Harvested by centrifugation	Volk´s procedure	TLC	[434]
Nostocarboline	*Nostoc 78-12A*	Inhibitor of butyrylcholin esterase	Mineral medium, 25ºC, continuously illumination, 15 µmol photon m^{-2} s^{-1}, CO^2enriched air (600 mL min^{-1}, 0.15 vol % CO_2	Lyophilized	60% methanol CH_3CN-H_2O,95:5 V/V Separated by HPLC	2D-NMR	[481,482]
[7.7] paracyclophanes	*Nostoc, Cylindrospermum, Anabaena Nodularia, Pseudanabaena*	Antibacterial, cytotoxic	BG-11, BG-11+0.5% NaCl, MBL medium, 20-28 ºC, 0.5-5% CO_2, 20-80 µmol photon m^{-2} s^{-1}	Freeze-dried after harvested by centrifugation	n-heptane, EtOAc, MeOH, and H2O (5:2:5:2, v/v/v/v;	UV–vis spectrophotometer, HPLC-UV, 2D NMR	[447]
Phenolic compounds	*Phaeodactylum tricornutum*	Antioxidant	f/2 medium [483], 24ºC, 8000 lux, 24:0 h	Freeze-dried after harvested by filtration	solid-phase extraction/ Methanol, acetone:hexane (1:4)	RP-HPLC	[448]
Sulphated polysaccharide	*Nostoc calcicola*	Antiviral	BG-11 medium, 25 ºC 35 µmol photon m^{-2} s^{-1}, 16:8 h	Harvested by centrifugation	Mouhim et al. (1993)	IR, GC	[484]

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