Universidade de Aveiro Departamento de Química
Ano 2016

Vítor Manuel Madureira Azevedo

Estudo lipidómico de macroalgas vermelhas como fonte de compostos bioativos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, ramo da Bioquímica Clínica realizada sob a orientação científica da Doutora Maria do Rosário Gonçalves dos Reis Marques Domingues, professora Associada com Agregação do Departamento de Química da Universidade de Aveiro e da Doutora Maria Helena Abreu, co-fundadora da empresa ALGAplus, Lda

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Palavras-chave

Macroalgas, Rhodophyta, *Porphyra dioica*, lípidos polares, composição química, perfil lipídico, atividades biológicas

Resumo

As macroalgas têm vindo a ganhar um interesse cada vez maior para o uso em diversas aplicações biotecnológicas, devido ao valor acrescentado dos seus diferentes constituintes. Entre estes, os glicolípidos e os fosfolípidos podem ser usados comercialmente em diferentes indústrias, tais como as indústrias alimentar, farmacêutica e cosmética. Com o objetivo de compreender melhor a composição lipídica das macroalgas, o presente trabalho relata, pela primeira vez, a caracterização do perfil de lípidos polares da macroalga vermelha *Porphyra dioica*, cultivada num sistema de aquacultura multi-trófica integrada (IMTA), utilizando para esse fim uma abordagem lipidómica baseada na espectrometria de massa (HILIC-ESI-MS). Foi também determinado o perfil de ácidos gordos da referida espécie de alga, tendo em consideração a variabilidade sazonal e o seu ciclo de vida. O perfil de lípidos polares da alga *P. dioica* revelou a presença de mais de 69 espécies moleculares diferentes, correspondendo a classes de glicolípidos (sulfoquinovosildiacilgliceróis, sulfoquinovosilmonoacilgliceróis e digalactosildiacilgliceróis), fosfolípidos (liso- e fosfatidilglicerol, liso- e fosfatidilcolinas) e derivados fitil. Alguns destes lípidos polares contêm ácidos gordos polinsaturados (PUFAs) na sua composição, nomeadamente o ácido araquidónico ($C_{20:4}$) e ácido eicosapentaenóico ($C_{20:5}$), revelando, assim, a capacidade da alga *P. dioica* em biossintetizar este tipo de ácidos gordos polinsaturados de cadeia longa. Considerando a variação sazonal do conteúdo em ácidos gordos, a *P. dioica* cultivada no inverno revelou ser mais rica em PUFAs, correspondendo a 37.0% do conteúdo total de ácidos gordos, contrariamente à *P. dioica* cultivada no verão (25.0%). O conteúdo em ácido eicosapentaenóico (EPA) é significativamente maior na estação de inverno (25.2%). O perfil em ácidos gordos também variou com o ciclo de vida *P. dioica*, sendo que na fase de conchocelis a quantidade de PUFA é significativamente mais elevada (47.0% de conteúdo de ácidos gordos), sendo o ácido araquidónico o ácido gordo mais abundante (21.2% de conteúdo de ácidos gordos).Várias classes de lípidos polares foram identificados como possuindo benefícios nutricionais e para a saúde, permitindo assim a valorização da alga vermelha *P. dioica* produzida em IMTA como uma fonte de compostos bioativos, adequados para o uso numa grande variedade de aplicações como um alimento funcional, rica em ácidos gordos polinsaturados ómega-3.

Keywords Macroalgae, Rhodophyta, *Porphyra dioica,* polar lipids, lipid profile, chemical composition, biological activities, bioactive compounds

Abstract

Marine macroalgae, or seaweeds, have gained an increased interest in recent times for the use in various biotechnological applications, due to the added-value of their chemical constituents. Among them, glycolipids and phospholipids display several commercial applications in a wide spectrum of industries, such as food, pharmaceutical and cosmetic. In an effort to further understand the lipid composition of macroalgae, the present work reports, for the first time, the isolation and characterization of the polar lipid profile of the red macroalgae *Porphyra dioica* cultivated on a land-based integrated multi-trophic aquaculture (IMTA) system, using a lipidomic-based approach employing hydrophilic interaction liquid chromatography-eletrospray ionization mass spectrometry (HILIC-ESI-MS). The fatty acid profile of this species of seaweed was also determined, accounting for season variability and its life cycle. The polar lipid profile of *P. dioica* revealed the presence of over 69 molecular species, corresponding to glycolipids (sulfoquinovolsyldiacylglycerols, sulfoquinovosylmonoacylglycerols, digalactosyldiacylglycerols) and glycerophospholipids (lyso- and phosphatidylglycerols), lyso- and phosphatidylcholines), as well as phytyl derivatives. Some of these polar lipids contain polyunsaturated fatty acids (PUFAs), namely arachidonic acid ($C_{20:4}$) and eicosapentaenoic acid ($C_{20:5}$), thus revealing the ability of *P. dioica* to biosynthesize this long chain PUFAs. *P.dioica* from the winter season revealed to be richer in PUFA content, accounting for 37.0% of total fatty acid (TFA) content, as opposed to *P. dioica* from the summer season (25.0% of TFA content). Eicosapentaenoic acid (EPA) content was revealed to be being significantly higher in the winter season (25.2% of TFA content). The diploid sporophyte conchocelis phase of *P. dioica* showed to possess the highest amount of PUFAs (47.0% of TFA content), with arachidonic acid being the most abundant fatty acid (21.2% of TFA content). Several of the lipids identified have been reported to possess nutritional and health benefits, thus allowing the valorisation of *P. dioica* from IMTA as a source of bioactive compounds, adequate for the use in a wide range of different applications and as a functional food, rich in omega-3 fatty acids.

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ABREVIATIONS

AA – arachidonic acid

ALA – α-linolenic acid

$CHCl_3$ - chlorophorm

DGDG – digalactosyldiacylglycerol

DGCC – 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline

DGTA - 1,2-diacylglyceryl-3-*O*-2'-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine

DGTS - 1,2-diacylglyceryl-3-*O*-4'-(*N*,*N*,*N*-trimethyl)-homoserine

DHA - docosahexaenoic acid

ESI – Electrospray ionization

EPA – eicosapentaenoic acid

FA – fatty acid

FAME – fatty acid methyl ester

GC-MS – gas chromatography–mass spectrometry

GL - glycolipid

HILIC-MS - hydrophilic interaction liquid chromatography–mass spectrometry

IMTA – Integrated Multi-trophic Aquaculture

LA - linoleic acid

LPC – lysophosphatidylcholine

LPG – lysophosphatidylglycerol

MeOH - Methanol

MUFA – monounsaturated fatty acid

PC - phosphatidylcholine

PG – phosphatidylglycerol

PL - phospholipid

PUFA – polyunsaturated fatty acid

SFA – saturated fatty acid

SQDG - sulfoquinovosyldiacylglycerol

SQMG – sulfoquinovosylmonoacylglycerol

TAG - Triacylglycerol

TFA – total fatty acid content

TLC - thin-layer chromatography

I. Introduction

I. Introduction

1. Macroalgae – General perspective and commercial importance

Marine algae are photosynthetic organisms that can be found in many forms and shapes. They can exist as unicellular microscopic organisms, referred to microalgae, or as macroscopic multicellular organisms, referred to macroalgae (or seaweeds) (1). Seaweeds form a diverse and complex group of organisms that can be found in a wide variety of habitats (2,3). Recent studies report the existence of nearly 9000 different seaweed species (4). The nomenclature of macroalgae is based on the various kinds and combinations of photosynthetic pigments present in the different algal species. As mentioned above, the systematic classification of algae is primarily based on their pigmentation. The largest macroalgae groups are Chlorophyceae (green algae), Phaeophyecae (brown algae) and Rhodophyceae (red algae) (2–5). The habitat where these types of seaweeds are found is intimately related to the presence of different pigments in their constitution. As a result, the majority of green macroalgae are found near coastal waters, since they are able to absorb large amounts of light energy, while brown and red macroalgae are mostly found at more profound depths, where the availability of sunlight is more limited (6).

Recently, the research on algae components and metabolites unravelled their importance as a source of bioactive compounds with pharmaceutical, biomedical and nutraceutical importance (7). Marine algae can be considered as low calorie food, that possess a high amount of minerals, vitamins, proteins and carbohydrates, as well as being an excellent source of a wide variety of compounds with biological activity, such as antioxidants. The main compounds responsible for antioxidant activity in macroalgae are carotenoids, vitamin E and chlorophylls, found in the lipidic fraction, as well as polyphenols, vitamin C and phycobiliproteins (1,8). Seaweeds are used as ingredients in the food industry in different regions across the world, traditionally in Asian countries such as China, Japan and Korea (8,9). They possess some interesting nutritional components, such as non-starch polysaccharides, like carrageenan and alginate, which are not degraded by mammalian enzymes, making seaweeds a fibre-rich material. They also biosynthesize fucose-containing sulfated polysaccharides, such as fucoidan, which was reported to possess various biological activities (10,11).

Several of the applications of macroalgae are related with their lipid components. Macroalgae lipids can be divided in two mains groups, the nonpolar lipids (acylglycerols, sterols, free fatty acids) and the polar lipids (glycolipids, phospholipids and betaine lipids) (2). Algae are natural source of bioactive lipids with pharmaceutical, biomedical and nutraceutical importance (12,13). Recent studies showed that some glycolipids, found in macroalgae, namely monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyldiacylglycerol (SQDG) possess antimicrobial, antiviral, anti-inflammatory and immunotropic properties (14–17). Although, macroalgae have been reported to have low lipid contents, their content in polyunsaturated fatty acids (PUFA) contents are equivalent to many terrestrial vegetables (18). Macroalgae also possess a considerable high amount of omega-3 PUFAs such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), which are amongst the fatty acids that have beneficial clinical and nutritional benefits, reducing the risk of coronary heart disease and preventing the risk of arteriosclerosis, inflammation and carcinomas by reducing the blood cholesterol (19). These compounds cannot be synthesized by humans and currently, the main commercial sources of PUFAs are fish oils and marine fishes. Macroalgae are a good alternative sources of high quality PUFAs, and some species are already approved to be used as food, such as the example of different genera of red algae, namely *Porphyra*, *Palmaria*, and *Gracilaria*, which justify the growing interest in the research on these species (20,21).

The *Porphyra* genus is one of the largest genera of red macroalgae, as well as one of the most commercially used, especially as a food ingredient (22,23). Due to that fact, the need to understand its chemical composition, and its potential benefits to human health has grown in recent times. The red seaweed *Porphyra dioica* is one of the major species of *Porphyra* found in Portugal (22,23) and is easily adaptable to cultivation on land-based integrated multi-trophic aquaculture (IMTA) system, that allows the replicability and control of biomass production (23). Until now, few studies have reported the chemical composition of this species, especially regarding its polar lipid and fatty acid composition, so for those reasons, this particular species was selected as the object of study for this work.

1.1. The red macroalgae *Porphyra dioica*

The genus *Porphyra* is one of the largest genera of red macroalgae (24). Even though the exact number of species within the *Porphyra* genus is still unknown, their number may surpass 150 different species (25). *Porphyra*, also known by the commercial name nori, is one of the most important cultured seaweeds in the world, representing, by weight, 12.5% of the world's seaweed mariculture (26). *Porphyra* is used primarily for food, especially in Asian countries like China and Japan, but also as a source of the red pigment r-phycoerythrin (23,26). *Porphyra dioica* (*P. dioica*), shown in figure 1, is one of at least 5 species of *Porphyra* described in Portugal (23) and is the most common species in North of Portugal together with *Porphyra umbilicalis* (23). In Portugal, *P. dioica* can be found all the way from Moledo, in the north, to Buarcos in the centre, near Figueira da Foz (23). *Porphyra dioica* inhabits the intertidal zone of rocky beaches throughout the year, with higher densities in late winter and spring months (22).

The life cycle of *P. dioica* is biphasic and heteromorphic, with a foliose haploid gametophyte phase, known as foliose/blade phase (figure 1 (a)) and a filamentous diploid sporophyte phase, known as conchocelis (figure 1 (b)) (27,28). Recent studies showed that *P. dioica* is a rich source of PUFAs, especially omega-3 PUFAs, that are proven to have health benefits, (29). So far, there are no reports of a detailed lipidomic study for this species of macroalgae, with characterization of the various compounds present in the lipid fraction, such as glycolipids and phospholipids. Thus, the study of the complete lipidome of the macroalgae would be fundamental due to the beneficial anti-inflammatory and anti-microbial bioactivity of this type of compounds, as well as their high nutritional value (17,30).

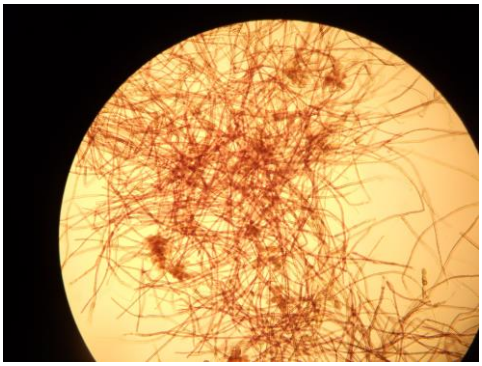
(a)  (b) 

Figure 1 – (a) *Porphyra dioica* haploid blade phase; (b) Dipoid conchocelis phase (© Andreia Rêgo, ALGAplus)

2. Chemical composition of red macroalgae (Rhodophyta)

Macroalgae produce a wide array of bioactive compounds with potential health benefits. These include peptides, phenolic compounds, active phytochemicals such as carotenoids and phycobilins, fatty acids, polysaccharides, vitamins, sterols, among others (21,31,32). Many of these compounds are known to possess biological activity and hence have potential beneficial use in healthcare (6,9). The following sections will cover the chemical composition reported in literature for marine macroalgae, with focus on species of red seaweeds (Rhodophyta) in general and the *Porphyra* genus in particular, regarding the content of proteins, polysaccharides, phenolic compounds, photosynthetic pigments, as well as detailed information of the lipidic composition.

2.1. Proteins, peptides and amino acids

The protein content of macroalgae experiences great variation between the different classes (33). The protein fraction of brown seaweeds is usually low, ranging from 3.0 to 15.0% of dry weight as opposed to that of the green or red seaweeds, that range from 10.0 to 47.0% of dry weight (34,35). For some of the most commercial important genera of red seaweeds, the total protein content has shown to possess considerable variety among the different genus, as well as from different species within the same genus. *Gracilaria córnea* possess 5.47 % (dry weight) of total protein content, in contrast of that from *Gracilaria cervicornis* that presents a total protein content of 23.0% (dry weight) (36). *Palmaria palmata* collected along the French Brittany coast has shown to possess protein content that varies from 11.9% to 21.9% (dry weight), with the highest content found in the winter-spring period (37), although earlier reports suggest that the total protein content of this red algae species can be as high as 47.5% (dry weight) (38). As for *Porphyra sp.* the total protein content was show to be in the range of 24.1% to 50.0% (dry weight) (34,39). The protein in macroalgae contains all essential and most of the non-essential amino acids, however, several studies report variations in their concentrations, possibly due to different seasonal periods and environmental conditions (34,35).

Tabarsa, *et al.* (21) reported that arginine, leucine, lysine, and methionine are the most abundant essential amino acids of *Gracilaria salicornia* (Rhodophyta) and their average levels are higher than those reported for cereals and legumes. In comparison, other

studies have shown that leucine, valine, and methionine are abundant essential amino acids of *Palmaria palmata* (Rhodophyta) and their average levels are close to those generally reported for ovalbumin (40).

Among the proteins present in algae, it is worth discussing the occurrence of protein-pigment complexes called phycobiliproteins, which are light-harvesting pigments found in the chloroplasts of red algae (41). Some of those compounds are currently used as fluorescent markers in clinical diagnosis and biotechnological applications (42,43).

Phycobiliproteins, in particular phycoerythrin, can constitute a major proportion of the red algal cell proteins, with reported levels of 1.2% of dry weight for *Palmaria palmata* (44) and 0.5% of dry weight for *Gracilaria tikvahiae* (45).

2.2. Polysaccharides

The overall content of carbohydrates present in macroalgae is relatively high, but even so they cannot be considered as potential source of energy rich food due to the fact that the digestibility of these carbohydrates is low on mammals (40). Carbohydrate content varies between different algae species. In red algae, the typical polysaccharides found consist of floridean starch, cellulose, xylan and mannan, and the water soluble fibre fraction formed by sulfur containing galactans such as agar and carrageenan (34). Since most of these polysaccharides are not digestible by the human gastrointestinal tract, they can be regarded as dietary fibres. The total dietary fibre content of seaweeds ranges from 29.3–62.3 g/100 g (34,46). Several studies report the total polysaccharide content and dietary fibre content of *Porphyra spp*, with the first ranging from 35.0% to 76.0% of dry weight, and the later from 18.0% to 49.0% of dry weight (47).The polysaccharides present in red macroalgae can be used for a wide variety of applications. Storage polysaccharides, such as agar and carrageenan, are the most commercially exploited components in seaweeds. These polysaccharides exhibit textural and stabilizing properties (46) and are, therefore, used in food applications such as thickening aqueous solutions, forming gels and water soluble films (48).

2.3. Phenolic compounds

Several studies have shown that marine macroalgae are rich source of phenolic compounds A wide array of polyphenolic compounds such as catechins, flavonols and flavonol glycosides have been identified in methanol extracts of red, green and brown algae, with brown algae generally possessing higher phenolic content then red and green algae (49,50). Regarding red macroalgae, Cox *et al.* (51) analysed the total phenolic content *C. crispus* and *Palmaria palmata* collected on the Irish coast, using the Folin-Ciocalteu method. *C. crispus* showed to possess a total phenolic content of 62.3 mg of gallic acid equivelents per gram (GAE/g), slightly higher than *Palmaria palmata* (42.8 mg GAE/G) (51). In, comparison, other species of red algae show different phenolic content values, such as *Meristiella echinocarpa* (28.5 mg GAE/g) (52), *Eucheuma cottonii* (22.5 mg GAE/g) or *Halymenia durvillae* (18.9 mg GAE/g) (53). Some reports determined lower values of total phenolic content for red algae species, ranging from 0.88 to 4.10 mg GAE/g (50,54).

2.4. Pigments

Photosynthetic organisms, from higher plants to aquatic plants, such as marine algae, possess several photosynthetic pigments, with chlorophyll *a* and carotenoids being the most common ones (55). Chlorophyll *a* plays a direct role in energy transduction, while carotenoids act as accessory pigments for harvesting light or as structural molecules that stabilize protein folding in the photosynthetic apparatus (55,56). In some species of red algae, namely *Palmaria palmata*, *Porphyra dioica* and *Chondrus crispus*, chlorophyll *a*, beta-carotene and zeaxanthin where the most abundant pigments found, with chlorophyll *a* ranging from 41.1% to 53.0% of total pigments and beta-carotene and zeaxanthine ranging from 19.2% to 30.2% and from 22.8% to 27.5%, respectively (29). Some carotenoids have shown to possess antioxidant activity, such antheraxanthin, lutein and zeaxanthin present in red algae (56).

2.5. Lipid Composition

Lipids are a chemically diverse group of compounds, which have in common their insolubility in water. Lipids in macroalgae can be divided in two main groups: apolar lipids, comprising mainly fatty acids and triacylglycerols, and the polar lipids, such as phospholipids, betaine lipids and glycolipids (57). Lipids possess a wide variety of different functions. Fats and oils are the principal form of stored energy, while phospholipids, glycolipids and betaine lipids are major structural elements of membranes (figure 2).

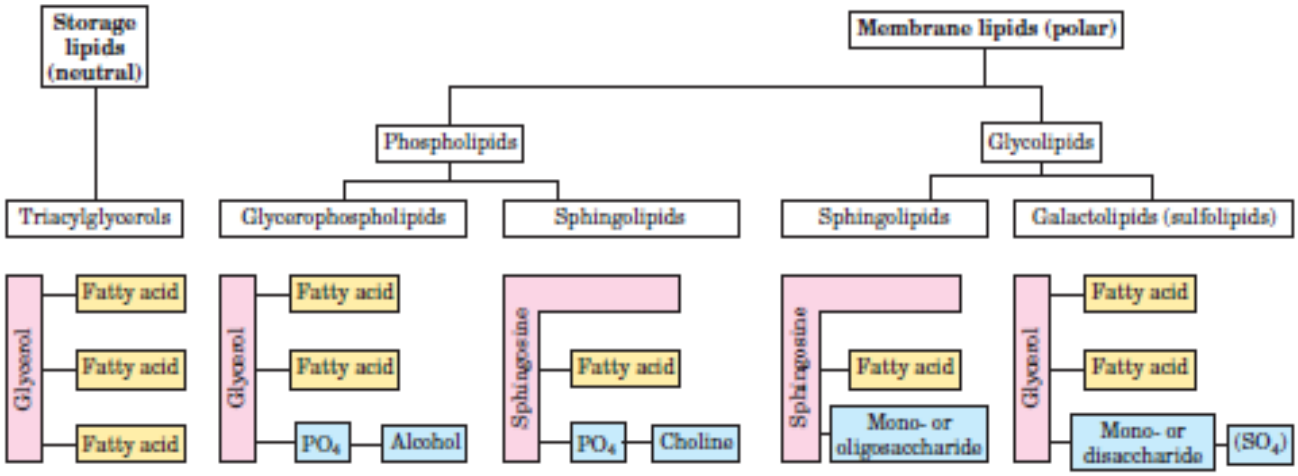


Figure 2 – Different classes of storage and membrane lipids (57).

The lipid content of marine macroalgae is relatively low, representing only 1–5% of the algal dry matter (3,43). Despite the low amount of total lipid content, macroalgae are an interesting source of PUFAs, glycolipids (GL) and phospholipids (PL) which possess many biological activities, such as antimicrobial, antiviral, anti-inflammatory and immunotropic properties (3). The following chapters will cover the composition in fatty acids, triacylglycerols and polar lipids reported in literature for species of red seaweeds, including *Porphyra*.

2.5.1. Fatty Acids

Fatty acids are carboxylic acids with hydrocarbon chains ranging from 4 to 36 carbons long (C_4 to C_{36}) (57). Fatty acids can be divided in 2 groups according to the number of double bonds in their hydrocarbon chain: Saturated fatty acids, possessing a fully

saturated hydrocarbon chain (with no double bonds) and unsaturated fatty acids, that can be either monounsaturated (one double bond) or polyunsaturated (multiple double bonds) (figure 3) (57).

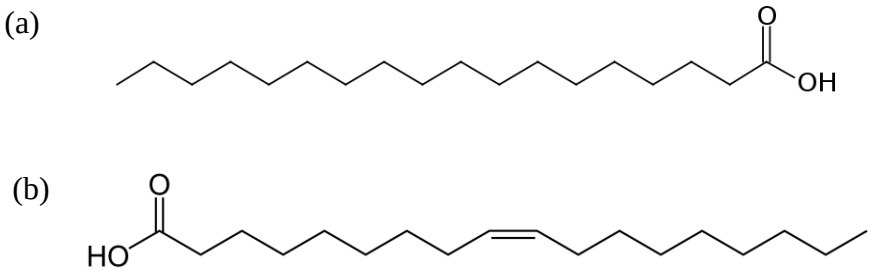


Figure 3 – Structure of: (a) Saturated fatty acid [Stearic acid ($C_{18:0}$)]; (b) Unsaturated fatty acid [Oleic acid ($C_{18:1}$)].

Essentially, there are two main groups of PUFAs, that are fundamental to human metabolism: the omega-3 (*n*-3) and omega-6 (*n*-6) fatty acids (58). Both *n*-3 and *n*-6 fatty acids are important components of cell membranes and are precursors to many other substances, such as eicosanoids, that are involved in inflammatory responses (58,59). Linoleic acid (LA), an *n*-6 fatty acid, and α-linolenic acid (ALA), an *n*-3 fatty acid are considered essential fatty acids due to the fact that they cannot be formed through mammalian metabolism, due to the lack of *n*-3 and *n*-6 desaturases, and therefore must consumed through the diet (58,60). Both of these fatty acids are needed to form other fatty acids within the body, such as the formation of arachidonic acid (AA) from LA (58). However, since the conversion to the *n*-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and the *n*-6 fatty arachidonic acid (AA) is limited, it is recommended that sources of these fatty acids should also be included in the diet, even though they are not considered essential fatty acids (5,58,59,61).

There are two pathways for the conversion of C_{18} PUFAs to long chain PUFAs in marine algae: Linoleic acid is converted to arachidonic acid in the *n*-6 series and *α*-linolenic acid is converted to EPA and DHA in the *n*-3 series (Figure 4) (5,61).

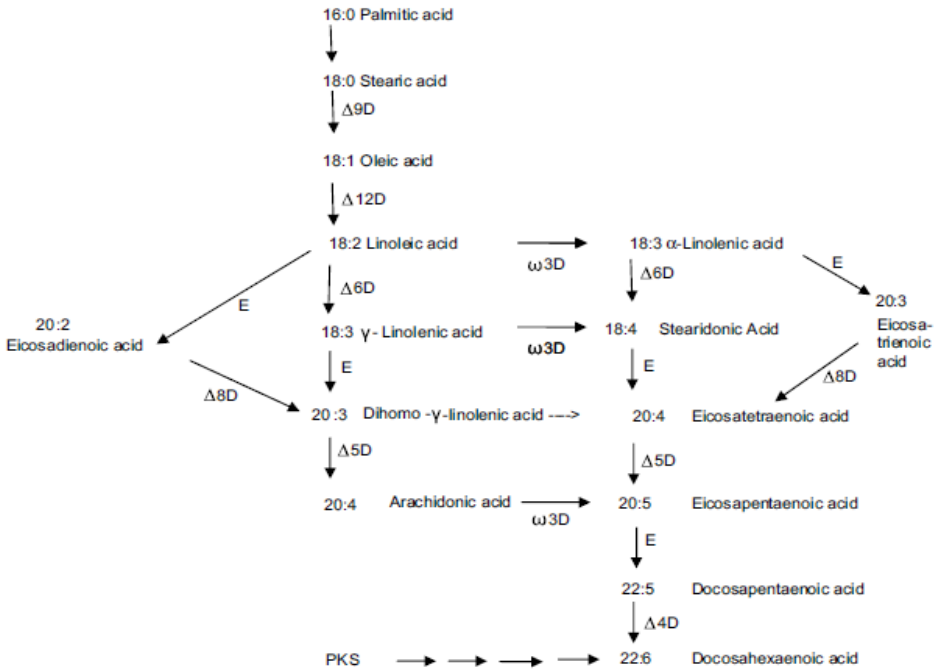


Figure 4 – Biosynthetic pathway of polyunsaturated fatty acids in eukaryotic algae. D - Desaturases. E - elongase; PKS - polyketide synthase (5)

Several studies report the fatty acid composition of several species of red macroalgae. Kumari *et al.* (18) studied the lipid and fatty acid composition of 27 tropical macroalgae form the three phyla (chlorophyta, rhodophyta and phaeophyta), 13 of which being red algae, using GC-MS to identify and analyse the fatty acids of each sample, as well as performing a multivariate analysis (PCA) in order to establish the relationship between and within different orders and families belonging to the same phyla. This study shown a large variety of total lipid content among different species and ranged from 0.57% to 3.50% of dry weight, with red algae in particular showing a minimum of 0.57% % of dry weight in total lipid content for the algae *Gracilaria furgosonii* and a maximum of 1.73% for *Laurencia papillosa*. Their overall fatty acid compositions had the characteristic pattern of rhodophyta with relatively higher levels of palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), AA and EPA (62). These FAs together accounted for 65.0–86.6% of total fatty acids whereas C_{18} PUFAs were present as minor components, ranging from 2.64% to 4.54% of total fatty acids, with the exception of *Amphiora anceps*, in which C_{18} PUFA content was 10.8% of total fatty acids. The present findings are in accordance with earlier studies where C_{20} PUFAs (AA and EPA) were recorded as the dominant fraction of fatty acids in red algae (18).

The *Gracilaria* genus is also extensively studied, with several reports showing that arachidonic acid ($C_{20:4}$ *n*-6) is the most abundant PUFA in *Gracilaria vermiculophylla (*also known as *Gracilaria verrucosa*), accounting for 41.2-45.4% of total lipid content and with palmitic acid ($C_{16:0}$) as the most abundant saturated fatty acid, representing 30.6-32.8% of total lipid content (63–66). The fatty acid composition of various species of algae belonging to the *Gracilaria* genus differs considerably, especially considering the content of AA ($C_{20:4}$ *n*-6) and EPA ($C_{20:5}$ *n*-3) (66). Other species of red algae, like *Palmaria palmata* and *Chondrus crispus*, share the same characteristic of the algae from the *Gracilaria* genus regarding palmitic acid as the most abundant saturated fatty acid (average of 22.3% and 27.3% of total fatty acids respectively), but instead of AA, EPA is the most abundant PUFA is these species, representing 57.97% and 33.47% of total lipid content, respectively (29). *Porphyra spp.* also share a similar fatty acid profile as of those from *Palmaria palmata* and *Chondrus crispus* species, with palmitic acid and EPA being the most abundant fatty acid (29,67), with some reports suggesting that *Porphyra sp.* also possess a considerable amount of oleic acid ($C_{18:1}$) (34,47). Palmitic, oleic, arachidonic and eicosapentaenoic acids were also the major fatty acids present in several other species of red macroalgae, as reported by a study conducted by Khotimchenko (68) about the fatty acid profiles of marine algae from the Pacific coast of North California. Moreover, the fatty acid composition not only varies with taxonomy, but is also strictly dependent on growth conditions and environmental effects, such as seasonality, temperature and light intensity (18,69,70). The table below (table 1) summarizes the fatty acid profile of some marine red algae.

Table 1 – Fatty acid composition of some red macroalgae (% of total fatty acid content)

Fatty acid	***Palmaria palmata*** (29)	***Chondrus crispus*** (29)	***Gracilaria verrucosa*** (65)	***Gracilaria salicórnia*** (21)	***Gracilaria furgosonii*** (18)	***Porphyra sp.*** (29,31,67)
14:0	4.51	1.98	4.60	5.50	2.58	0.53-13.76
16:0	22.3	27.3	32.8	33.4	26.4	28.1-63.19
16:1	1.17	2.71	2.60	2.46	0.99	0.90-6.22
18:0	1.69	0.58	2.0	3.04	2.50	1.23-2.16
18:1	2.91	6.06	8.40	11.7	6.24	1.84-2.46
18:2 *n*-6	0.65	1.58	1.30	1.45	3.23	0.69-1.64
18:3 *n*-3	0.65	0.23	Not detected	1.65	1.43	0.23-1.34
20:4 *n*-6	0.67	19.9	41.2	8.05	28.0	1.45-14.8
20:5 *n*-3	57.9	33.5	0.50	1.53	0.39	36.2-46.4
∑ Saturated FA	28.6	32.4	39.4	48.9	58.6	34.1-64.9
∑ Unsaturated FA	70.7	65.2	56.3	33.7	41.4	35.1-66.0

Seaweed products represent an important source of PUFAs (*n*-3; *n*-6), that are crucial for the formation of important structural lipids and elements of cell membranes. Additionally, these PUFA are precursors of eicosanoids, such as prostaglandins, leukotrienes and thromboxanes, which influence inflammation processes and immune reactions (71). Eicosanoids derived from *n*-6 PUFA are generally pro-inflammatory and proaggretory, meaning that they promote platelet aggregation, whereas those derived from *n*-3 are predominately anti-inflammatory and promote the inhibition of platelet aggregation (72). Therefore, it is imperative to balance the intake of *n*-3 an *n*-6 fatty acids, in order to prevent the development of cardiovascular diseases and other chronic diseases, such as diabetes, hypertension, and autoimmune diseases (59). The World Health Organization have recommended that human diet should have a *n*-6/*n*-3 ratio less than 4:1, in order to prevent inflammatory, cardiovascular and neural disorders (31). Several species of red algae have been reported to possess a *n*-6/*n*-3 fatty acid ratio less than 2:0, ranging from 0.46 to 1.80 in *Porphyra spp.* (34), 0.13 in *Palmaria spp.* (31) and from 0.42 to 1.02 in *Chondracanthus canaliculatus* (73).

2.5.2. Triacylglycerols

Triacylglycerols (TAG), also referred to as triglycerides, fats, or neutral fats, are one of the simplest lipids derived from fatty acids (57). Triacylglycerols are composed of three fatty acids each in ester linkage with a single glycerol. Those containing the same kind of fatty acid in all three positions are simply called simple triacylglycerols and are named after the fatty acid they contain, although most naturally occurring triacylglycerols are mixed, containing two or more different types of fatty acids (57).

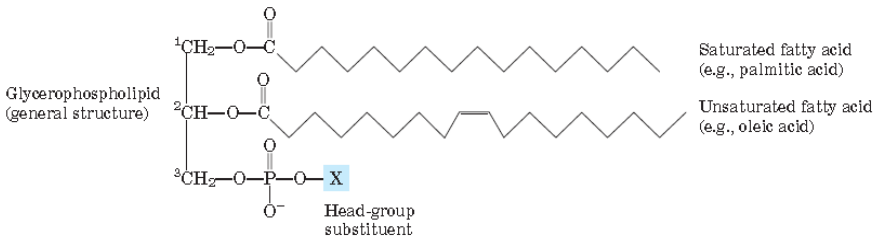
In red macroalgae, TAG are not usually found in large amounts, with few reports stating the composition of TAG in these organisms. The red macroalgae *Gracialria verrucosa* has shown to possess 5.50% of total lipids in the form of TAG (65) and with *Palmaria palmata*, *Porphyra dioica* and *Chondrus crispus* displaying 11.1%-13.4% of total lipid content as TAG (29).

2.5.3. Polar lipids

2.5.3.1. Glycerophospholipids and glycolipids

Glycerophospholipids and glycolipids comprise the 2 main groups of polar lipids present in red macroalgae (64,67,68,74,75). Glycerophospholipids, also called phosphoglycerides or phospholipids, are membrane lipids in which two fatty acids are linked by an ester bond to the first and second carbons of a glycerol backbone, a highly polar or charged group is attached through a phosphodiester linkage to the third carbon of the glycerol molecule. Glycerophospholipids are named as derivatives of the original compound, phosphatidic acid, according to the polar alcohol in the head group. For example, phosphatidylcholine and phosphatidylserine have choline and serine in their polar head groups (57). The general structure of glycerophospholipids, as well as some examples of the different polar head groups is shown in figure 5 (57).

(a)



(b)

Name of glycerophospholipid	Name of polar head group (X)	Formula of polar head group (X)
Phosphatidic acid	————	— H
Phosphatidylethanolamine	Ethanolamine	— CH_2—CH_2—$\overset{+}{N}H_3$
Phosphatidylcholine	Choline	— CH_2—CH_2—$\overset{+}{N}(CH_3)_3$
Phosphatidylserine	Serine	— CH_2—$\underset{COO^-}{CH}$—$\overset{+}{N}H_3$
Phosphatidylglycerol	Glycerol	— CH_2—$\underset{OH}{CH}$—CH_2—OH

Figure 5 - General structure of glycerophospholipids; (b) Some examples of the different polar head groups (adapted from (57)).

Glycerolipids lacking the phosphate group on the third carbon of the glycerol backbone, but instead possessing a simple sugar or complex oligosaccharide at their polar ends, are known as glycolipids (57). In red macroalgae, the most abundant glycolipids are monogalactosyldyacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyodiacylglycerol (SQDG) (figure 6) (3,64,68,75). Glycolipids are important structural membrane lipids of chloroplasts and thylakoids, with an essential role as markers for cellular recognition and also as energy providers (15,17).

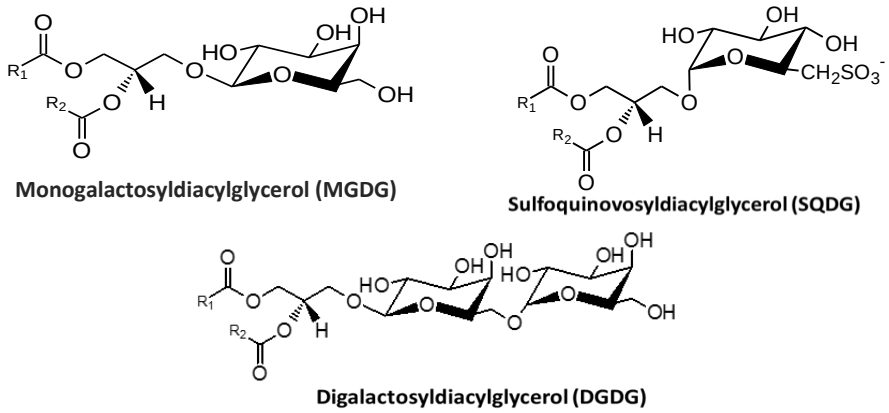


Figure 6 - General structures of the three major classes of glycolypids: Monogalactosyldyacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and Sulfoquinovosyodiacylglycerol (SQDG).

Glycolipids account for more than half of total lipid content, with certain species of red algae containing from 50.3 to 75.1% of total lipids (67,74,76), brown algae from 47.2 to 83.1% (77,78) and green algae from 68.0 to 75.1% (79). A summary of the glycolipid content of some species of red macroalgae, with results expressed in both (%) of total glycolipid content and (%) of total fatty acid content can be found in table 2.

Table 2 – Glycolipid composition of some species of red marine algae: (1) (% of total glycolipids); (2) (% total fatty acids).

Red Algae species	Glycolipids			Reference
	MGDG	DGDG	SQDG	
Porphyra veriegata	39.0 (1)	34.2 (1)	26.5 (1)	(64)
Nemalion vermiculare	45.1 (1)	30.8 (1)	24.1 (1)	
Tichocarpus crinitus	49.4 (1)	26.0 (1)	24.5 (1)	
Gracialria verrucosa	31.9 (1)	41.5 (1)	26.6 (1)	
Ahnfeltia tobuchiensis	36.1 (1)	44.2 (1)	19.4 (1)	
Chondrus pinnulatus	34.1 (1)	26.0 (1)	39.3 (1)	
Palmaria Stenogona	27.7 (1)	38.8 (1)	33.4 (1)	
Laurencia nipponica	21.2 (1)	49.3 (1)	29.4 (1)	
Porphyra perfonata	14.8 (2)	31.4 (2)	15.7 (2)	(67)
P. Palmata, C. crispus, P. dioica	17.0-23.1 (2)	17.4-21.7 (2)	16.8-25.4 (2)	(29)

Khotimchenko (64) studied the composition of glycolipids, namely monolgalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG) and sulfoquinovosyodiacylglycerol (SQDG), from 28 different seaweed species collected in Peter the Great Bay (Sea of Japan), 10 of which belonging to the Rhodophyta phyla. The lipid extract was obtained using the Bligh and Dyer method, lipid classes were separated by two-dimensional thin-layer chromatography (TLC) and fatty acids were analysed by gas chromatography coupled with flame ionization detector (GC-FID). For red algae, MGDG content ranged from 21.2% of total glycolipids from *Laurencia nipponica* to 49.4% from *Tichocarpus crinitus*, while DGDG content varied from a minimum of 24.1% for *Antithamnion sparsum* to a maximum of 49.3%, for the algae *Laurencia nipponica*. SQDG represented, on average, the lowest glycolipid present in the red algae analysed in that study, with a minimum of 19.4% of total glycolipid content, up to a maximum of 44.6%. Fatty acid composition of the studied glyceroglycolipids was also determined. In red algae, C_{20} PUFAs are concentrated in the MGDG, with eicosapentaenoic acid being the most predominant, accounting for 69.0% of total fatty acid content in MGDG. The amount of $C_{20:5}$ *n*-3 and $C_{20:4}$ *n*-6 decreases from MGDG, to DGDG and SQDG whereas the content of saturated and monounsaturated acids, mainly $C_{16:0}$, increased simultaneously, reaching 63.2% in SQDG (64).

The glycolipid and phospholipid profile of the red algae *Gracilaria verrucosa* was reported by Khotimchenko (65), with DGDG being the most abundant glycolipid,

representing 25.0% of total lipids, and phosphatidylcholine (PC) the most abundant phospholipid, accounting for 22.9% of total lipid content. MGDG turned out to be the most unsaturated glycolipid due to their high concentration of AA (67.2% of total fatty acids) and PC being the phospholipid with the highest amount of PUFA in their composition, owing that to the high level or AA (56.5% of total lipids) (65).

According to the study conducted by Robertson *et al.* the polar lipid fraction of three species of red algae (*P. palmata, P. dioica* and *C. Crispus*) is composed essentially of MGDG (17.0%-23.1%), DGDG (17.4%-21.7%) and SQDG (16.8%-25.4%), representing 71.6-76.9% of total fatty acids, with phospholipids, such as PC, PG and PE, accounting for 11.1%-16.4% of total fatty acids. In that study, reverse phase silica gel columns were used to separate the neutral and polar lipids, and thin-layer chromatography was performed in order to separate and identify the different polar lipid classes (29).

Another study performed by Sanina *et al.* (3) reported the fatty acid composition of polar lipid classes for some marine algae species, including the red algae *A. tobuchiensis* . In this study, major glycolipids (MGDG, DGDG and SQDG) and phospholipids, namely phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), whose structure is shown in figure 7, were isolated from 3 species of macroalgae, one of each phylum. The results showed that, for the red algae *A. tobuchiensis*, PC was the major phospholipid found in its composition, accounting for 70.9% of total phospholipids and DGDG was the most abundant glycolipid, representing 64.0% of total glycolipids. The percentage of *n*-6 PUFAs, as well as the sum of *n*-3 and *n*-6 PUFAs decreased in the sequence PC-PE-PG and MGDG-DGDG-SQDG.

Regarding *Porphyra* spp., *Porphyra perforata*, MGDG was the most unsaturated lipid due to a high concentration of C_{20} polyunsaturated fatty acids, namely EPA (68.5% of the total FAs) and AA (14.8%). The content of these acids in DGDG was as high as in MGDG, although their proportion was smaller. In the series MGDG, DGDG, and SQDG, the level of $C_{20:5}$ *n*-3 and $C_{20:4}$ *n*-6 decreased and simultaneously increased the proportion of saturated and monounsaturated acids, primarily $C_{16:0}$, which amounted to 63.2% in SQDG (67). A similar distribution pattern of fatty acids in glycolipids has been reported for other red algae, particularly in *Palmaria stenogoma*, as well as for *Gracilaria gigas* (75). Regarding phospholipid content of *Porphyra perfonata*, PG was observed to be the most abundant class, representing 13.8% of lipid content, and was also the most saturated

phospholipid due to the high amount of palmitic acid (35,9%) of total fatty acid content in its composition) (67).

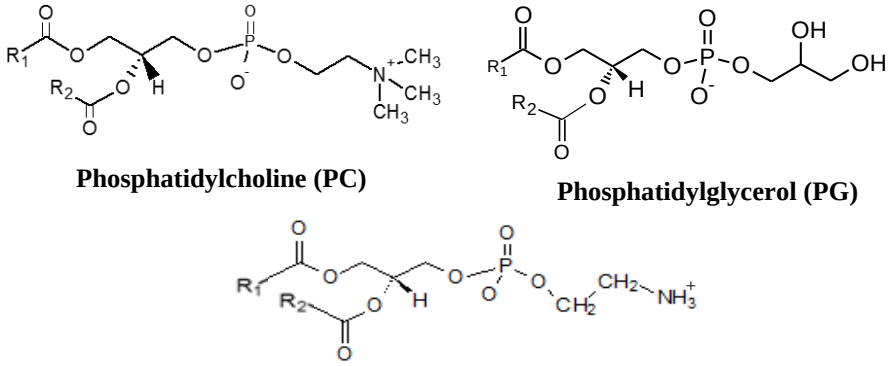


Figure 7 – General structure of phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE)

Melo *et al.* (80) reported, for the first time, a thorough lipidomic study of the red seaweed *C. crispus*, using hydrophilic interaction liquid chromatography – electrospray ionization mass spectrometry (HILIC-ESI-MS), in order to characterize the fatty acid and polar lipid composition of the mentioned algae, as an approach to better understand the valuable properties provided from lipidic components. The use of mass spectrometry not only allows the identification of different polar lipid classes, but also provides information about the composition of each molecular species within each class of lipids, including the structure of the different polar head groups and the fatty acid acyl chains. The main polar lipid groups identified include glycolipids (sulfoquinovosyldiacylglycerols and digalactosyldiacylglycerols), glycosphingolipids bearing ceramide backbones (galactosylceramides), inositolphosphoceramides, glycerophospholipids (phosphatidylcholines, lyso-phosphatidylcholines, phosphatidic acids, phosphatidylglycerols and lyso-phosphatidylglycerols), and betaine lipids, as well as some phytyl derivatives, as chlorophylls and pheophytins. In all of these lipid classes, several molecular species bearing $C_{20:4}$ *n*-6 (arachidonic acid) and $C_{20:5}$ *n*-3 (eicosapentaenoic acid) PUFA's were identified, that are known for their beneficial effects in human health. The knowledge that this detailed study provided on the lipid composition of this particular seaweed opened the way for new studies that are able to validate the use of seaweeds as healthy food products.

2.5.3.2. Betaine lipids

Among the large variety of polar lipids that can be found in marine macroalgae, betaine lipids represent a common class of both functional and structural lipids (81,82).

Betaine lipids are glycerolipids containing a quarternary amine alcohol ether-linked to the *sn*-3 position of the glycerol moiety, with the esterified fatty acids in the *sn*-1 and *sn*-2 positions (figure 8) (83,84). Unlike other classes of polar lipids, such as phospholipids or glycolipids, betaine lipids do not possess any phosphorus or carbohydrate group, which lead to their alternative classification as complex lipoamino acids (85). This class of lipid occurs naturally not only in algae, but also in other lower eukaryotic organisms, such as bryophytes, fungi and some primitive protozoa, as well as in photosynthetic bacteria (83,86).

Currently, there are three related betaine lipids that have been described, each possessing different trimethylated hydroxyamino acids. They have a positively charged trimethylammonium group and a negatively charged carboxyl group, therefore, making them zwitterionic at neutral pH. The three types of betaine lipid are 1,2-diacylglyceryl-3-*O*-4'-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS), 1,2-diacylglyceryl-3-*O*-2'-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine (DGTA) and 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline (DGCC) (figure 5) (83–86).

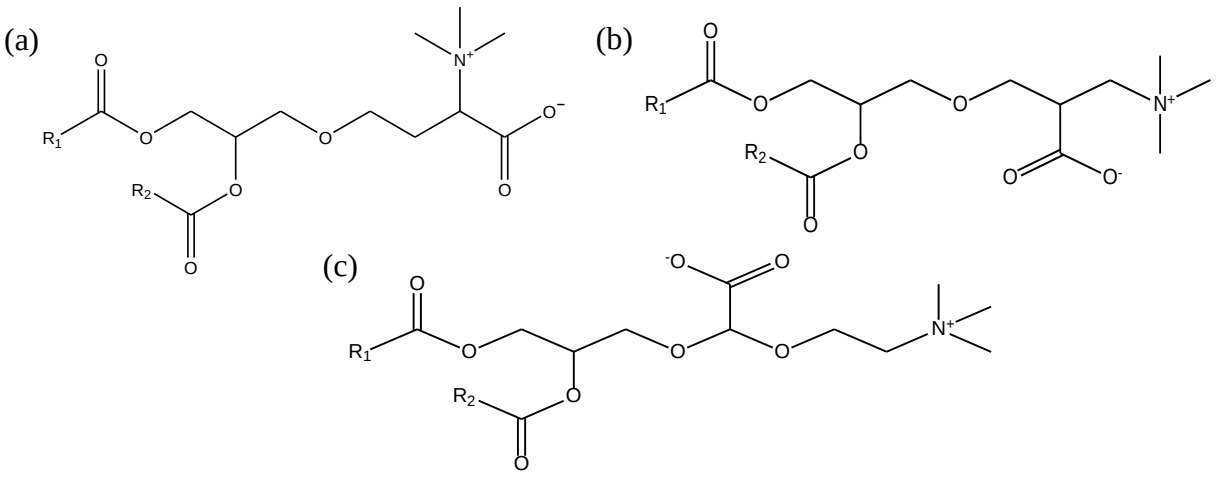


Figure 8 – Structure of the three different known betaine lipids. (a) 1,2-diacylglyceryl-3-*O*-4'-(*N*,*N*,*N*-trimethyl)-homoserine (DGTS); (b) 1,2-diacylglyceryl-3-*O*-2'-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine (DGTA); (c) 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline (DGCC).

In macroalgae, several studies suggest different betaine lipid profiles depending on the macroalgae group. It has been shown that DGTS is more predominant in green algae (83,87) whereas in brown algae, DGTA is more abundant (83,88). In red algae, betaine lipids are not generally found in high amount, but some species of red algae (*Lomentaria articulata Mastocarpus stellatus*, *Phyllophora pseudoceranoides*, *Membranoptera alata* and P*hycodrys rubens*) have been reported to possess betaine lipids (89). In addition, several molecular species of DGTS were identified in *C. crispus* (80). However, it is important to underline that the amount of betaine lipids found in macroalgae not only varies with the different type of algae, but also with the growth conditions and the external environment that they are subjected to (82,83). For instance, marine green algae are more prone to possess higher amounts of DGTS then fresh water green algae, mainly due to the fact that in a marine environment there is a much lower availability of phosphate than in fresh water environments (86).

Due to the characteristic zwitterionic structure shared by both betaine lipids and the membrane phospholipid phosphatidylcholine (PC), it has been proposed that betaine lipids may perform the same function within the cell membranes as PC (86,89). This hypothesis has been further corroborated by a number of reports showing that most betaine lipid-containing algae, especially the ones that hail from habitats where phosphorous is a limiting nutrient, either do not contain PC at all or, if they do, it is in a very small amount (86,89).

3. Methods for lipid analysis

The analysis of lipid content requires a number of sequential steps of experimental procedure, as demonstrated in figure 9.

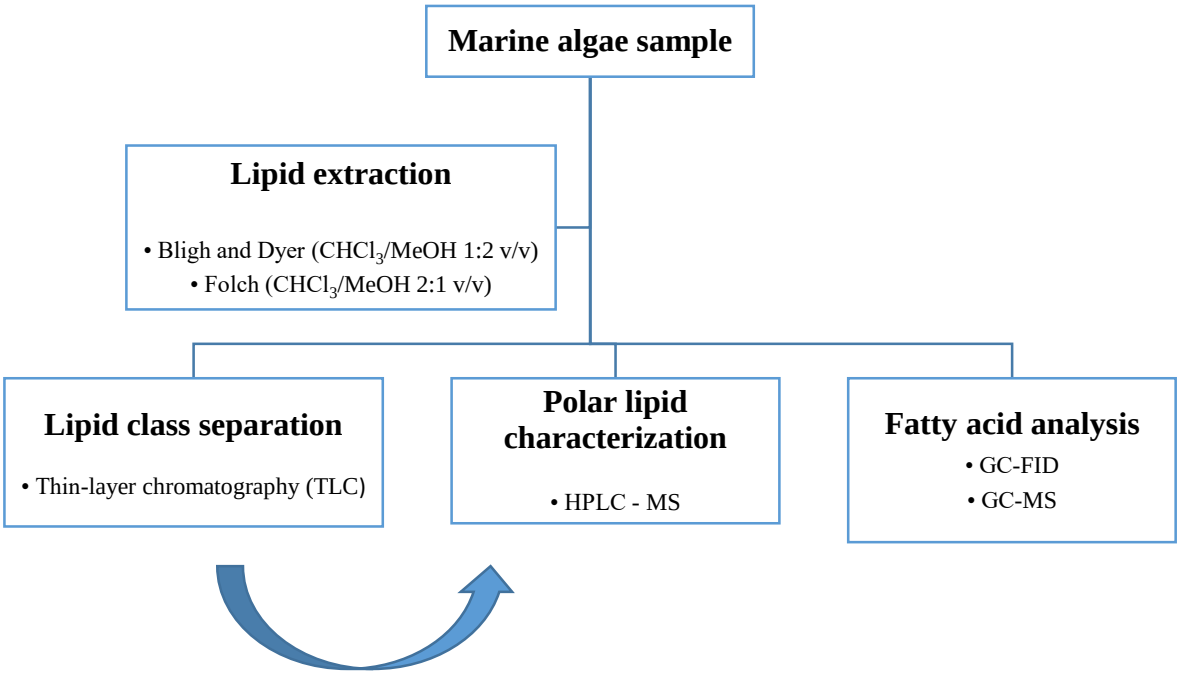


Figure 9 – Flow diagram of different procedures used in lipidomic analysis.

Lipid extracts can be obtained from macroalgae samples by applying different extraction methods, revolving around the combination of different organic solvents, in different proportions. The most commonly used methods for lipid extraction are Bligh and Dyer and Folch methods, that use a mixture of chloroform/methanol as solvent, but differing in the solvent proportions [(1:2 v/v) and (2:1 v/v) respectively] (90). Neutral lipids and polar lipids present in the crude lipid extract can be separated using such techniques as solid phase extraction that separates different lipid classes according to their polarity (91). Thin-layer chromatography is a vastly used technique to perform lipid class separation and identification, in order to determine the different polar lipid classes present in the lipid extract. Polar lipids are eluted with a mixture of different solvents, and are separated based on their polarity.

For fatty acid determination, it is common to resort to gas chromatography techniques (GC-FID and GC-MS) to analyse the fatty acid methyl esters (FAME), obtained by transmethylation of an aliquot of total lipid extract, or by direct-transmethylation of the silica powder containing the different lipid fractions (18,29,31,61).

Mass spectrometry techniques have grown in interest in recent years, due to the detailed information they provide about the identification and composition of different molecular species present within each class of polar lipids, bringing new insights to lipidomic analysis of many substances, including macroalgae. There are few studies providing detailed information regarding polar lipid composition of marine algae using mass spectrometry (80,92–94), mainly due to the fact that mass spectrometry equipment is expensive, meaning not everyone can have easy access to it, and because data interpretation requires specialized staff. Thus far, only one study has reported the complete analysis of the lipidome of one red algae species (*C. crispus*), through hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry (HILIC-ESI-MS) (80). HILIC allows the separation of lipids depending on their polar head group and fatty acyl composition, allowing the identification of polar lipid classes, as well as the different molecular species. This lipidomic-based approach on lipid analysis through mass spectrometry is a step forward in the understanding of algae lipid metabolism, as well as in the valorisation of macroalgae as edible products and a source of bioactive lipid compounds.

4. Biological properties of red macroalgae

Marine macroalgae have become, in recent years, increasing attractive as new sources of bioactive compounds with potential beneficial properties. Marine macroalgae have been used for direct human consumption since ancient times as food and as a source of medicinal drugs (31,95). Several studies reported macroalgae as a source of anti-inflammatory, antioxidant, anti-viral, antimicrobial, antitumor and anti-coagulant compounds, among others (3,95,96). These findings provided, from the nutritional and health viewpoints, a growing interest in the promotion of healthy dietary habits in western countries, with evidence suggesting that certain dietary changes may play a key role as long-term alternative to pharmaceutical compounds, making algae emerging as an important functional food (47,97). An overview of the different biological properties of macroalgae constituents is explained in the topics below.

4.1. Anti-inflammatory activity

Marine algae contain several bioactive lipid compounds that possess anti-inflammatory activity, such as *n*-3 PUFAs, namely eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Metabolites derived of these fatty acids, such as resolvins, produced from EPA and DHA, and protectins produced from DHA have demonstrated to possess a wide range of anti-inflammatory actions, with studies reporting, for example, that resolvin E1, resolvin D1 and protectin D1 inhibited transendothelial migration of neutrophils, thus preventing the infiltration of neutrophils into sites of inflammation. Resolvin D1 and protectin D1 have also shown to inhibited IL-1β production, with the later also inhibiting tumour necrosis factor (TNF)-α production (98–101). These observations suggest that *n*-3 PUFAs promote an environment of reduced inflammation, lowered cell responsiveness and increased resolution of inflammation. On the contrary, *n*-6 PUFAs, such as AA, promote a pro-inflammatory condition, with the formation of pro-inflammatory eicosanoids, such as prostaglandins, thromboxanes and leukotrienes (72,102). The eicosanoids derived from AA, unlike the ones derived from EPA and DHA are biologically active in small quantities, due to a higher affinity of AA-derived eicosanoids to eicosanoid receptors and thus, if formed in larger quantities, can cause the formation of atheromas, and promote the development of allergic and inflammatory disorders (72). Therefore, it is important to balance the ratio between *n*-6 and *n*-3 PUFAs in order to prevent the development of cardiovascular diseases, as well as other chronic diseases (59).

A study conducted by Robertson (29) characterized the lipid class composition of three red algae, *P. palmata, P. dioica* and *C. crispus,* as well as the distribution of the major long chain PUFAs within the different lipid classes, in order to investigate the anti-inflammatory potential of the lipid extracts of these algae in lipopolysaccharide (LPS)-stimulated human THP-1 macrophages. Each algal species contained high proportions of *n*-3 PUFAs, ranging from 34.0% to 62.0% of total fatty acid content, with EPA being the most abundant fatty acid in all extracts (28.0%-58.0% of total fatty acid content). The *n*-3 PUFAs within the algae were found to be primarily incorporated MGDG, DGDG and SQDG, corroborating previous studies that have shown that polar lipids isolated from red algae demonstrate higher anti-inflammatory activity than pure EPA isolated from the same species (103), which leads to believe that the entire polar lipid structure contributes to anti-inflammatory activity. When exposed to human THP-1 macrophages for 24 hours, lipid

extracts of *P. palmata* significantly suppressed LPS-induced production of the pro-inflammatory cytokine IL-6 and IL-8, compared with the untreated control. However, all lipid extracts inhibited the expression of a number of inflammatory genes in THP-1 macrophages, including those involved in toll like receptor activity and cytokine-linked signalling pathways. Out of the three red algae species studied, the lipid extract of *C. crispus* displayed the lowest anti-inflammatory potential, probably due to its high content of *n*-6 arachidonic acid, which has been associated with pro-inflammatory properties (29). Previous studies showed that methanolic extracts of this red algae species was capable of inhibiting lipopolysaccharide-induced nitric oxide (NO) production in macrophage RAW 264.7 cells (103). Lipidic extracts from *Palmaria palmata* were the most potent at inhibiting inflammatory cytokine production, whereas those from *porphyra dioica* inhibited the expression of more inflammatory genes than any of the other species tested. These results suggest that the algal lipid extracts have potential to suppress inflammation through inhibition of inflammatory cytokine production and down-regulation of several genes involved in a number of inflammatory signalling pathways (29).

Some species of macroalgae from Jeju Island, Korea, such as *Laurencia okamurae* and *Grateloupia elliptica*, were reported as potent inhibitors of the production of pro-inflammatory mediators such as nitric oxide (NO), prostaglandin E_2 (PGE_2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) (104).

4.2. Antioxidant activity

Antioxidant activity has been identified in various marine algae, including several red seaweeds (50,51,105). Methanolic extracts of *P. palmata* and *C. crispus* collected on Ireland were shown to possess antioxidant activity, with efficient concentration (EC_{50}) values of 25 µg/ml and 5 µg/ml, respectively (51). These results were obtained from the use of DPPH radical scavenging method, with EC_{50} being defined as the concentration of extract required to reduce DPPH radical by 50% (51). Methanolic extracts of the red seaweeds *Euchema kappaphycus, Gracilaria edulis and Acanthophora spicifera* collected from the coast of India were also reported to possess antioxidant activity with DPPH scavenging activities ranging from 5,20% to 11,9% (50).

The methanolic extract (2mg/ml) of the red algae *Grateloupia Filicina* has shown to exhibit a good effect on the scavenging reactive oxygen species (DPPH, $^{\bullet}OH$, H_2O_2 and O_2^-), resulting in the scavenging of 82.0% of DPPH radicals, nearly three times higher than that of BHT, which is one of the tested commercial antioxidants. The same methanolic extract scavenged 65.0% of superoxide anion, almost two times higher than that of BHT and α-tocopherol. Interestingly enough, the chloroform and carbon tetrachloride extracts inhibited lipid peroxidation more effectively than any of the commercial antioxidants tested, based on the results using the linoleic acid model system (106).

4.3. Antimicrobial activity

Some lipid extracts of macroalgae have been reported to possess antimicrobial and antibiotic properties (107,108). A study conducted by Mendes, et al. (108) investigated the antimicrobial activity of seaweed extracts from three red algae, *G. vermiculophylla*, *P. dioica* and *C. crispus*, both from wild and from IMTA. In that study, the seaweed extracts were prepared with different solvent with different polarity: diethyl ether, ethyl acetate and methanol. Results revealed that test organisms (Gram negative and Gram positive bacteria as well as one yeast species) were more sensitive to extracts obtained with dried algae, processed continuously at higher temperatures. Results from antimicrobial activity of wild and IMTA seaweed extracts showed stronger antimicrobial activity in extracts of ethyl acetate when compared with those from methanol and diethyl ether. Among the type of microorganisms tested, there was tendency for higher inhibition of the Gram positive ones, when compared to the Gram negative ones, which corroborate the results from previous studies (109). One possible explanation for this fact resides in the differences in the cell wall structure and composition between Gram positive and Gram negative bacteria, with the later possessing an outer membrane that acts like a barrier, preventing the entrance of environmental substances, such as antibiotics (109). However, some red algae, such as *Gracilaria corticata* has shown to be highly active against *Proteus mirabilis*, a Gram negative pathogenic bacterium (110).

Eicosapentaenoic acid (EPA) has proven to be effective antimicrobial agent against food spoilage microorganisms, such as *Bacillus subtilis, Listeria monocytogenes, Staphylococcus aureus and Pseudomonas aeruginosa* (111).

Bansemir, *et.al* (107) conducted a study on the potential antibacterial activity of several species of marine macroalgae against fish pathogenic bacteria. Results showed that dichloromethane-extracts of the red algae species submitted to test have proven to be effective in inhibiting the action of several bacteria, with *Asparagopsis armata* and *Gracilaria* spp. displaying the highest inhibition zones in the agar diffusion assay, and therefore, possessing the highest antimicrobial potential.

5. Aims

Marine macroalgae are increasingly viewed as a potential natural source of bioactive compounds with application in the pharmaceutical, biomedical and nutraceutical fields. Many macroalgal species have been used as ingredients in food in many countries around the world, thus the interest as well as the need to study the chemical composition of different algae, and their beneficial biological activities has increased in recent years.

Thus, the present work aims at the study of the lipidic composition of the red macroalga *Porphyra dioica*, cultivated on a land-based IMTA system. The polar lipid and fatty acid profiles of *Porphyra dioica* will be determined, as well as the potential effect of seasonality on the variation of the fatty acid profile. Furthermore, the fatty acid profile of the sporophyte, filamentous and diploid phase of *P. dioica*, named as the conchocelis phase, will be unravelled, considering the potential changes of fatty acid content in this stage of *Porphyra* life cycle.

This red algae was selected as object of study for this work mainly due to the fact that this is one of the most commercialized species of red algae, being primarily used as a food ingredient consumed in human diet in several countries worldwide (22,26), is one of the most abundant species of *Porphyra* in the north of Portugal and it easily adapts to cultivation on land-based IMTA (23). Regarding the aforementioned reasons, it is important to perform detailed studies of its chemical composition and nutritional value. The samples of *Porphyra dioica* will be provided by the Portuguese company ALGAplus, cultivated in IMTA, with controlled growth conditions.

For the lipidomic study, hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry (HILIC-ESI-MS) will be used in order to determine the polar lipid profile of the aforementioned specie of red algae, providing detailed information about the composition of each molecular species within each class of lipids, including the structure

of the different polar head groups and the fatty acid acyl chains. The fatty acid profile of *P.dioica* cultivated in the winter and summer seasons, as well as of the conchocelis phase will be determined by gas chromatography coupled to mass spectrometry (GC-MS).

II. Materials and Methods

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1. Algal material

Dried samples (25 °C, up to 12% moisture content) of cultivated *P. dioica* were provided by ALGAplus Ltd. (production site in Ria de Aveiro, mainland Portugal, 40°36′43″N, 8°40′43″W). Two samples from the red macroalga *P. dioica* (blade) were submitted to analysis, one harvested on the winter season (March 2014) and one harvested in the summer season (July 2012). One sample of the sporophyte conchocelis phase of *P. dioica* life cycle collected on the winter season (November 2015) was also analysed.

2. Reagents

HPLC grade chloroform and methanol were purchased from Fisher Scientific Ltd. (Loughborough, UK). All other reagents were purchased from major commercial sources. Milli-Q water (Synergy, Millipore Corporation, Billerica, MA, USA) was used. TLC silica gel 60 glass plates (20 × 20 cm) with concentrating zone were purchased from Merck (Darmstadt, Germany).

3. Lipid Extraction procedure

The algal material was extracted using a modified Bligh and Dyer method (112). Two hundred and fifty milligrams of algal material was weighed and put in a glass PIREX tube, where 3 mL of a mixture of MeOH:$CHCl_3$ (2:1) was added. After a 2 minute homogenization process by using a vortex, the sample was incubated on an orbital shaker for 2h. Then, after a 10 minute centrifugation at 1500 rpm, the supernatant was transferred to another glass PIREX tube, and the solid sample was re-extracted with 1 mL of a mixture of MeOH:$CHCl_3$ (2:1), that was homogenized for 2 minutes and then transferred to the aforementioned tube. This procedure was executed 2 times. Next step was to induce phase separation by adding 2.3 mL of Mili-Q water to the tube containing the supernatant, followed by 10 minute incubation at room temperature and 10 minute centrifugation at 1500 rpm. The lower phase (organic phase) was collected and transferred to another tuber. Lipid extracts were dried under vacuum and stored at -20 °C prior to analysis by LC–MS and GC–MS. The extractions and analyses were performed in different days.

4. Thin-layer chromatography

Polar lipids classes were separated by TLC using silica gel plates. Prior to separation, the plates were washed with chloroform/MeOH (1:1, V/V) and activated (sprayed or impregnated) with 2.3% boric acid in ethanol and placed in an oven at 100 °C for 15 min. Plates with spots containing 20 µg of lipids were developed in a solvent mixture of chloroform/ethanol/water/ triethylamine (30:35:7:35, V/V/V/V). Spots corresponding to polar lipids were visualized with a UV lamp (254 and 366 nm; Camag, Berlin, Germany) after exposing the plates to primuline (50 mg in 100 mL of acetone/water, 80:20, V/V), were scrapped off from the silica and gathered for further extraction. After silica extraction, the different phospholipid and glycolipid classes were analysed by mass spectrometry (HILIC-ESI-MS) in positive and negative modes, after dilution with methanol.

5. Silica extraction

In order to analyse the different phospholipid and glycolipid classes by mass spectrometry, TLC spots corresponding to all identified classes were scraped off from the plates with the help of a spatula to a piece of aluminium paper, and then transferred to a glass tube. To all tubes were added 2 mL of $CHCl_3$/MeOH (2:1), vortex well for 2 mins, in order to be able to extract the phospholipids from the silica. Then, the samples were centrifuged for 5 mins at 1000 rpm. The organic phase was transferred to a new glass tube and the silica was re-extracted according to the procedure described above. Then, 1.3 mL of $CHCl_3$ and 2.4 mL of mili-Q water were added to the organic layer, vortexed well and centrifuged for 5 mins at 1000 rpm. The organic layer was recovered to a new tube and dried under nitrogen stream. In order to remove some vestiges of silica, samples were filtered using a syringe (Hamilton, Supelco, 1 mL) containing filters (Millex-GV, 0,22 µL). In order to recover some vestiges of sample, after filtration of samples the syringe was washed twice with 200 µL of chloroform. After each sample filtration, syringe and filter was thoroughly washed with methanol, and filter was changed every two filtered samples. The filtered samples were dried under nitrogen stream and were re-suspended in 100 µL of $CHCl_3$ to mass spectrometry analysis, or stored at -4°C for further analysis.

6. Fatty acid analysis by gas chromatography–mass spectrometry (GC–MS)

The total fatty acyl substituents were analysed after transesterification of total lipid extracts (30 μg of total phospholipid). The fatty acid methyl esters (FAMEs) were prepared in triplicate using a methanolic solution of potassium hydroxide (2.0 M) according to the methodology described by Aued-Pimentel et al. (113). The methylated FAs were submitted to analysis by gas chromatography-mass spectrometry (GC–MS) on an Agilent Technologies 6890 N Network (Santa Clara, CA) equipped with a DB-FFAP column with 30 m of length, 0.32 mm of internal diameter, and 0.25 μm of film thickness (J&W Scientific, Folsom, CA). The GC equipment was connected to an Agilent 5973 Network Mass Selective Detector operating with an electron impact mode at 70 eV and scanning the range m/z 40–500 in a 1 s cycle in a full scan mode acquisition. The oven temperature was programmed from an initial temperature of 80 °C, a linear increase to 155 °C at 15 °C min^{-1}, followed by a linear increase at 8 °C min^{-1} to 210 °C, then at 30 °C min^{-1} to 250 °C, standing at 250 °C for 18min. The injector and detector temperatures were 220 and 280 °C, respectively. Helium was used as carrier gas at a flow rate of 0.5 mL min^{-1}. The relative amounts of FAs were calculated by the percent area method with proper normalization considering the sum of all areas of the identified FAs.

7. Hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–ESI-MS)

Hydrophilic interaction liquid chromatography analysis of total lipid extracts was performed on a Waters Alliance 2690 HPLC system (Waters Corp.,Milford,MA, USA) coupled to a Finnigan LXQ electrospray linear ion trap mass spectrometer (Thermo Fisher, San Jose, CA, USA).

Mobile phase A consisted of 25% water, 50% acetonitrile and 25% MeOH, with 1 mM ammonium acetate, and mobile phase B consisted of 60% acetonitrile and 40% MeOH with 1 mM ammonium acetate.

The lipid extracts (12,5 μg), prepared in triplicate, were diluted in mobile phase B (90 μL) and 10 μL of the reaction mixture was introduced into an Ascentis Si HPLC Pore column (15 cm × 1.0mm, 3 μm; Sigma-Aldrich). The solvent gradient was programmed as follows: gradient started with 0% of A and 100% of B, linearly increased to 100% of A in

20 min, and isocratically held for 35 min, returning to the initial conditions in 5 min. The flow rate through the column was 7.5 μL min^{-1} obtained using a pre-column split (Accurate, LC Packings, San Francisco, CA, USA). Polar lipid analysis was carried out by negative- and positive-ion electrospray ionization mass spectrometry (ESI-MS). The electrospray voltage was 4.7 kV in the negative-ion mode and 5.0 in the positive-ion mode. The capillary temperature was 275 °C, and the sheath gas (He) flow rate was 25 units. A precursor ion isolation width of 0.5 *m/z* units was used, with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a maximum ionization time of 50 ms and 200 ms, respectively. The normalized collision energy (CE) varied between 17 and 20 (arbitrary units) for MS/MS. To obtain the product-ion spectra of the major components during LC experiments, cycles consisting of one full scan mass spectrum and three data-dependent MS/MS scans were repeated continuously throughout the experiments with the following dynamic exclusion settings: repeat count 3; repeat duration 30 s and exclusion duration 45 s. Data acquisition and treatment of results were carried out with the Xcalibur® Data System 2.0 (Thermo Scientific, San Jose, CA, USA). Analysis of LC–MS and MS/MS data was performed to identify the molecular species of the polar lipids of *P. dioica*. Identification of molecular lipid species was based on the assignment of the molecular ions observed in the LC–MS and by manual interpretation of the LC–MS/MS obtained for each molecular ion. All the analyses were performed in triplicate.

8. Statistical analysis

The experiments were done independently for all conditions and the results were expressed as the means ± SD. Two way analysis of variance (ANOVA) with the Bonferroni post-hoc (for quantification of FA) was used to determine significant differences among samples. A value of $p < 0.05$ was considered significant. Statistics was done using PRISM® GraphPad Software.

III. Results

III. Results

The fatty acid profile of the red macroalgae *P. dioica* (blade) harvested in two different seasons (winter and summer), as well as the fatty acid profile of the conchocelis phase, which is a sporophyte and diploid stage of the *Porphyra* life cycle, were performed using gas chromatography coupled with mass spectrometry (GC-MS). The detailed polar lipid profile of *P. dioica* (blade) was also unravelled, using a lipidomic approach employing hydrophilic interaction liquid chromatography-eletctrospray ionization mass spectrometry (HILIC-ESI-MS). The LC-MS spectra allowed the identification of which polar lipid classes were present in *P. dioica* (blade), as well the molecular species of each class. Analysis of tandem mass spectra (MS/MS) provided the confirmation of the structure of the molecular species of each lipid class, including polar head structure and the fatty acyl substitutions.

1. Phospholipid and glycolipid class separation by TLC of *P. dioica* (blade) and of the conchocelis phase

The separation of the different phospholipid (PL) and glycolipid (GL) classes was accomplished by thin layer chromatography (TLC). The spots of each class were visualized after spraying with primuline, and each PL and GL class was identified by comparison with pure standards applied in the same TLC plate. This procedure allowed the identification of phosphatidylcholine (PC), phosphatidylglycerol (PG), classes in distinct spots, as well as digalactosyldiacylglycerol (DGDG) (Figure 10). This technique allowed the identification of the PL and GL class present in both *P. dioica* and the conchocelis phase. The different phospholipid and glycolipid classes were extracted from the silica with $CHCl_3$:MeOH (2:1) and analysed by mass spectrometry (ESI-MS/MS) in positive and negative modes, after dilution with methanol.

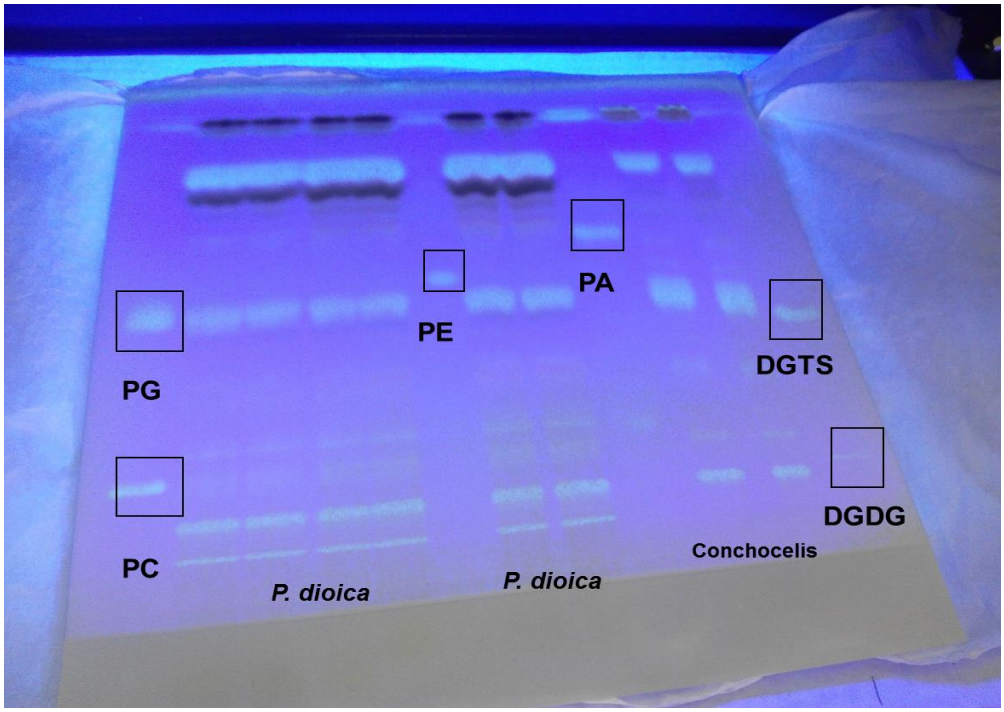


Figure 10 - Thin-layer chromatography of total lipid extract from *P. dioica* and conchocelis. Phospholipid and glycolipid standards were also applied: PC, PG, PE, PA, DGTS, DGDG.

2. Fatty acid profile of *P. dioica* (blade) harvested in winter and summer seasons and of the conchocelis phase

The identification and quantification of the FA profile of the total lipidc extract of the red macroalgae *P. dioica* (blade), as well as that of its conchocelis phase, was performed by GC-MS analysis of the FA methyl esters. Analysis was performed on total lipid extracts from two samples of *P. dioica* harvested on different seasons (winter and summer), represented in figure 11 as well from a conchocelis phase sample (figure 12). The fatty acid profile of all the aforementioned samples is summarized in figure 13. All samples were cultivated in an IMTA system. The analysis of each sample was performed in duplicate.

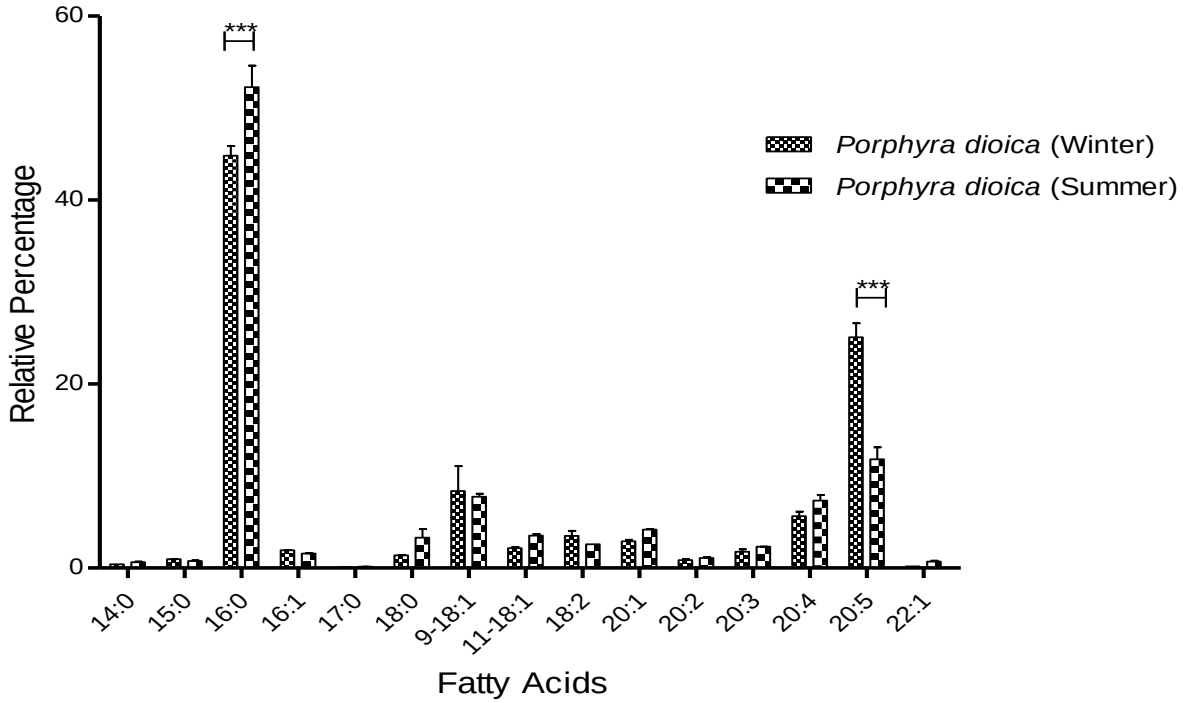


Figure 11 – Relative FA content (%) in total lipid extract of *P. dioica* (blade) harvested in the winter and summer seasons. Values were the means ± SD. ***, $p < 0.001$ comparing both samples.

Results obtained by GC-MS analysis allowed the identification of 15 different fatty acids in both *P. dioica* samples. The identified fatty acids were: $C_{14:0}$ (myristic acid), $C_{15:0}$ (pentadecanoic acid), $C_{16:0}$ (palmitic acid), $C_{16:1}$ (palmitoleic acid), $C_{17:0}$ (heptadecanoic acid), $C_{18:0}$ (stearic acid), $C_{18:1\ (n\text{-}9)}$ (oleic acid; *n*-9), $C_{18:1\ (n\text{-}7)}$ (oleic acid; *n*-7), $C_{18:2}$ (linoleic acid), $C_{20:1}$ (eicosenoic acid), $C_{20:2}$ (eicosadienoic acid), $C_{20:3}$ (eicosatrienoic acid), $C_{20:4}$ (arachidonic acid), $C_{20:5}$ (eicosapentaenoic acid), $C_{22:1}$ (docosenoic acid).

In general, the most abundant fatty acid present in both samples is palmitic (16:0) acid, followed by eicosapentaenoic (20:5), oleic (18:1 *n*-9) and arachidonic (20:4) acids.

It is interesting to note that besides the generic fatty acid profile being similar in the samples from both seasons, there are some significant differences observed, mainly in the amount of palmitic acid (16:0), that is slightly lower on the winter season sample (45.0% of total fatty acid content in winter season, as opposed to 52.2% in the summer season), and also in the amount of eicosapentaenoic acid (20:5), that is higher in the winter season (25.2% of total fatty acid content in the winter season, in contrast with the 11.8% in the summer season).

Regarding de *n*-6/*n*-3 ratio of *P. dioica* from both seasons, it can be observed that it is lower than 1:1, with the ratio for *P. dioica* from the winter season (ratio of 0,35) being slightly lower the one from the summer season (ratio of 0,8).

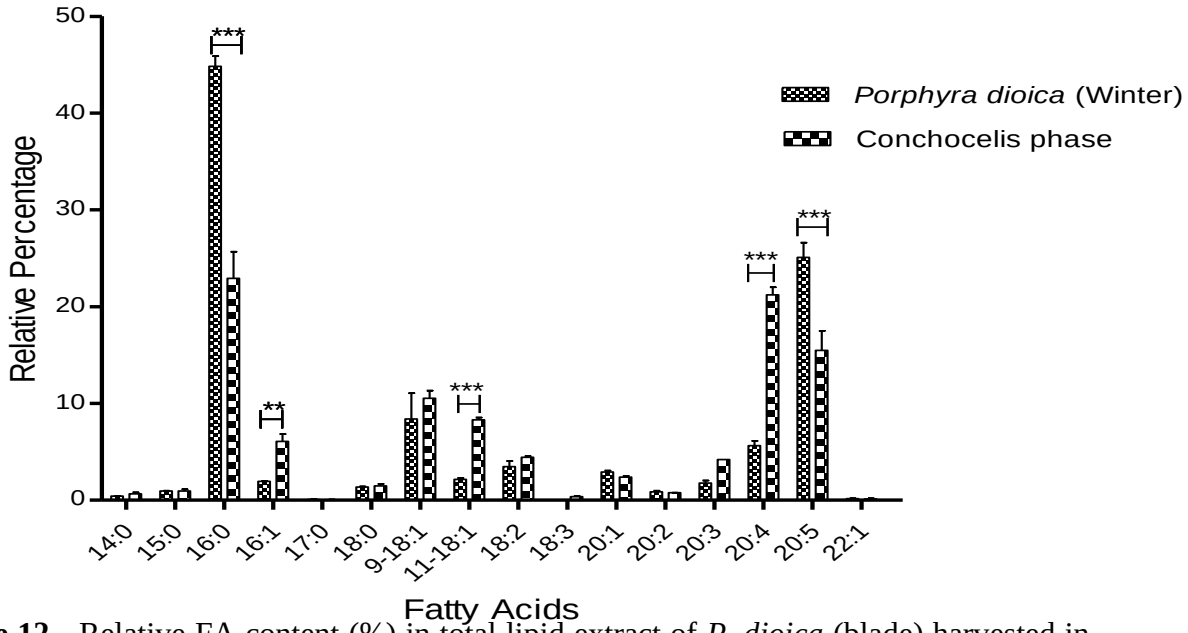


Figure 12 - Relative FA content (%) in total lipid extract of *P. dioica* (blade) harvested in the winter season and the conchocelis phase. Values were the means ± SD. ***, significantly different from control group (p <0.001); **, significantly different from control group (p <0.01).

The fatty acid profile of the conchocelis phase shows the same FAs identified for *Porphyra*, but with some significant differences in the content of some fatty acids. The most notorious changes are in the amount of palmitic acid (16:0), which significantly lower in conchocelis (22.9% of the total fatty acid content, as opposed to the 45.0% of *P. dioica*), and in the amount of arachidonic acid (20:4) that is significantly higher in conchocelis (21.2% of total fatty acid content, while in *Porphyra* stands at 5.70%). The amount of eicosapentaenoic acid (20:5) in conchocelis stands at about 15.5% of total fatty acid content, slightly lower than in *P. dioica* (25.2%).

It is also worth mentioning the increased amount of MUFAs in the conchocelis phase, especially palmitoleic acid (16:1) and oleic acid (18:1), with $C_{16:1}$ accounting for 6.09% of total fatty acid content in conchocelis and $C_{18:1}$ for a total of 18.8% of total fatty acid content (considering both 9-18:1 and 11-18:1).

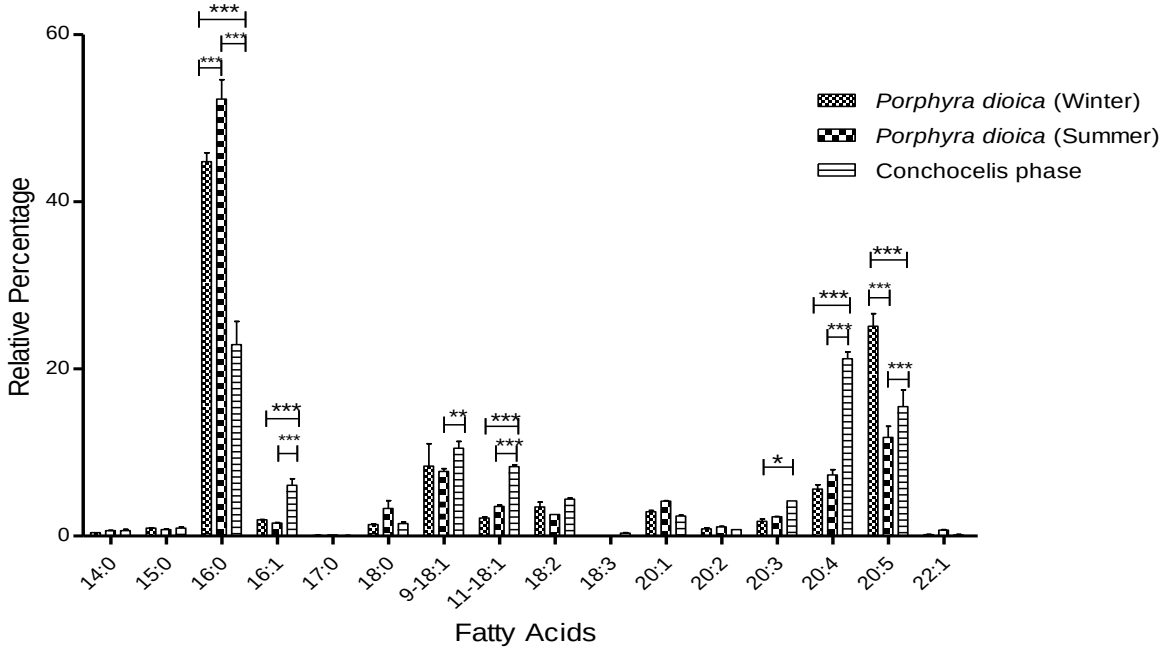


Figure 13 - Relative FA content (%) in total lipid extract of *P. dioica* (blade) harvested in the winter and summer seasons and the conchocelis phase. Values were the means ± SD. ***, significantly different from control group (p <0.001); **, significantly different from control group (p <0.01); *, significantly different from control group (p <0.05).

Comparing the FA profile of conchochelis with that of *P. dioica* (blade) from both seasons, a significant decrease in palmitic acid (16:0) content can be observed, as well as increased content in arachidonic acid (20:4). Eicosapentaenoic acid content is slightly lower than in *P. dioica* from winter season. Significant differences between palmitoleic acid ($C_{16:1}$) content can also be observed, with the conchocelis phase containing higher amount of this fatty acid (6.10% of total fatty acid content), as opposed to *P. dioica* (1.50-1.90% of total fatty acid content). The essential fatty acid α-linolenic acid ($C_{18:3}$ *n-3*) was also present in the conchocelis phase, even though in a very small amount (0.35% of total fatty acid content).

Regarding *P. dioica* from winter and from summer seasons, significant differences in fatty acid content are mainly due to the increase of EPA ($C_{20:5}$) and decrease of palmitic acid ($C_{16:0}$) in *Porphyra* from winter.

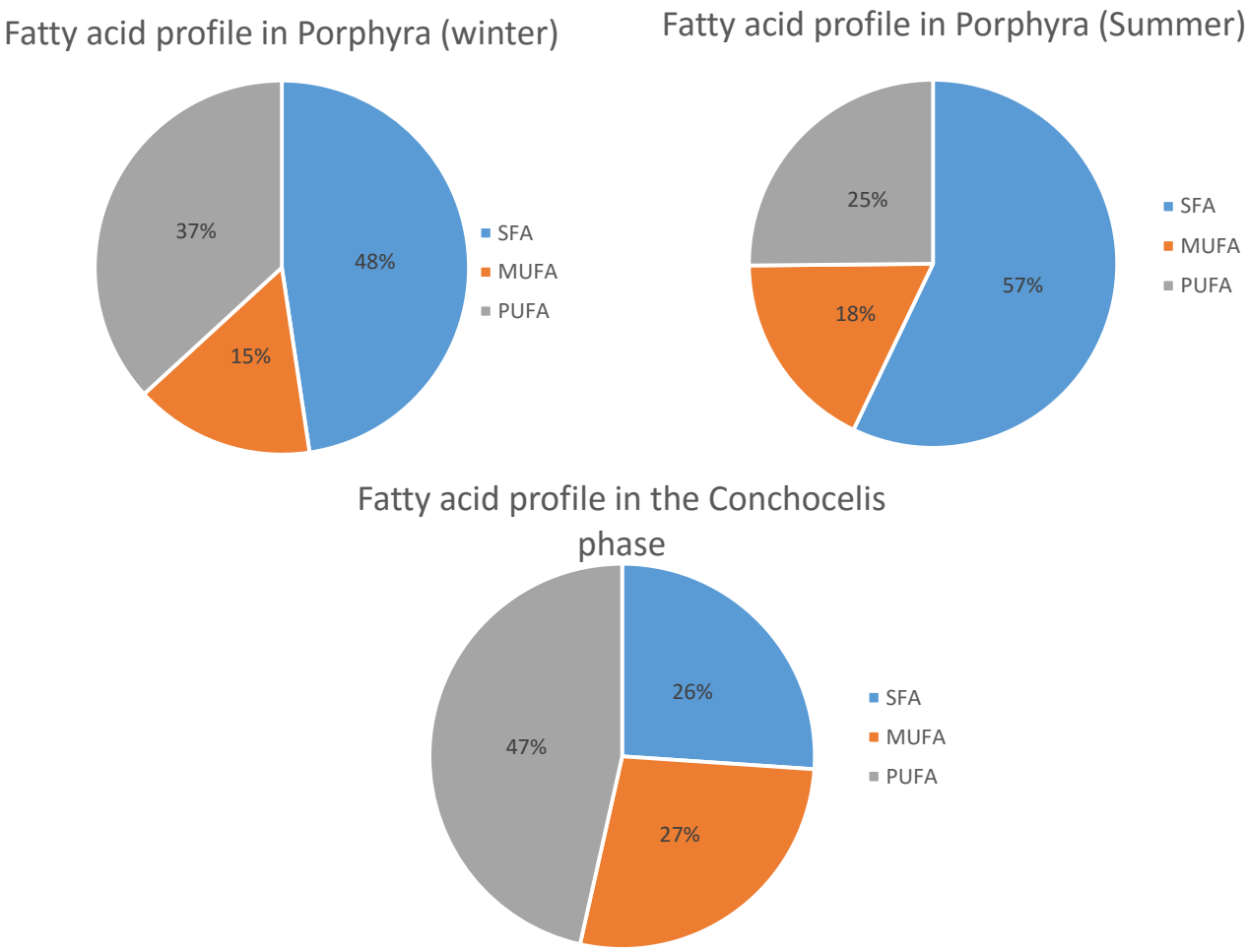


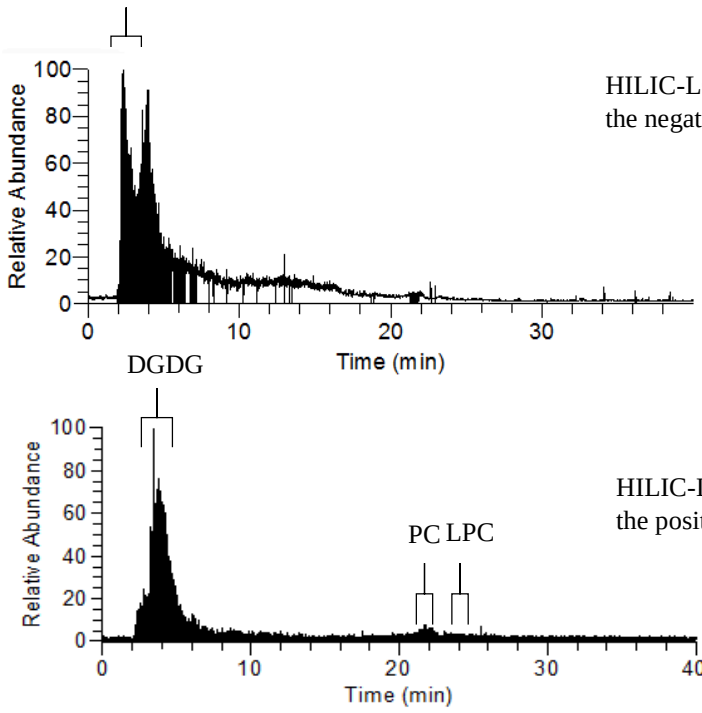
Figura 14 – Relationship between saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) in: (a) *P. dioica* (blade) harvested in the winter season; (b) *P. dioica* (blade) harvested in the summer season; (c) conchocelis phase.

The relationship between saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids in both samples of *P. dioica*, as well as in conchocelis was determined (figure 14). *P. dioica* from the summer season possessed the highest amount of SFA, accounting for 56,0% of the total fatty acid content. Conchocelis presented the highest content of PUFA (47,0%). Between the two samples of *P. dioica*, the one from winter season has shown to be richer in PUFA (37,0%), as well as having a total of 52,0% of total lipid content in the form of unsaturated fatty acids.

Considering the interesting potential of this macroalgae as a source of omega-3 fatty acids and other bioactive compounds, the lipidome of the winter batch of *P. dioica* was studied by HPLC-MS/MS.

3. Analysis of polar lipid profile of *P. dioica* (blade) from the winter season by HPLC-MS/MS

HPLC-MS/MS was used to analyse the total polar lipid profile of the *Porphyra* extracts, since LC-MS allows the separation of phospholipid (PL) and glycolipid (GL) classes, thus making possible the analysis by MS and MS/MS of each class. The molecular species of each class were identified in order to provide a detailed knowledge of the polar lipid profile of the *P. dioica*. Through this analysis, several classes of PL (PC, LPC, PG and LPG) and GL (SQMG, SQDG and DGDG) were identified. The PC, LPC, and DGDG classes were analysed by HPLC-MS and MS/MS in the positive-ion mode, on the other hand, SQDG, SQMG, PG and LPG classes were analysed by HPLC-MS and MS/MS in the negative-ion mode. The chromatogram showing the elution of each PL and GL class is shown in figure 15.



HILIC-LC-MS Chromatogram of *P. dioica* in the negative-ion mode

HILIC-LC-MS Chromatogram of *P. dioica* in the positive-ion mode

Figure 15 - LC-MS chromatograms in negative and positive modes, showing the retention times where each PL and GL class eluted. PG, LPG, SQDG and SQMG classes were identified in the HILIC-LC-MS Chromatogram in the negative-ion mode, co-eluting around 2-3 minutes range. DGDG; PC and LPC were identified in HILIC-LC-MS Chromatogram of in the positive-ion mode, with DGDG eluting at 2-4 minutes range, whereas PC and LPC eluted at the 20-24 minutes range.

3.1. Glycolipids

In the lipid extract of *P. dioica*, three different classes of glycolipids were identified and analysed. The basic structure of glycolipids is characterized by a 1,2-diacyl-sn-glycerol moiety with a mono- or oligosaccharide attached to the *sn-3* position of the glycerol backbone. The classes of glycolipids identified in *P. dioica* were sulfoquinovosyldiacylglycerols (SQDGs), sulfoquinovosylmonoacylglycerols (SQMG) and digalactosyldiacylglycerols (DGDGs). In the following topics, the detailed profile of the molecular species obtained through HPLC-MS and MS/MS will be presented.

3.1.1. Analysis of sulfoquinovosyldiacylglycerols (SQDG) and sulfoquinovosylmonoacylglycerols (SQMG)

The LC–MS spectra obtained for the SQDG and SQMG classes are represented in figure 16, showing both SQDG and SQMG molecular species as $[M–H]^-$ ions and allowed the identification of their molecular weight. The analysis of the LC-MS/MS data was essential for the confirmation of the composition of the molecular species belonging to this class of glycolipids, including the polar head group and the fatty acyl chain substitutions. The typical fragmentation pattern obtained through MS/MS spectra of SQDG as $[M– H]^-$ ions is the presence of ions at *m/z* 225 corresponding to the sulfoquinovosyl group, thus confirming the polar head of these polar lipids. It was also observed the presence of ions formed due to the loss of fatty acyl chains, both as acid and keto derivatives, as well as the carboxylate anions ($RCOO^–$) (figure 17). SQMG structure is similar of that of SQDG, with the difference of only possessing one fatty acid acyl substitution. Thus the fragmentation pattern obtained through MS/MS spectra of SQMG as $[M-H]^-$ is similar of that of SQDG (figure 18).

(a) SQDG

(b) SQMG

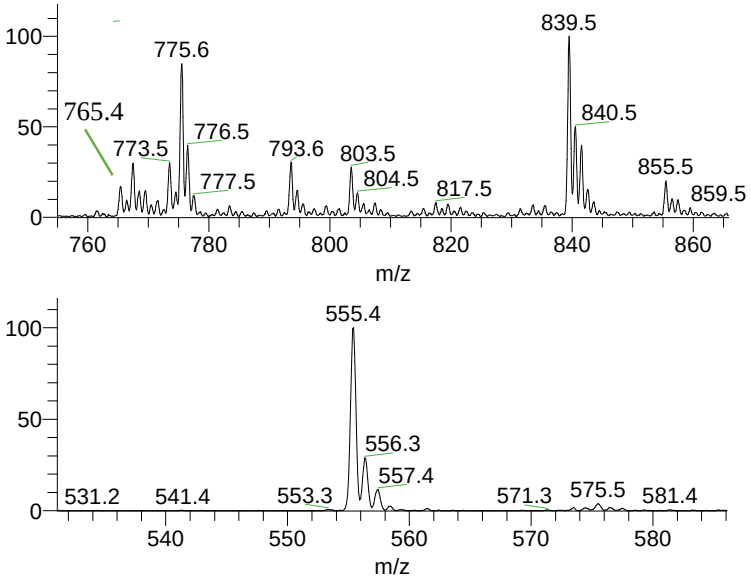


Figure 16 - HPLC-MS spectra of: (a) SQDG class; (b) SQMG class. Both spectra were obtained in the negative-ion mode with formation of $[M-H]^-$ ions, in *P. dioica* (blade). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

(a)

(b)

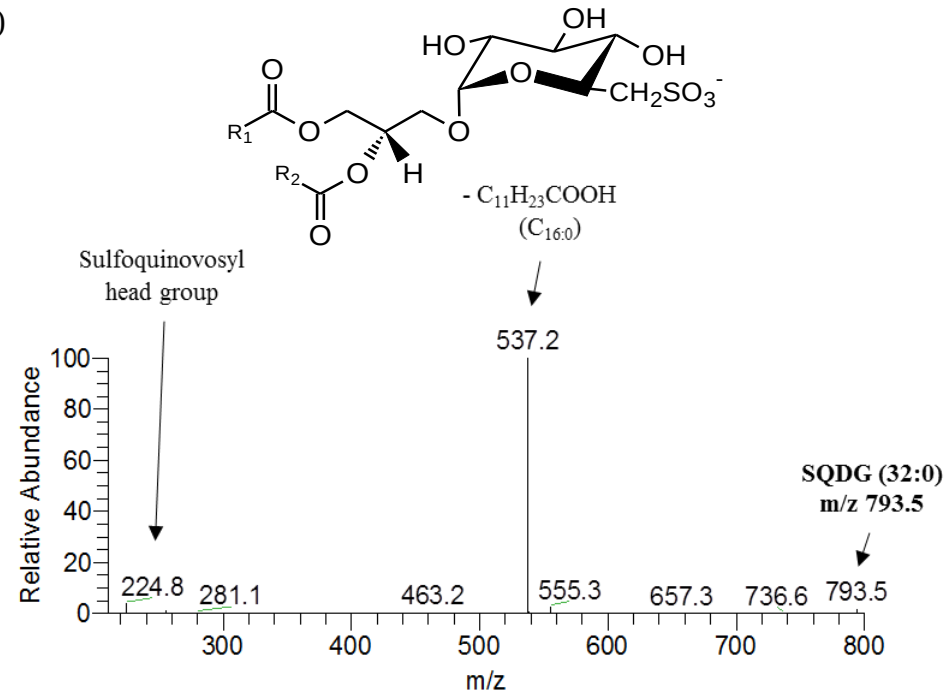


Figure 17 – (a) General structure of SQDG. (b) MS/MS spectrum of SQDG in $[M-H]^-$ at *m/z* 793.5 (16:0/16:0) with the fragmentation patterns of SQDGs.

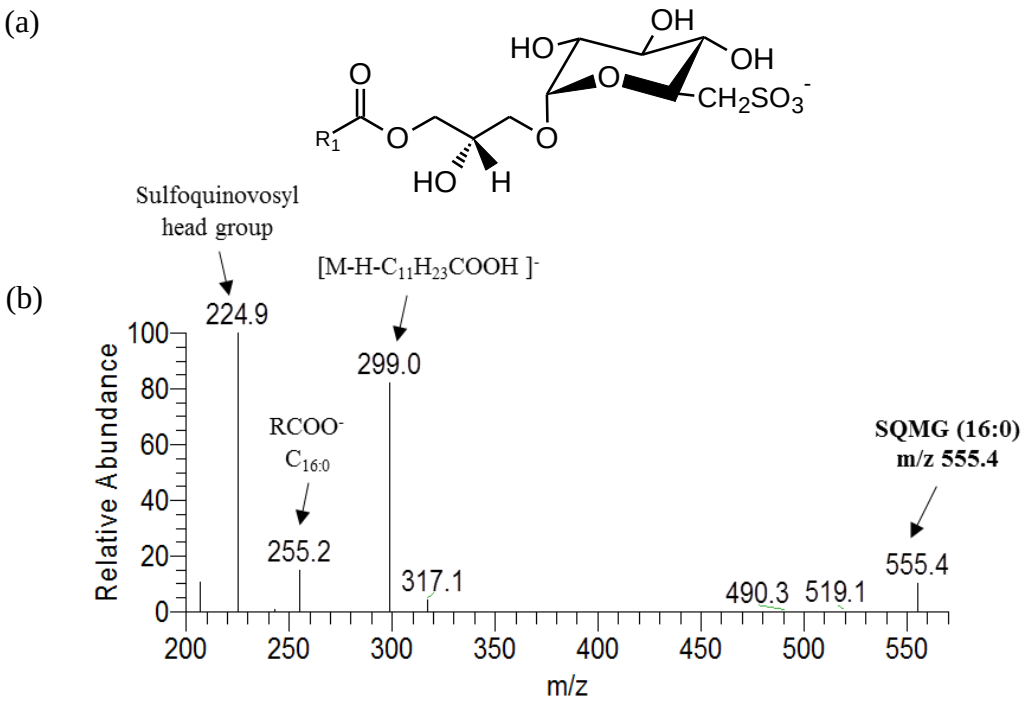


Figure 18 – (a) General structure of SQMG. (b) MS/MS spectrum of SQMG in $[M-H]^-$ at *m/z* 555.4 (16:0) with the fragmentation patterns of SQMGs.

Table 3 – Identification of the $[M–H]^-$ ions of SQMG and SQDGs observed in the HPLC-MS spectra.

$[M-H]^-$ m/z	Lipid species (C:N)	Fatty acyl chains
761.5	SQDG (30:2)	14:1/16:1 and 14:0/16:2
763.4	SQDG (30:1)	14:0/16:1 and 16:0/14:1
765.4	SQDG (30:0)	14:0/16:0 and 18:0/12:0
779.6	SQDG (30:1-OH)	14:0/16:1-OH and 16:0/14:1-OH
785.4	SQDG (32:4)	14:0/18:4
791.5	SQDG (32:1)	16:0/16:1
793.5	SQDG (32:0)	16:0/16:0
807.5	SQDG (32:1-OH)	16:0/16:1-OH
813.4	SQDG (34:4)	16:0/18:4
815.5	SQDG (34:3)	16:0/18:3
817.5	SQDG (34:2)	16:0/18:2
819.5	SQDG (34:1)	16:0/18:1
833.4	SQDG (34:2)	16:0/18:2-OH
839.5	SQDG (36:5)	16:0/20:5
841.5	SQDG (36:4)	16:0/20:4 and 16:1/20:3
843.5	SQDG (36:4)	16:0/20:3
855.5	SQDG (36:5-OH)	16:0/20:5-OH
857.5	SQDG (36:4-OH)	16:0/20:4-OH
$[M-H]^-$ m/z	**Lipid species (C:N)**	
555.4	SQMG (16:0)	

The assignment of the fatty acyl composition of each lipid molecular species was performed according to the interpretation of the correspondent MS/MS spectra. Numbers in parentheses (C:N) indicate the number of carbon atoms (C) and double bonds (N) in the fatty acid side chains.

Based on the LC–MS/MS analysis, several molecular species of SQDGs were identified, being the most abundant species found at *m/z* 839.5, correspondent to SQDG 16:0/20:5. The second most abundant species was observed at *m/z* 793.5, which can be assigned to SQDG 16:0/16:0. Other species with long fatty acyl chains and bearing polyunsaturated fatty acids can be seen, such as the case of the ions at *m/z* 841.5 (SQDG 16:0/20:4 bearing the AA fatty acid), and ions at *m/z* 855.5 and *m/z* 857.5 (SQDG 16:0/20:5-OH and SQDG 16:0/20:4-OH respectively). The complete list of all the SQDG molecular species detected are present in table 3. Along with the wide array of SQDG molecular species detected, one molecular specie of sulfoquinovosylmonoacylglycerol (SQMG) was also found at *m/z* 555.5, corresponding to SQMG (16:0). This glycerolipids are the monoacyl form of sulfoquinovosyldiacylglycerol (SQDG), both sharing a similar fragmentation pattern under MS/MS (figure 18).

3.1.2. Analysis of digalactosyldiacylglycerols (DGDG)

The digalactosyldiacylglycerides (DGDGs) were analysed by LC–MS in the positive-ion mode and the molecular species were identified as $[M+NH_4]^+$ ions (figure 19). The confirmation of the structural composition of the different molecular species of DGDG, including the polar head group and the fatty acyl substitutions was performed by the analysis of the MS/MS spectra of the $[M+Na]^+$ ions of the extracts obtained from the scrapped off silica of the TLC spot correspondent to the DGDG class.

The fragmentation pattern under MS/MS of $[M+Na]^+$ ions of DGDGs (figure 20) showed the loss of one hexose residue (neutral loss of 162 Da) as well as the presence of the ions at *m/z* 347,1 referring to the loss of two hexose residues (hexose minus H_2O) plus Na^+ $[Hex_{2res}+Na]^+$, *m/z* 365, referring to the loss of two hexoses plus Na^+ $[Hex_2+Na]^+$ and *m/z* 405,3 corresponding to the sodiated digalactosyl glycero head group $[C_{15}H_{26}O_{11}+Na]^+$, thus confirming the presence of the digalactosyl head group. Further confirmation the structures of the different molecular species of DGDG was possible through the observation of product

ions, corresponding to the loss of fatty acyl chains as acid derivatives (-RCOOH) and ions formed due to the combined loss of the hexose residue and the fatty acyl chain. For example, the molecular species of DGDG at *m/z* 961.6 (figure 20) showed the neutral loss of the hexose residue (-162 Da) corresponding to the ion at *m/z* 799.5 and loss of the fatty acyl chains as acid derivatives, with the ion at *m/z* 705.4 corresponding to the loss of $C_{16:0}$ and the ion at *m/z* 659.5 to the loss of $C_{20:5}$, thus confirming that the ion at *m/z* 961.6 corresponds to DGDG (16:0/20:5).

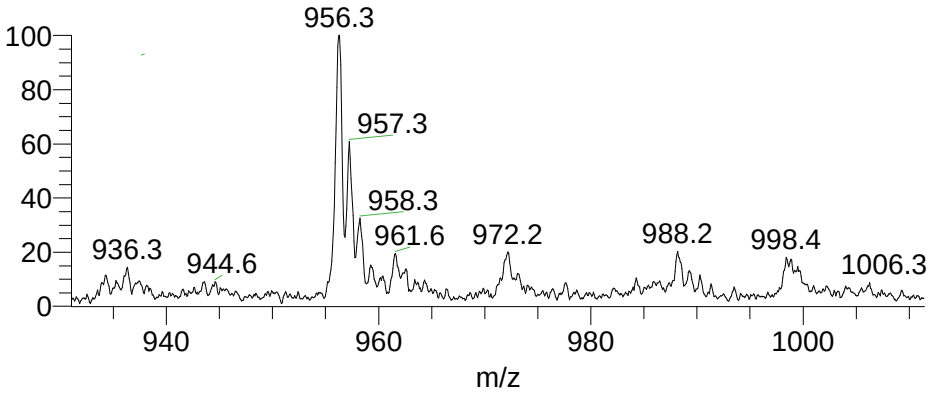


Figure 19 - HPLC-MS spectra of the DGDG class. The spectra was obtained in the positive-ion mode with formation of $[M+NH_4]^+$ ions, in *P. dioica* (blade). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

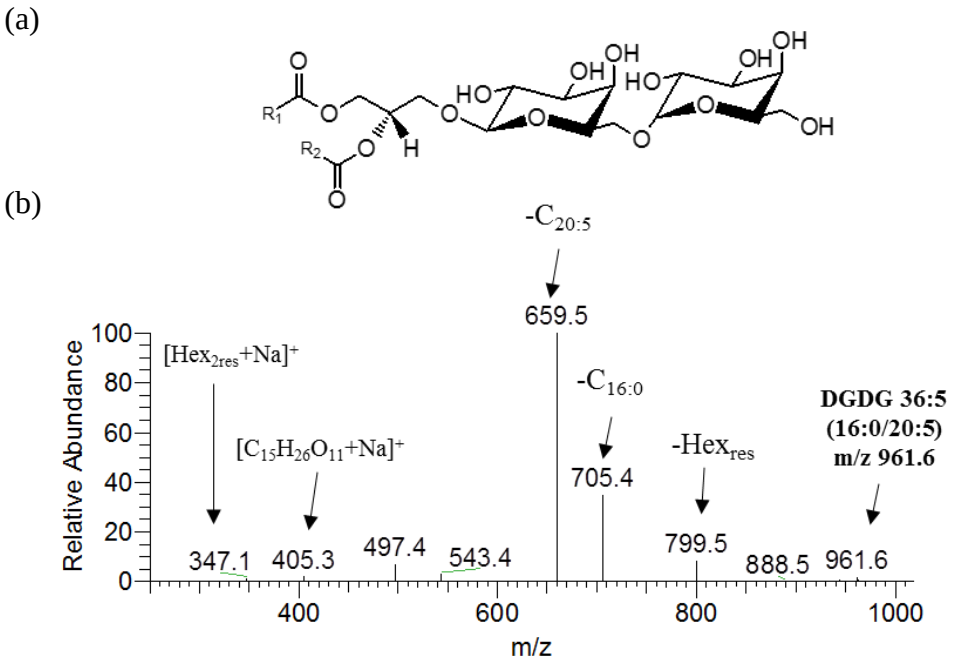


Figure 20 – (a) General structure of DGDG. (b) MS/MS spectrum of DGDG in $[M+Na]^+$ at *m/z* 961.6 (16:0/20:5) with the fragmentation patterns of DGDGs.

Table 4 – Identification of the $[M+NH_4]^+$ and $[M+Na]^+$ ions of DGDG observed in the HPLC-MS spectra.

$[M+NH_4]^+$ m/z	$[M+Na]^+$ m/z	Lipid species (C:N)	Fatty acyl chains
934.3	939.7	34:2	18:2/16:0
936.3	941.7	34:1	16:0/18:1
956.3	961.6	36:5	16:0/20:5
958.6	963.7	36:4	16:0/20.4
966.4	971.7	36:0	18:0/18:0
972.4	977.7	36:5-OH	16:0/20:5-OH
986.4	991.7	38:4	16:0/22:4 and 18:1/20:3
988.4	993.7	38:3	16:0/22:3 and 18:1/20:2

The assignment of the fatty acyl composition of each lipid molecular species was performed according to the interpretation of the correspondent MS/MS spectra. Numbers in parentheses (C:N) indicate the number of carbon atoms (C) and double bonds (N) in the fatty acid side chains.

Based on the LC–MS/MS analysis of the $[M+Na]^+$ ions of DGDGs, several molecular species this glycolipid class were identified (shown in table 4) being the most abundant species found at *m/z* 961.6, correspondent to DGDG 16:0/20:5. Other species with long fatty acyl chains and bearing polyunsaturated fatty acids can be seen, such as the case of the ions at *m/z* 963.7, (DGDG 16:0/20:4 bearing the AA fatty acid), and at *m/z* 977.7 (DGDG 16:0/20:5-OH).

3.2.Phospholipids

Phospholipids are characterized by the presence of a phosphate group linked to the *sn-3* position of a glycerol backbone, with the later bearing fatty acid acyl chains in the *sn-1* and *sn-2* positions. The phosphate group is further linked to and hydrophilic head group that provides the classification of the different PL classes.

The analysis of phospholipids (PLs) profile of *P. dioica* by LC-MS allowed the identification of four classes: phosphatidylglycerols (PGs), lysophosphatidylglycerols (LPGs), phosphatidylcholines (PCs) and lysophosphatidylcholines (LPCs). In the following topics, the detailed composition of the different molecular species of the aforementioned PL detected in *P. dioica* will be presented.

3.2.1. Phosphatidylglycerol (PG) and lysophosphatidylglycerol (LPG)

PGs and LPGs were identified by LC–MS as $[M–H]^-$ ions (figure 21). The analysis of LC–MS/MS spectra of PGs showed typical product ions formed due to the loss of fatty acyl chains, as both acid and keto derivatives, as well as the carboxylate anions of the fatty acyl chains. Ions corresponding to the neutral loss of 74 Da (loss of glycerol head group as an oxirane, $C_3H_6O_2$) and to the combined loss of 74 Da and the fatty acyl chain were also found (figure 22). The LC–MS/MS spectra of LPGs showed ions formed due to the loss of 74 Da (loss of glycerol head group as an oxirane, $C_3H_6O_2$), ions at *m/z* 171 ($[C_3H_7O_2OPO_3H]^-$, glycerol phosphate), *m/z* 152.8 (glycerol phosphate-H_2O), *m/z* 226.9 ($[C_6H_{12}O_7P]^-$, loss of water from glycerophosphate glycerol) and the carboxylate anion of fatty acyl chain (figure 23). The PG and LPG molecular species identified in *P. dioica* as a result of the MS/MS data analysis is summarized in tables 5 and 6.

(a) PG

(b) LPG

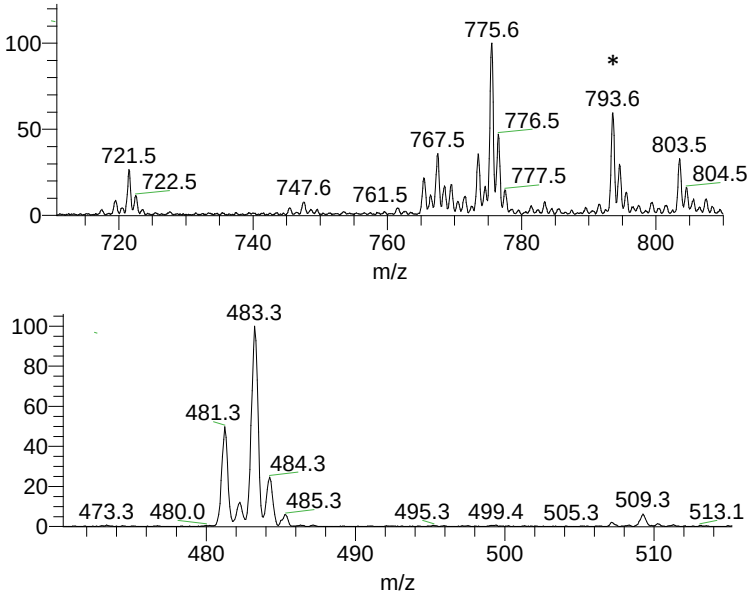


Figure 21 - HPLC-MS spectra of: (a) PG class; (b) LPG class. Both spectra were obtained in the negative-ion mode with formation of $[M-H]^-$ ions, in *P. dioica* (blade). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion. * - contaminant

(a)

(b)

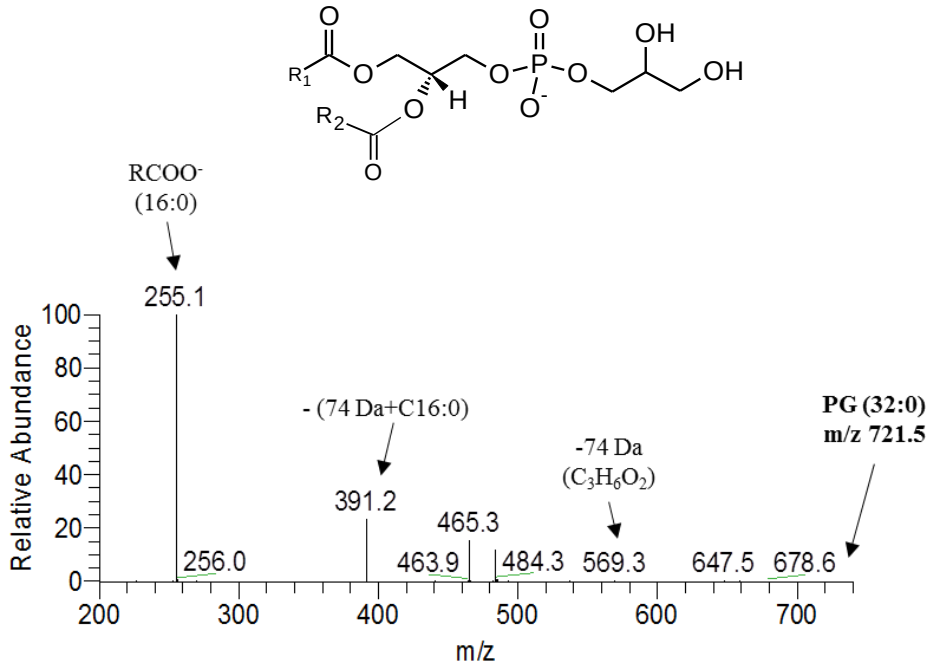


Figure 22 - (a) General structure of PG. (b) MS/MS spectrum of PG in $[M-H]^-$ at *m/z* 721.5 (16:0/16:0) with the fragmentation patterns of PGs.

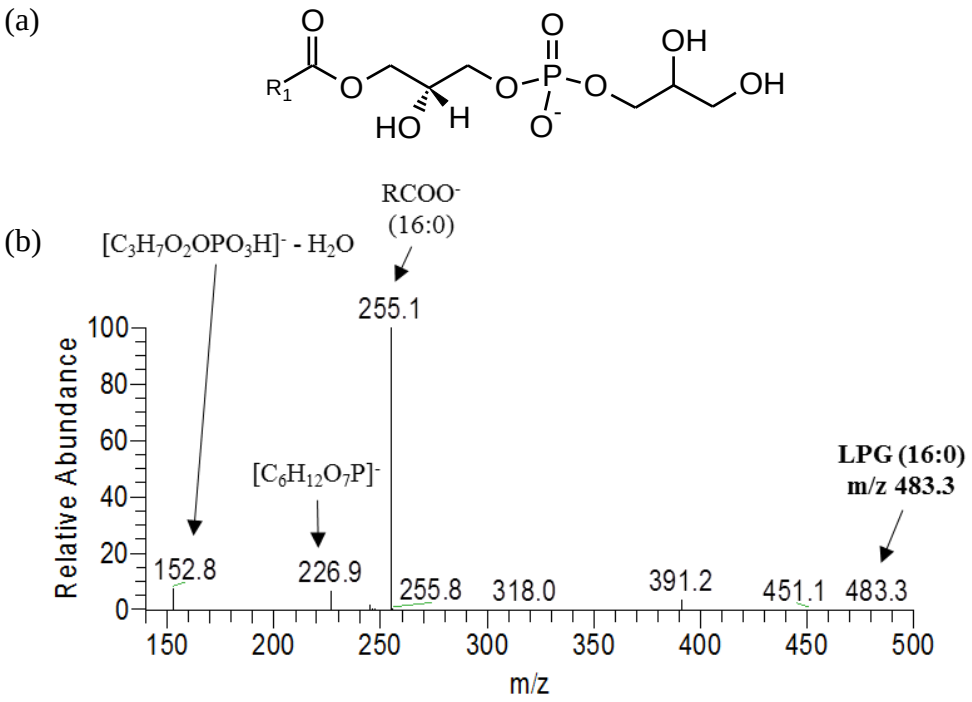


Figure 23 - (a) General structure of LPG; (b) MS/MS spectrum of LPG in $[M-H]^-$ at *m/z* 483.3 (LPG 16:0) with the fragmentation patterns of LPGs.

Table 5 – Identification of the $[M-H]^-$ ions of PG observed in the HPLC-MS spectra.

$[M-H]^-$ m/z	Lipid species (C:N)	Fatty acyl chains	$[M-H]^-$ m/z	Lipid species (C:N)	Fatty acyl chains
717.5	PG (32:2)	16:0/16:2	775.6	PG (36:1)	16:0/20:1
719.5	PG (32:1)	16:0/16:1	783.4	PG (37:4)	19:1/18:3
721.5	PG (32:0)	16:0/16:0	785.4	PG (37:3)	19:1/18:2
			787.4	PG (37:2)	19:1/18:1
745.4	PG (34:2)	16:1/18:1 and 16:0/18:2			
		16:0/18:1	789.5	PG (37:1)	19:0/18:1 and 18:0/19:1
747.5	PG (34:1)	18:0/16:1	791.5	PG (37:0)	18:0/19:0
761.5	PG (35:1)	16/0:19:1			
765.4	PG (36:6)	16:1/20:5	795.5	PG (38:5)	18:0/20:5 and 18:1/20:4
		16:0/20:5 and			
767.5	PG (36:5)	16:1/20:4			18:0/20:4 and
769.5	PG (36:4)	16:0/20:4	797.5	PG (38:4)	18:1/20:3
		18:1/18:2 and			
771.5	PG (36:3)	16:0/20:3	799.5	PG (38:3)	18:0/20:3 and 18:1/20:2
		18:1/18:1 and	801.5	PG (38:2)	16:0/22:2
		16:1/20:1 and			
773.5	PG (36:2)	16:0/20:2	803.5	PG (38:1)	16:0/22:1

Table 6 – Identification of the $[M-H]^-$ ions of LPG observed in the HPLC-MS spectra.

$[M-H]^-$ m/z	Lipid Species (C:N)
481.3	LPG (16:1)
483.3	LPG (16:0)
509.3	LPG (18:1)

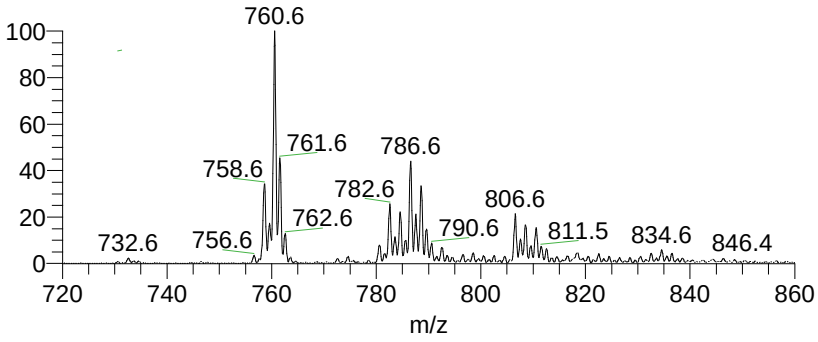
Attribution of the fatty acyl composition of each lipid molecular species was performed according to the interpretation of the correspondent MS/MS spectra. Numbers in parentheses (C:N) indicate the number of carbon atoms (C) and double bonds (N) in the fatty acid side chains.

Several molecular species were identified by the LC–MS/MS analysis of PGs and LPGs. The most abundant molecular species of PG were found at *m/z* 775.6 corresponding to PG (16:0/20:1). Other species of PG are listed in table 4, with some species bearing PUFAs in their fatty acid acyl chain composition, namely EPA and AA, such as the PG (16:1/20:5) at *m/z* 765.4 and PG (16:0/20:5 and 16:1/20:4) at *m/z* 767.5. LPGs were also identified, and the LC–MS spectra obtained for the $[M-H]^-$ ions are presented in figure 21. The most abundant species were found at *m/z* 483.3, corresponding to LPG (16:0), followed by LPG (16:1) at *m/z* 481.3. A third molecular species of LPG was detected at *m/z* 509.3, which can be assigned to LPG (18:1).

3.2.2. Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC)

PCs and LPCs ionize preferentially in the positive-ion mode, forming $[M+H]^+$ ions in the MS spectra (figure 24). The confirmation of the composition the different molecular species of PC and LPC was accomplished by MS/MS. The MS/MS spectra analysis of PCs and LPC shows a characteristic product ion at *m/z* 184, corresponding to the polar head group ($H_2PO_4(CH_2)_2N^+(CH_3)_3$, phosphocholine), another product ion due to neutral loss of polar head (loss of 183 Da) and ions that are formed due to the loss of the fatty acyl chains. The PC and LPC molecular species identified in *P. dioica* as result of the MS/MS data analysis is summarized in tables 7 and 8.

(a) PC



(b) LPC

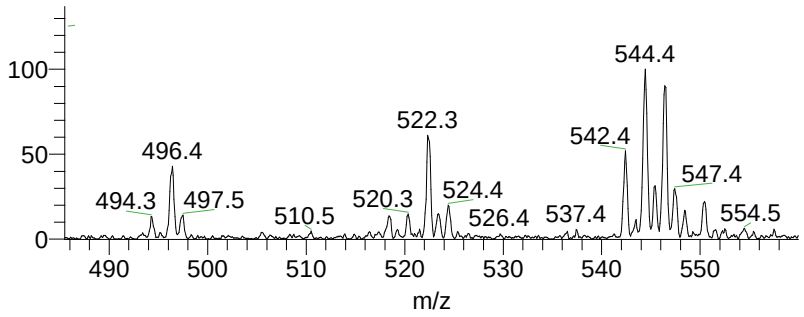


Figure 24 - HPLC-MS spectra of: (a) PC class; (b) LPC class. Both spectra were obtained in the positive-ion mode with formation of $[M+H]^+$ ions, in *P. dioica* (blade). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

Table 7 – Identification of the $[M+H]^+$ ions of PC observed in the HPLC-MS spectra.

$[M+H]^+$ m/z	Lipid species (C:N)	Fatty acyl chains
732.6	PC (32:1)	16:0/16:1
734.5	PC (32:0)	16:0/16:0
758.6	PC (34:2)	16:0/18:2
760.6	PC (34:1)	16:0/18:1
780.6	PC (36:5)	16:0/20:5 e 16:1/20:4
782.6	PC (36:4)	16:0/20:4
784.5	PC (36:3)	18:1/18:2 e 16:0/20:3
786.6	PC (36:2)	18:0/18:2 e 18:1/18:1
788.6	PC (36:1)	18:0/18:1
806.5	PC (38:6)	18:2/20:4

Table 8 – Identification of the $[M+H]^+$ ions of LPC observed in the HPLC-MS spectra.

$[M+H]^+$ m/z	Lipid Species (C:N)
494.4	LPC (16:1)
496.4	LPC (16:0)
518.3	LPC (18:3)
522.3	LPC (18:1)
542.4	LPC (20:5)
544.5	LPC (20:4)
546.42	LPC (20:3)

Attribution of the fatty acyl composition of each lipid molecular species was performed according to the interpretation of the correspondent MS/MS spectra. Numbers in parentheses (C:N) indicate the number of carbon atoms (C) and double bonds (N) in the fatty acid side chains.

Several molecular species were identified by the LC–MS/MS analysis of PCs and LPCs. The most abundant molecular species of PC were found at *m/z* 760.6 and *m/z* 786.6, corresponding to PC (16:0/18:1) and a mixture of PC (18:0/18:2) and (18:1/18:1), respectively. Other species can be seen in table 7, with some species bearing PUFAs, namely EPA and AA fatty acids, such as PC (16:0/20:5) and (16:1/20:4) at *m/z* 780.6 and PC (16:0/20:4) at *m/z* 782.6. LPCs were also identified, and the LC–MS spectra showing the $[M+H]^+$ ions are presented in figure 24. The most abundant species were found at *m/z* 544.5, corresponding to LPC (20:4), followed by LPC (16:0) at *m/z* 496.4. Other molecular species of LPC are presented in table 8, also bearing PUFAs, namely EPA (LPC 20:5).

3.3. Phytyl derivatives

Chlorophyll and pheophytin derivatives were identified in the lipid extracts of *P. dioica* by LC–MS in positive-ion mode as $[M+H]^+$ ions (figure 25). The LC–MS/MS spectra of chlorophyll derivatives (figure 26) showed product ions formed due to the neutral losses of 278 Da, corresponding to the loss of the phytyl side chain as an alkene, $[M-C_{20}H_{38}]^+$), 310 Da due to the loss of 278 (phytyl side chain) plus 32 ($HOCH_3$) and 338 (loss of 278 plus 60, $[M-CH_3COOC_{20}H_{39}]^+$). In the case of pheophytin derivatives, the LC-MS/MS spectra (figure 26) showed ions formed by the losses of 278 Da (loss of phytyl side chain) and 338 (loss of 278 plus 60, which means loss of phytyl plus $HCOOCH_3$). The interpretation of the LC-MS/MS data showed the presence of several phytyl derivatives in *P. dioica* with the most abundant being phophytin *a*, at *m/z* 871.7. The full list of phytyl derivatives detected trough the interpretation of LC-MS and LC-MS/MS is presented in table 9.

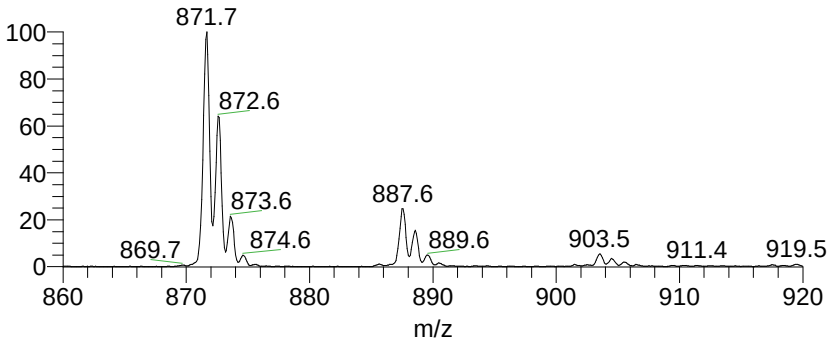
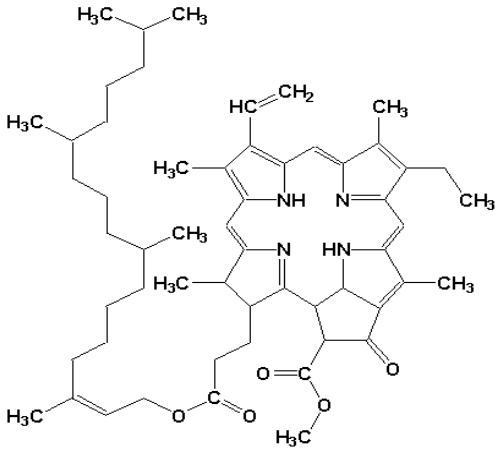


Figure 25 - HPLC-MS spectra of phytyl derivatives identified in the positive-ion mode with formation of $[M+H]^+$ ions, in *P. dioica* (blade). Y-axis: Relative abundance considering the highest abundant ion as 100 %; x-axis: *m/z* for each ion.

(a)



Pheophytin A

(b)

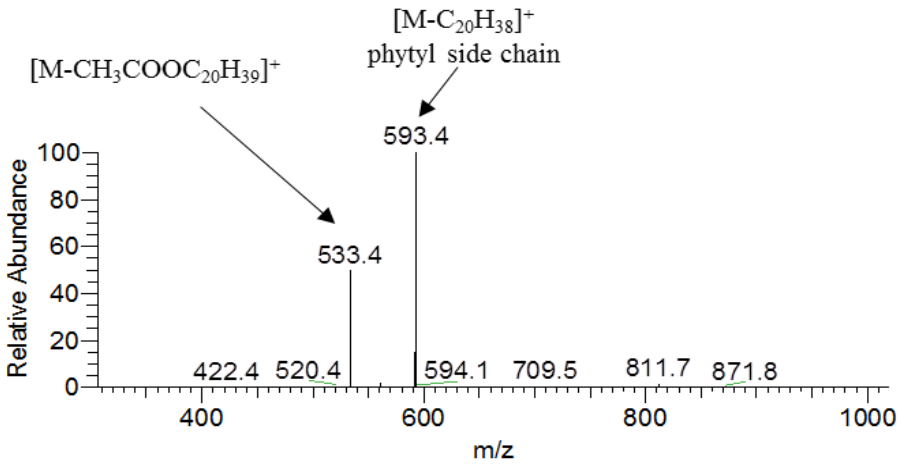


Figure 26 – (a) General structure of pheophytin a. (b) MS/MS spectrum of pheophytin *a* in $[M+H]^+$ at *m/z* 871.7 with the characteristic fragmentation patterns of phoephytin derivatives.

Table 9 – Identification of the $[M+H]^+$ ions of phytyl derivatives observed in the HPLC-MS spectra.

$[M+H]^+$ m/z	Fatty acyl chains
871.7	Pheophytin a
873.6	Pheophytin b
887.6	hidroxyPheophytin a
889.6	hidroxyPheophytin b
903.6	lactone
905.6	divinyll clhorophyl a

IV. Discussion

IV. Discussion

Macroalgae have been used as food ingredient since ancient times in many countries around the world, especially in Asian countries, but the interest on this organisms for other applications has grown in recent times (12,13). Macroalgae have shown to possess a wide variety of compounds that are known to be beneficial to human health, such as proteins, soluble dietary fibres, lipids, minerals, vitamins and phytochemicals, increasing their popularity as a heathy food product across the world (6,9,21,31,32). Regardless, the use of macroalgae in the food industry is just the tip of the iceberg, with the continuous investigation on their numerous constituents showing that these organisms are a prominent source of bioactive compounds that enables them to possess antioxidant, anti-inflammatory, antimicrobial, antitumor and anti-coagulant activities (18,47,95,114). This range of beneficial biological activities promotes the increased demanding of the use of these organisms in a wide array of applications, ranging from pharmaceutical and cosmetic industries, to the use on agriculture and animal feed (115,116).

Among the aforementioned bioactive compounds found in macroalgae, lipids are very important constituents of macroalgae. Phospholipids, such as PC, PG and PE are the major structural components of extra-chloroplast membranes, with PG also being located in significant amounts in thylakoid membranes (117). Glycolipids are predominately located photosynthetic membranes, and are essential to provide energy and function as markers for cellular recognition, due to their association with cell membranes (15,17). PLs and GLs are also of the utmost importance to human metabolism, mainly due to their role in cell membrane composition, energy storage capacity within cells and metabolic pathway signalling (58,117–119).

The main lipid classes present in marine macroalgae are glycolipids, phospholipids and betaine lipids, all of which are polar lipids. (2,81,89,118). Polar lipids, such as phospholipids and glycolipids, have demonstrated to possess several beneficial health effects, such as antibacterial, antifungal, antioxidative, anti-inflammatory and antitumor activities (15,16,117,120). However, is important to notice that the biological activity that this type of compounds present depends largely on their fatty acid content and structure, mainly due to omega-3 (*n-3*) FA being essential precursors of eicosanoids, that are involved in important metabolic pathways in human metabolism, such as inflammation (71,102,121).

With that in mind, it becomes clear that, with a detailed knowledge of the polar lipid profile of marine algae, it is possible to emphasize the need for the bioprospecting of the marine organisms, in order to acknowledge their role as a reliable source of lipids with important biological activities.

Following this line of thought, the present work had two major goals. The first objective was to characterize the fatty acid profile, using the GC-MS technique, of the red macroalgae *P. dioica*, which is one of the most commercially used species of red algae, mainly in the food industry (23–26). The fatty acid profile of the conchocelis phase of *P. dioica*, which is a microscopic, filamentous and diploid phase of *P. dioica* life cycle, cultivated on land-based IMTA conditions, was performed for the first time. *P. dioica* samples were cultivated in a land-based sustainable integrated Multi-Trophic aquaculture (IMTA) system, by the Portuguese company ALGAplus, sediated in Ílhavo, Aveiro. This company specializes in the production and commercialization of macroalgae cultivated in a sustainable and controlled IMTA environment. Two different batches of *P. dioica* (July 2012 and March 2014) were analysed, one harvested during the winter season and one during summer season. The second goal was to provide a detailed characterization of the polar lipid profile of *P. dioica* using the modern lipidomic approach based on HILIC-LC-MS and MS/MS. This method allows the identification and structural characterization of individual molecular species within each class of polar lipids. This lipidomic approach is a novelty in the studies on lipid content in seaweeds, with only a few reports that provide detailed insight of the lipidome of macroalgae using this method: *C. crispus* (80), *Codium tomentosum* (122) and *Ulva lactuca* (92,123). Previous polar lipid analysis of *Porphyra* species relied solely on techniques like thin-layer chromatography (TLC), that only allowed the separation and identification of the different polar lipid classes present, but is unable to provide the same level of detailed analysis of each polar lipid class (29,64,67).

Regarding the fatty acid analysis by GC-MS, we identified fifteen different fatty acids in *P. dioica* of both winter and summer seasons. In terms of saturated fatty acids (SFA), palmitic acid ($C_{16:0}$) is the most abundant fatty acid overall in both *P. dioica* samples, accounting for 52.2% of total fatty acid content (TFA) in *P. dioica* in the summer season and 45.0% of TFA in *P. dioica* of the winter season. Other SFA, such as myristic acid (C14:0), pentadecanoic acid ($C_{15:0}$) and heptadecanoic acid ($C_{17:0}$) and stearic acid ($C_{18:0}$) were also detected, but in much lower levels. The sum of SFA ranged from 48.0% of TFA in *P. dioica* form the winter

season to 57.0% of TFA in *P. dioica* of the summer season. Studies regarding the fatty acid profiling in *Porphyra* species from various proveniences show a similar SFA content, with palmitic acid being the most abundant (29,67,70). Regarding unsaturated fatty acids in *P. dioica*, they account for the majority of TFA content in the winter season (total of 55.0% of TFA combining both monounsaturated and polyunsaturated fatty acids), with long chain polyunsaturated fatty acids (PUFAs) comprising of 37.0% of TFA content. Eicosapentaenoic acid (EPA, $C_{20:5}$ *n-3*) and arachidonic acid (AA, $C_{20:4}$ *n-6*) are the most abundant PUFAs in *P. dioica*, with EPA accounting for 25.2% of TFA content. Comparing the PUFA content between both seasons, it is possible to see that in the winter season the total amount of PUFAs is slightly higher than in the summer season (37.0% in winter as opposed to 25.0% in summer). It is also important to notice that there is a significant decrease EPA in the later season, accounting for only 11.8% of TFA content, as opposed to the winter season (25,2% of TFA content). Linoleic acid ($C_{18:2}$ *n-6*), which is an essential fatty acid that cannot be synthesised in human metabolism and therefore needs to be included in the diet is also present in *P. dioica*, although in relatively low amount (3.50% of TFA content in the winter sample and 2.50% of TFA content in the summer one). The other essential fatty acid, α-linolenic acid ($C_{18:3}$ *n-3*), needed to produce *n-3* fatty acids such as EPA in the human body (72,102), was not found in these particular samples of *P. dioica*, but their absence is compensated by the high amount of EPA observed, since direct consumption of this fatty acid through diet is also recommended (59,102,119,121). These findings are, in generic terms, consistent with other reports in the literature about the fatty acid profile of *P. dioica* and other red algae (31,34,64,67,70), some even accounting for the season variability of fatty acid content (70). That said, it is important to take into consideration that the macroalgae usually employed for bioprospecting are commonly harvested from the wild, where they are subjected to different growth conditions and various biotic and abiotic factors that have an impact on their chemical composition, including their fatty acid and polar lipid contents (13). These factors lead to some variability when it come to the fatty acid profiles of macroalgae, even from the same species. As an example, *P. dioica* harvested from Japan and Korea show a slightly different fatty acid profile then *P. dioica* harvested from China, with the first possessing double the content in EPA and slightly less palmitic acid (34). The use of macroalgae cultivated under controlled conditions, using IMTA, such as the alga *P. dioica* studied in the present work, is a way to work-around the natural variability of the chemical

composition of macroalgae, obtaining large biomass yields with the supply of the desired metabolites suitable for the required needs of different applications (122).

PUFAs accounted for almost half of the total fatty acid content in conchocelis phase (47.0% of total fatty acids), which is significantly higher than in *P. dioica* (37.0% of TFA content in *P. dioica* from the winter season and 25.0% of TFA in *P. dioica* from the summer season). Arachidonic acid ($C_{20:4}$) was the most abundant PUFA in conchocelis phase (21,2 of TFA content), followed by eicosapentaenoic acid ($C_{20:5}$), that accounted for 15.5% of TFA content. Regarding saturated fatty acids, palmitic acid ($C_{16:0}$) was the most abundant in the conchocelis phase sample (22.9% of TFA content), though being significantly less abundant in the then in *P. dioica.* It is also worth mentioning the presence of the essential linolenic ($C_{18:2}$ *n-6*) and α-linolenic ($C_{18:3}$ *n-3*) fatty acids in the conchocelis phase, even though in very low amounts (4.40% and 0.36% of TFA content, respectively).

The molecular profile of polar lipids from *P. dioica* was identified for the first time using the modern lipidomic approach based HILIC-LC-MS/MS. The results obtained allowed the identification of sixty-nine molecular species from seven classes of glycolipids and phospholipids, as well as six molecular species of phytyl derivatives. Glycolipids are mostly located in photosynthetic membranes, in the thylakoid membranes of the chloroplasts (124), serving as an energy source and as markers for cellular recognition (16). Among the glycolipids identified in *P. dioica*, eighteen molecular species of SQDGs were found, containing fatty acyl substitutions from 14:0 to 20:5, whereas for DGDGs, eight molecular species were identified, containing FAs from 14:0 up to 22:3. The most abundant molecular species were SQDG (16:0/20:5) and DGDG (16:0/20:5). Interestingly, previous report concerning the glycolipids of the Rhodophyta *C. crispus* harvested in the summer season (63,80), profiled by using a mass spectrometry-based approach, appear to preferably biosynthesize DGDG and SQDG molecular species containing saturated and monounsaturated C_{16} and C_{18} fatty acids and minor molecular species including C_{20} fatty acids such as AA. In contrast, *P. dioica* contains important glycolipids from DGDG and SQDG classes that primarily incorporate *n*-3 PUFA, namely EPA. Glycolipids such as DGDG (16:0/20:5), DGDG (16:0/20:4) and SQDGs incorporating PUFAs, isolated from red algae, have recognised anti-inflammatory and antitumoral properties (15,103). One species of sulfoquinovosylmonoacylglycerol (SQMG), was also identified in *P. dioica*, bearing palmitic acid ($C_{16:0}$) as the fatty acyl substituent. SQMGs found in spinach have been

reported to possess antitumoral effect (125) and recently have been identified as well in the green macroalgae *C. tomentosum* (122). Hydroxylated FAs, or oxylipins, were also identified in the glycolipidome of *P. dioica*. This kind of metabolites occur naturally in plants and other marine organisms, as a result of the action of lipoxygenases, with an important role in defence mechanisms (126,127). Glycolipids contain molecular species with potential bioactive properties and high content of *n-3* PUFAs, thus enhancing the value of *P. dioica* as a functional food, as well as the application in the cosmetic and pharmaceutical industries (14,17,124).

Concerning the extraplastidic membrane phospholipids (PLs) from *P. dioica*, four main classes were identified and accounted for PCs (10 molecular species), LPCs (7 molecular species), PGs (22 molecular species) and LPGs (3 molecular species). PLs in *P. dioica* enclose FAs from 16:0 to 22:2 in PGs and 16:0 to 20:5 in PCs. For the lyso classes, LPG only showed to possess fatty acyl substitutions with C_{16} and C_{18}, whereas in LPC, C_{20} unsaturated acyl chains were identified, namely $C_{20:4}$ and $C_{20:5}$. Comparing with previously reported *C. crispus* (80), a lower number of classes were found in the phospholipidome of *P. dioica*. However, a huge number of PG molecular species were identified, containing PUFAs, namely C_{20} fatty acids such as AA and EPA. The beneficial effects of algae phospholipids have been recently documented, displaying antitumor, antiviral and antimicrobial activities (117,128). PLs containing *n-3* PUFAs have shown to possess anti-inflammatory activity, namely PG molecular species (103), as well as beneficial effects on cognitive functions and in alleviating senescence (117).

V. Conclusion

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Using a lipidomic-based approach employing hydrophilic interaction liquid chromatography-electrospay ionization mass spectrometry (HILIC-ESI-MS), the characterization of the polar lipid profile of the red macroalga *Porphyra dioica* (blade) cultivated in an IMTA system was carried out. The fatty acid profile of this alga, cultivated under controlled conditions on two different seasons (winter and summer) as well as its sporophyte conchocelis phase was also analysed. The results obtained allowed to conclude that:

- The on land-based IMTA cultivated *P. dioica* (blade) is rich in PUFAs, namely *n-3* and *n-6* fatty acids such as eicosapentaenoic acid ($C_{20:5}$ *n-3*) and arachidonic acid ($C_{20:4}$ *n-6*);
- *P. dioica* harvested during winter season possess, overall, a slightly higher amount of PUFAs in comparison to *P. dioica* cultivated during the summer season, especially regarding the EPA content;
- The sporophyte conchocelis phase of *P. dioica* fatty acid profile, studied for the first time, revealed to possess almost half of its total fatty acid content in the form of PUFAs (47.0% of TFA content). AA and EPA were identified as the two major PUFAs in conchocelis, accounting for 21.2% and 15.5% of TFA content, respectively;
- Sixty-nine molecular species from seven classes of phospholipids and glycolipids were identified in HPLC-ESI-MS/MS analysis of *P. dioica* (blade);
- SQDGs (18 molecular species), SQMG (1 molecular species), DGDGs (8 molecular species), PCs (10 molecular species), LPCs (7 molecular species), PGs (22 molecular species) and LPG (3 molecular species) were identified in *P. dioica* (blade), bearing fatty acids from $C_{14:0}$ up to $C_{22:3}$, with predominance of $C_{16:0}$, $C_{20:4}$ and $C_{20:5}$.
- *P. dioica* contains several molecular species of glycolipids and phospholipids with potential bioactive properties.

Overall, the results obtained in this work provide a complete and detailed analysis of the lipidome of the IMTA produced seaweed *P. dioica*, highlighting the potential use of this algae in a wide spectrum of applications, from the use as a functional food with beneficial health effects, up to the use in several industries, such as pharmaceutical and cosmetic. Macroalgae production on land-based IMTA systems has some advantages over the harvest

of wild seaweeds, allowing the production of large biomass volumes with known chemical composition, suitable to the needs for different applications.

Future work should focus on understanding the relation between the polar lipid structures and the bioactive properties that they demonstrate, in order to understand the mechanisms that allow polar lipids to display this kind of properties, further enhancing the important benefits of these compounds.

VI. References

VI. References

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