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by Mohamed Nadir Keddar

Supramolecular solvents extraction of bioactive compounds from *Scenedesmus sp*. biomass

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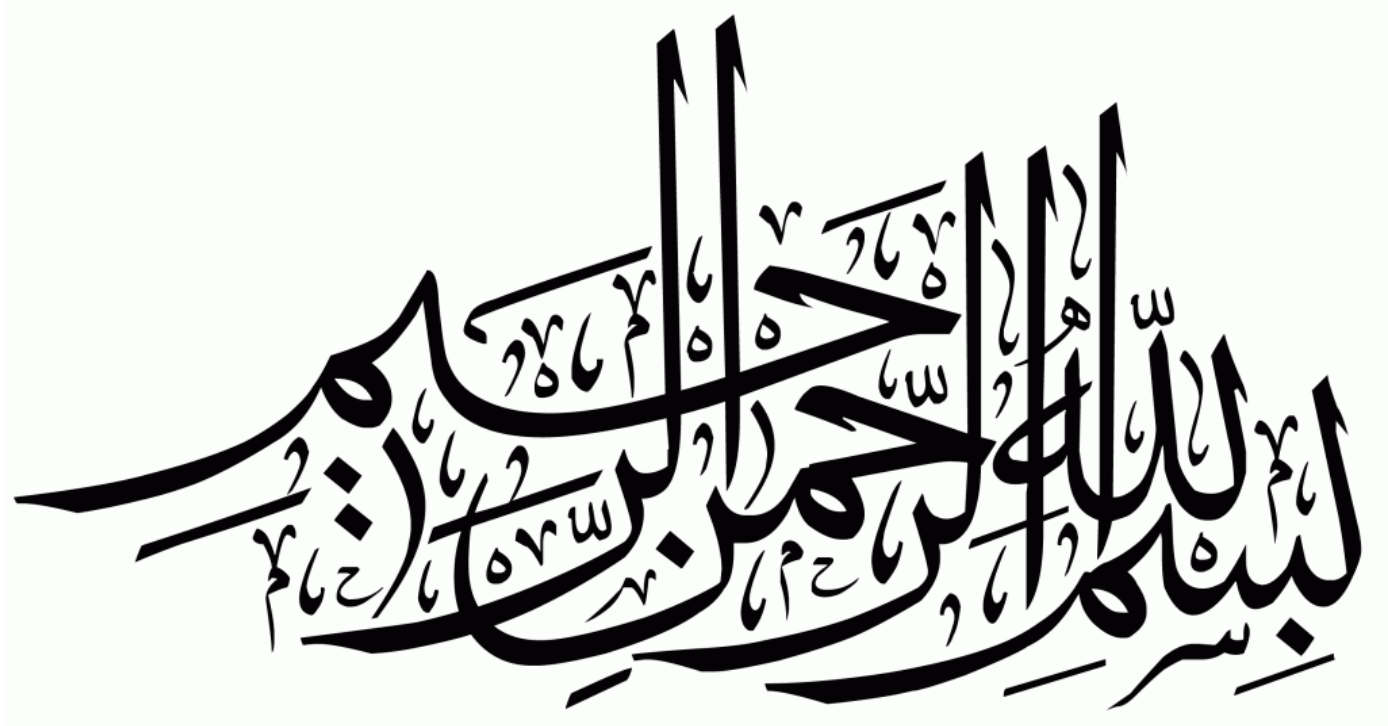
بسم الله الرحمن الرحيم

This work is

dedicated to my

family and my

friends for giving

me all the

inspiration and

support I need.

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List of Appendices

$	Dollars
%	Percent
€	Euros
µL	Microlitre
µM	Micromolar
AA	Ascorbic acid
ABTS	2,2´-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AE.g_{DB}^{-1}	Ascorbic acid equivalent per gram of dry biomass
AEAC	Ascorbic acid equivalent antioxidant capacity
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ARA	Arachidonic acid
BHT	Butylhydroxytoluene
C8	Octanoic acid
Car	Carotenoids
Chla	Chlorophyll *a*
Chlb	Chlorophyll b
cm	Centimetre
CO_2	Carbone dioxide
CP	Cloud point
d	Day
DHA	Docosahexaenoic acid
DPPH	2,2 diphenyl-1-picrylhydrazyl
dw	Dry weight
EPA	Eicosapentaenoic acid
EqS	Equilibrium solution
ESI	Electrospray ionization
ET	Electron transfer
EtOH	Ethanol
eV	Electronvolt
FA	Fatty acid
FAME	Fatty Acid Methyl Esters
g	Gram
GAE	Gallic acid equivalent
GC	Gaz chromatography
GRAS	Generally recognized as safe
ha	Hectare
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
IA	Index of atherogenicity
IC50	Median inhibitory capacity
kg	Kilogram

L	Litre
LC	Liquid chromatography
M	Molar
mg	Milligram
min	Minute
mL	Millilitre
mm	Millimetre
mM	Millimolar
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
N	Normal
NaOH	Sodium hydroxide
nm	Nanometre
ºC	Degree Celsius
pH	Potential hydrogen
PUFA	Polyunsaturated fatty acids
RAM	Restricted access materials
rpm	Round per minute
RT	Retention time
s	Second
SD	Standard deviation
SE	Supramolecular extract
SFA	Saturated fatty acid
SP	Scavenging percentage
SUPRAS	Supramolecular solvents
TAGs	Triacylglycerols
TEAC	Trolox equivalent antioxidant capacity
TIC	Total ion chromatogram
TP	Total polyphenols
UFA	Unsaturated fatty acid
USA	United states of America
UV	Ultraviolet
V	Volt
v/v	Volume per volume
yr	Year
α-ALA	Alpha linolenic acid
µA	Microampere
µm	Micrometre
$\mu mol_{trolox.eq.}\ g^{-1}$	Micromolar Trolox equivalent per gram
ω-3	Omega-3
ω-6	Omega-6

Abstract

The harvesting of natural bioactives such as lipids (polyunsaturated fatty acids PUFA) and antioxidants (carotenoids and polyphenols mainly) from algae biomass is very challenging and may determine their potential use in nutraceutical industries as food additives for human health. The present work aimed at the effective extraction of bioactives mainly antioxidants and lipids from the microalga *Scenedesmus sp.*, by means of applying innovative green solvent process prior to their incorporation as natural antioxidants in food formulations. Firstly, a supramolecular solvent was synthesized by a mixture in different proportions of three elements allowed in food, namely water, octanoic acid (C8) and ethanol, where a composition of 5:36:59% v/v/v C8:EtOH:water was found to be optimal for the simultaneous extraction of carotenoids (1.04 ± 0.07 mg/g dw) and polyphenols (10.3 ± 0.3 mg/g dw). Screening of supramolecular extracts by LC-MS revealed lutein as the major carotenoid and caffeic acid as the main phenolic compound. The antioxidant activity of the supramolecular extracts was also evaluated *in vitro* by means of the ABTS and DPPH tests, where high inhibitory values of these two radicals were recorded, showing the effectiveness of the supramolecular solvent as a natural antioxidant. On the other hand, lipid extraction was performed using a mixture of conventional solvents, after which the extract was analysed by GC-MS. Indeed, linolenic acid (C18:3) represents almost half of the fatty acid fraction of the biomass, which endorse the hypothesis of its abundance in the supramolecular solvent network due to its marked amphiphilic character. According to the reported results, SUPRAS may be approved as an effective, ecological and economical food supplement or additive with a double effect, both as an antioxidant and ω3 fatty acid enrichment material.

Keywords: Microalgae, Supramolecular solvents, antioxidants, lipids, green solvent

Résumé

L'extraction de composés naturels bioactifs à partir de la biomasse microalgale constitue un challenge vers la substitution de molécules chimiques de synthèse s'avérant néfaste pour la santé humaine. De ce fait l'objectif général de cette étude est de développer une technique innovante verte d'extraction de composés bioactifs à partir de la biomasse de la microalgue *Scenedesmus* sp. en vue de leur incorporation comme antioxydants naturels dans les formulations alimentaires. En premier lieu, un solvant supramoléculaire a été synthétisé par un mélange à différentes proportions de trois éléments autorisés en alimentation, à savoir l'eau, l'acide octanoïque (C8) et l'éthanol où une composition de 5:36:59% v/v/v C8 :EtOH :eau s'est avérée optimale pour extraire simultanément les caroténoïdes (1.04 ± 0.07 mg/g MS) et polyphénols (10.3 ± 0.3 mg/g MS). Le screening des extraits supramoléculaires par LC-MS révèle la lutéine comme principal caroténoïde et l'acide caféique comme composé phénolique majeur. L'activité antioxydante des extraites supramoléculaires a été également évaluée *in-vitro* par le biais des tests ABTS et DPPH, où de fortes valeurs inhibitrices de ces deux radicaux ont été enregistrées, montrant ainsi l'efficacité du solvant supramoléculaire en tant qu'antioxydant naturel. D'autre part, l'extraction des lipides a été réalisée par un mélange de solvants conventionnels, ensuite l'extrait a été analysé par CG-MS. En effet, l'acide linolénique (C18:3) représente presque la moitié de la fraction acides gras de la biomasse, ce qui solidifie l'hypothèse de son abondance dans le réseau des solvants supramoléculaires en raison de leur caractère amphiphile marqué. A travers les résultats obtenus, les solvants supramoléculaires développés ont montré leur preuve d'efficacité en tant qu'additifs écologique et économique à double effet, à la fois antioxydant et en même temps enrichissant en acide gras essentiel ω3.

Mots clés : Microalgues, solvants supramoléculaires, antioxydants, lipides, solvant vert

في السنوات الأخيرة، ركزت البحوث على استخدام المركبات الحيوية الطبيعية من مصادر غير تقليدية مثل الطحالب المجهرية. بيد أن استخراج هذه المركبات من الكتلة الحيوية يشكل تحديا للاستغناء عن المركبات الكيميائية الاصطناعية الضارة بصحة الإنسان. ولذلك فإن الهدف العام لهذه الدراسة هو تطوير تقنية بيئية مبتكرة لاستخراج المركبات النشطة حيويا من الكتلة الحيوية للطحالب الدقيقة (*Scenedesmus* sp.) لإدراجها كمضادات أكسدة طبيعية في تركيبات الأغذية. في هذا السياق، تم تصنيع مذيب فوق **جزيئي** من خلال المزج بنسب مختلفة من ثلاثة عناصر مسموح بها غذائيا، وهي الماء والإيثانول وحمض الأوكتانويك (C8) حيث وجد أن تركيبة 59:36:5% v/v تسلسليا كانت مثالية للاستخراج المتزامن للكاروتينات (1,04 ±0,07 ملغ/غرام) وعديد فينولات (10,3 ±0,3 ملغ/غرام). كشف الفحص الكيميائي للمستخلصات فوق الجزيئية بواسطة LC-MS عن لوتين باعتباره الكاروتينويد الرئيسي وحمض الكافيين كمركب فينوليك رئيسي. كما تم تقييم النشاط المضاد للأكسدة في المستخلصات فوق الجزيئية مخبريا بواسطة اختبارات ABTS وDPPH، حيث تم تسجيل قيم مثبطة قوية لهذين الراديكالين، مما يبين كفاءة المذيب فوق الجزيئي كمضاد أكسدة طبيعي. ومن ناحية أخرى، تم استخراج الدهون بواسطة خليط من المذيبات العضوية التقليدية، ثم تم تحليل المستخلص بواسطة CG-MS. تبين إن حمض اللينولينيك (C18:3) يمثل تقريباً نصف جزء الأحماض الدهنية من الكتلة الحيوية، وهو ما يعمل على تقوية فرضية وفرته في شبكة المذيب فوق **جزيئي** بسبب طابعه الامفيفيلي البارز. ومن خلال النتائج التي تم الحصول عليها، أظهرت المذيبات فوق الجزيئية فعاليتها المتقدمة بوصفها مضافات إيكولوجية واقتصادية ذات تأثير مزدوج، سواء مضاد للأكسدة أو في الوقت نفسه مساهمة في اثراء الأحماض الدهنية الأساسية اوميغا 3.

الكلمات المفتاحية: الطحالب المجهرية، مذيب فوق **جزيئي**، مضادات أكسدة، دهون، مذيب بيئي

Introduction

In order to satisfy increasing human demand for natural-like foods with an appreciated nutritional quality and without any toxicity, the use of synthetic components in food formulations steadily reduced during recent years. Several highly productive sources of natural chemicals have been explored at various scale: among these microalgae are viewed as a sustainable and renewable feedstock for various classes of valuable compounds including lipids, polyunsaturated fatty acids, antioxidants, proteins, carbohydrates, etc (Levasseur et al., 2020). Antioxidants are presumed to have several positive health effects, including prevention of cardiovascular disorders, of certain ageing related diseases such as Alzheimer and of certain types of cancer (Goiris et al., 2012). Besides, such chemicals play also an important role in the maintenance of food products quality by preventing lipid peroxidation and other deterioration oxidative processes (Lourenço et al., 2019). The global food antioxidants market size is estimated to be valued 1.3 billion $ in 2020 and is expected to reach a value of 1.8 billion $ by 2025, growing at a compound annual growth rate of 6.0% during the forecast period. The natural segment is the most dominant as well as fastest growing type due to the increased need for natural antioxidants in food products (MarketsandMarkets, 2020). Carotenoids and phenolics, as representative of lipophilic and hydrophilic antioxidants, are usually extracted from their matrices using different organic solvents, through procedures that require long extraction times (up to 12 h) and high energy consumption (Fattore et al., 2016). Moreover, most of used solvents for extraction are toxic or even generate pollutant residues, which may offset their use in food and nutraceuticals (Pinela et al., 2016).

The development of new analytical methods and techniques is highly advised for the simultaneous recovery of lipophilic and hydrophilic antioxidants from biomass in a cost-effective and eco-friendly way with respect to their applications in the food industry. In this respect, supramolecular solvents (SUPRAS) are nanostructured liquids

spontaneously formed by adding a coacervation-inducing agent to colloidal suspensions of amphiphiles (Ballesteros-Gómez et al., 2010). The ability of SUPRAS for efficient extraction derives from the high concentration of amphiphiles they contain (0.1-1 mg/ µL) and consequently the high number of binding sites available, and from the possibility of establishing different types of interactions (e.g. dispersion, dipole-dipole, hydrogen bonding, etc.). The capability of SUPRAS for quick extraction emanates from their discontinuous character, and consequently their high surface area, which facilitates solute mass transfer from sample to SUPRAS (Ballesteros-Gómez et al., 2010). The SUPRAS have been already proved to efficiently extract alkaloids and polyphenols from vegetal biomass such as coffee by-products (Torres-Valenzuela et al., 2020; Torres-Valenzuela et al., 2019).

Otherwise, microalgae are known also to accumulate high amount of ω-3 polyunsaturated fatty acids (PUFA) like linolenic acid (α-ALA), and thereby emerged as an alternative to oily fish which unfortunately tend to be depleted (Bhalamurugan et al., 2018). α-ALA is an essential fatty acid that have been proven to reduce cholesterol levels, prevent cardiovascular diseases and Alzheimer's, delay aging, and more (Khan et al., 2018). Also, its anticancer, antitumor and anti-inflammatory effects have been proven (Cavina et al., 2020; Li et al., 2017). In addition, α-ALA is the precursor of other long-chain omega-3 fatty acids such as EPA and DHA (Punia et al., 2019). However, due to their great sensitivity, a considerable part of PUFA may be lost with food processing, hence a special care should be taken while handling fatty food.

In this thesis work we aimed to develop an efficient supramolecular solvents (SUPRAS) method for simultaneous extraction of carotenoids and phenolic in *Scenedesmus sp* microalgae biomass. We hypothesize that SUPRAS have the potential to extract efficiently and quickly both types of antioxidants on the basis of their valuable

intrinsic properties. Moreover, we explore the capacity of these SUPRAS to harvest the lipidic fraction especially the ω-3 linolenic acid (C18:3). By the way, we estimate the total lipid content gravimetrically, then we evaluate the fatty acid profile of the lipidic extract for the detection and quantification of PUFA (and other FA) by chromatographic techniques (*i.e*. gas chromatography (GC) coupled to mass spectrometry).

This manuscript is divided into five chapters:

The first chapter is devoted to collating valuable information about microalgae including their upstream and downstream processes for bioactives production with special focus on carotenoids, polyphenols and lipids.

In the second chapter we develop an innovative technique for simultaneous extraction of lipophilic and hydrophilic antioxidants from *Scenedesmus sp*. by supramolecular solvents. Thereby, SUPRAS composition and extraction parameters were optimized for maximum yield of total carotenoids and phenolic content.

The third chapter describes the antioxidant profile of supramolecular extracts resulting from the optimal extraction condition. Accordingly, major antioxidant compounds of each class were identified by characteristic transitions in LC-MS/MS.

The fourth chapter consists to evaluate *in vitro* the antioxidant activity of SUPRAS extracts (SE) in order to assess their ability to be incorporated as GRAS antioxidant additive in food formulations.

Finally, the last chapter elucidate the extraction of the lipidic fraction by conventional solvents and the determination of the total lipid content. In addition, the fatty acid profile was analysed by GC-MS in order to detect and quantify linolenic acid content.

Chapter I:

Literature review

I.1. Definition

The term algae (originated from the Latin word "alga") designate a diverse group of photosynthetic organisms possessing chlorophyll a and a thallus not differentiated into roots, stems, and leaves (Siu & Reese, 1953). This word defined initially only macroscopic seaweeds; then microscopic microalgae were also incorporated. The latter occur mainly as single or grouped (in colonies) cells with large diversity of sizes (few microns 0.2–2.0 μm) and shapes (coccoid, filamentous, flagellate…etc) (Siu & Reese, 1953). Such cells appear either with a nucleus plus typical membrane-bound organelles as in green, red, brown algae and diatoms (eukaryotes), or even lacking a membrane bound nucleus in the case of blue-green algae or cyanobacteria (prokaryotes) (Figure I.1) (Friedl et al., 2012).

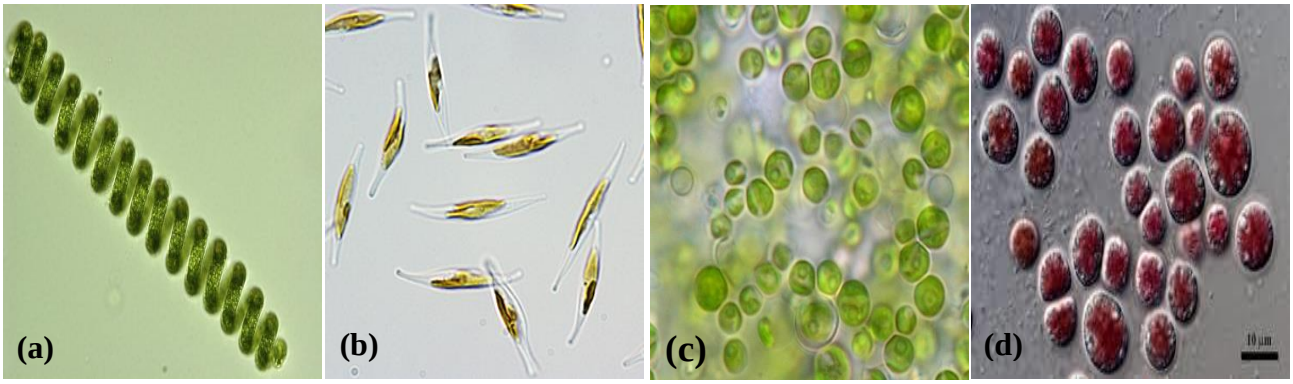

Figure I.1: Some microalgal species: (a) *Spirulina platensis* (Cyanobacteria), (b) *Phaeodactylum tricornutum* (Diatom), (c) *Chlorella vulgaris* (Chlorophyceae), *(d) Porphyridium cruentum* (Rhodophyceae).

Microalgae species form an ubiquitous group mostly aquatic, but few can grow on land (terrestrial), or also as an epiphyte, endophyte, and as well as in extreme conditions (Andersen, 2013). For centuries, microalgae have been recognized as the basis of the food chain in aquatic ecosystems (Christaki et al., 2015). They are capable of photosynthetically capture, fix and transform carbon dioxide into organic compounds,

thereby, they are responsible for at least 32% of global photosynthesis and nearly half of the atmospheric oxygen (Safi et al., 2014; Suresh et al., 2015). To date, there are approximately between 350,000 and 1,000,000 estimated species of microalgae representing different cellular structures ordered in different phylogenetic lineages, among which less than 10% (ca. 30,000) have been described and analysed (Andersen, 2013). The three most important classes of microalgae in terms of abundance are the diatoms (Bacillariophyceae), the green algae (Chlorophyceae), and the golden algae (Chrysophyceae) (Venkatesan et al., 2015).

I.2. Microalgae cultivation for bioactives production

Algal cultivation is advantaged over terrestrial crops in terms of phototrophic efficiency, higher productivity and process sustainability, which make microalgae the perfect organism utilized as next generation feedstock for valuable products such as lipids and antioxidants (Chen et al., 2012). Commercial large-scale production of microalgae started in the early 1960s in Japan with the culture of *Chlorella* as a food additive, which was followed in the 1970s and 1980s by expanded world production in countries such as USA, India, and Australia (Borowitzka, 2013).

Currently, about a dozen of microalgae species including the eukaryotic algae *Chlorella vulgaris*, *Dunaliella salina*, *Haematococcus pluvialis* and the cyanobacterium *Arthrospira platensis* are being extensively cultivated at an industrial scale for commercial use in foodstuff and Human nutrition (see Table I.1) (Cuellar-Bermudez et al., 2015; Goiris et al., 2015). The general requirements for successful microalgal cultivation include light (autotrophic and mixotrophic), carbon, macronutrients such as nitrogen, phosphorus, magnesium and several micronutrients (species dependant). Furthermore, additional factors of salinity, pH-value, turbulence, temperature and

presence of other microorganisms can decisively affect cellular growth and product formation (Liu et al., 2011).

Table I.1. The main microalgae produced in the world for food purposes (Pulz & Gross, 2004; Spolaore et al., 2006)

Microalgae	Production tonnes/ year	Principal producers	Key bioproduct	Applications
Spirulina	3000	China, USA, Myanmar, India, Japan	Biomass Phycocyanin	Human nutrition, cosmetic, pharmaceutic
Chlorella	2000	Taiwan, Germany, Japan	Biomass Carotenoids	Human nutrition, Aquaculture
Dunaliella salina	1200	Australia, USA, Japan	β-carotene	Human nutrition, cosmetic, pharmaceutic
Haematococcus pluvialis	300	USA, India	Astaxanthin	Human nutrition, cosmetic, pharmaceutic

The main cultivation technologies available for commercial production of algal biomass are open ponds and closed systems called “photobioreactors”.

I.2.1. Open pounds systems

This cultivation system is widely used in algae farming due to its relatively low financial and energy inputs and its simple configuration. Open ponds can be divided into natural waters (lakes, lagoons, ponds) and artificial ponds. Currently, four open pond systems are used in microalgae farming, namely raceway ponds, shallow big ponds, circular ponds and paddle wheel ponds (Figure I.2). The application of open ponds for microalgae production brings with it a high risk of contamination from other

microorganisms (e.g., bacteria, fungi or predators) and the low cell density that is possible to achieve. Therefore, only some microalgae species can be cultivated in open systems, for example: *Dunaliell*a with high salt tolerance, *Spirulina* with high pH tolerance and *Chlorella* and *Scenedesmus* with high nutrition demands (Singh & Dhar, 2011). The operating parameters of open pond systems are not easy to control because the effect of the local climate will have a large impact, such as temperature, light intensity, wind and others (Rocha et al., 2003).

Figure I.2: Photos of open ponds for algae farming: (a) raceway ponds at Cyanotech algae farm (Hawaii, USA), (b) circular ponds at Aqualia All Gas (Sevilla, Spain), (c) paddle wheel ponds at Biorizon Biotech (Almeria, Spain).

I.2.2. Closed culture systems

To overcome the drawbacks of the open ponds system, three types of closed photobioreactors have been proposed such as tubular, flat panel or column types (Figure I.3). In these systems, microalgae are grown either photoautotrophically and mixotrophically using external light supplying, or even under heterotrophic conditions. The main advantages of photobioreactors are the ease to control culture parameters such as temperature and pH, with no risk of contamination from other microorganisms, and high productivity. Tubular and Flat panel bioreactors offer high photosynthetic efficiencies due to their large surface area for illumination while column type has poor

light efficiency but gives a very good mixing of the culture, and high gas transfer rates (Acién et al., 2017).

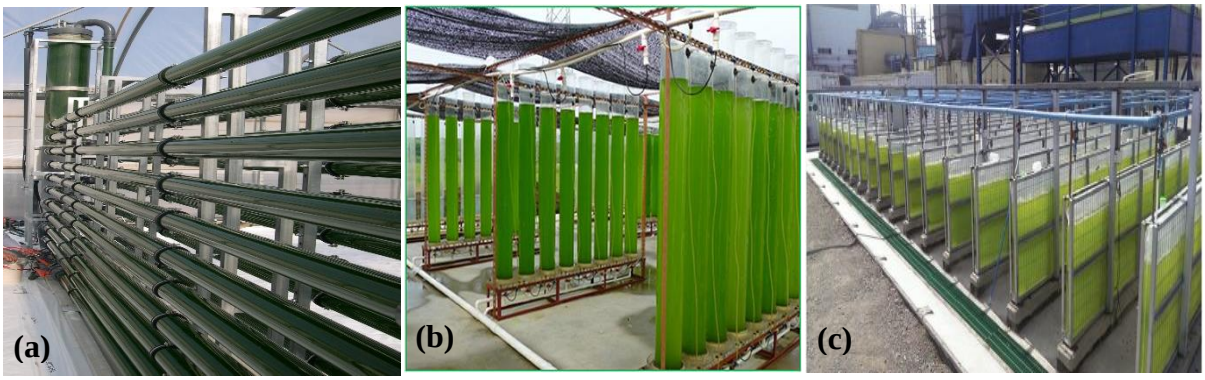

Figure I.3: (a) Horizontal tubular serpentine photobioreactor (Almeria, Spain), (b) Vertical bubble column photobioreactor (Guangdong, China), (c) Flat panel photobioreactor (Tocopilla, Chile).

Recently, in economical approach, hybrid systems combining open ponds and photobioreactors have emerged (e.g.., floating photobioreactors) to reduce cultivation process costs (Norsker et al., 2011; Zittelli et al., 2013).

Basically, *Scenedesmus sp*. is recognized among the most frequently used microalgae species to be suitable for commercial cultivation, due to their ease of culture, lack of toxicity, high nutritional value, and presence of digestible cell walls to make the biocomponents available (Borowitzka, 2013).

I.2.3. Morphological and physiological features of *Scenedesmus*

The genus *Scenedesmus* assembles the plankton of freshwaters belonging to the order *Sphaeropleales* of the family *Scenedesmaceae* which form occasionally dense populations not typically regarded as nuisance growths (Guiry et al., 2014). Molecular sequence analyses link the ancestry of *Scenedesmus* to that of the coenobial taxa *Hydrodictyon* and *Pediastrum* (Graham & Wilcox, 2000).

It is a very common and sometimes abundant genus specially found in eutrophic and hypertrophic waters including standing and running waters, lakes and ponds (Bellinger & Sigee, 2015). It is also recognized for its universal adaptability to various environment including saline conditions in salt lakes and estuaries, where salt levels range from low (brackish waters) to high levels as well as in waste waters (Okcu, 2019). As a matter of fact, *Scenedesmus* is widely used as biomonitor to assess the nutrient or toxin status of natural fresh waters (Graham & Wilcox, 2000).

Scenedesmus forms usually non-motile flat coenobial colonies of 4, 8, or 16 linearly arranged cells (Fig.I.4), but sometimes one- or two-celled forms occur. Cells are cylindrical in shape and have rounded or pointed ends. Typically, terminal cells are often ornamented with short spines and tufts of chitinous hairs or bristles (up to 200 μm long) that are believed to reduce settling in the water column or to deter herbivores, or to space the algae for optimum light and nutrient availability (Barsanti & Gualtieri, 2014). Each of the more than 100 described species is characterized by distinctive cell size, cell shape, and wall ornamentation patterns (Alam et al., 2019).

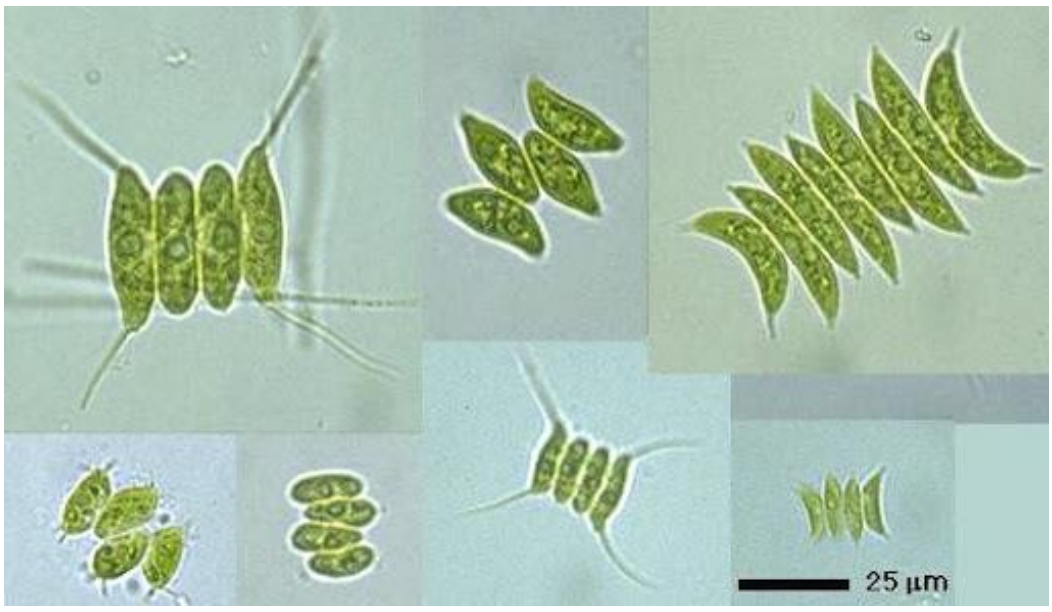

Figure I.4. Morphological diversity of *Scenedesmus* species

Scenedesmus is among the most common members of freshwater green algae that have fossil records, containing algaenans (fatty acid polymers) and also some silicon in the cell wall which may play an important role in adhesion of cells into (Gelin et al., 1997). The cell wall of *Scenedesmus* is made of three layers (Figure I.5): an inner cellulosic layer delimiting individual cells, a thin middle algaenan-based layer, and an outer pectic layer joining the cells into coenobium (Mathad et al., 2001).

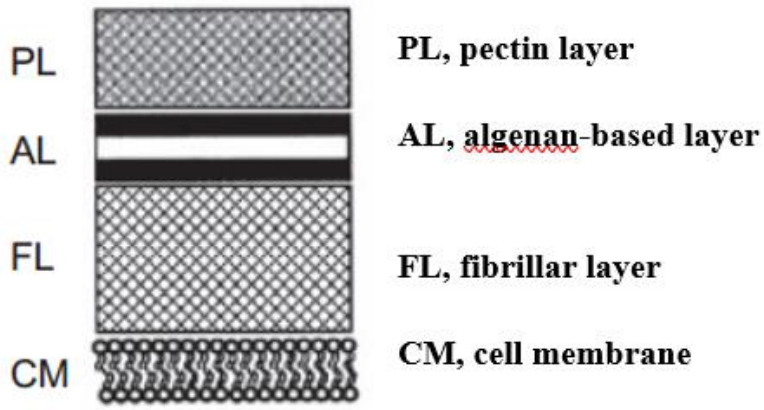

Figure I.5. Schematic view of cell wall structure of *Scenedesmus quadricauda* (Blsalputra & Weier, 1963)

Each *Scenedesmus* cell contains a single laminate plastid with pyrenoid (which are uninucleate) used to concentrate CO_2 around RuBisCO to decrease oxygenase activity and subsequent photorespiration (Mackinder et al., 2017; Zhang et al., 2019). For instance, high CO_2 fixation ability and biomass productivity were observed for *Scenedesmus sp*. reaching a consumption rate of 408.9 mg L^{-1} day^{-1} with 217.5 mg L^{-1} day^{-1} biomass productivity (Yoo et al., 2010).

Besides, *Scenedesmus* is characterized by high asexual reproduction rate achieved by autocolony formation without the involvement of flagellate zoospores (Sandgren, 1988). Parental cells divide to form non-flagellate cells that align themselves laterally, though often curled tightly within the confines of the parental wall. Cleavage occurs after

at least four nuclei have been produced by successive mitoses (Vuuren et al., 2006). Autocolonies are released by breakdown of the parental wall. A single colony is capable of producing as many autocolonies as there are coenobial cells (Graham & Wilcox, 2000).

All these characteristics make *Scenedesmus* highly suitable for mass production of high added-value biomass.

I.2.4. Mass production of Scenedesmus for commercial uses

The *Scenedesmus* own a huge tolerance to wide-ranging culture conditions and contamination along with fast growth and reproductivity. Therefore, many strains of the genus are widely exploited for large-scale outdoor production. It has been reported that this kind of specie is able to convert 15% to 25% of atmospheric CO_2 into a variety of valuable compounds (Ho et al., 2010).

Fast-growing species of *Scenedesmus* have demonstrated a strong ability to be cultivated in photobioreactors and even in pond systems because these algae will outgrow most of their competitors under optimal growth conditions (Mandal & Mallick, 2009). The mass culture of *Scenedesmus* was initiated in Germany in the 1960s, using both small circular and also paddle wheel mixed raceway ponds, and in the 1970s, Germany carried out several projects using raceway cultures in India, Peru, and Egypt (Weissman & Nielsen, 2016). For instance, the company NOVAgreen (GmbH, Germany) is producing *Scenedesmus* in 4600 L modular units made of 144 V-shaped sleeve (Polyethylene bags) reactors hung on a metal frame, which are bubbled with an air: CO_2 mixture (CO_2 originates from biogas) and placed under a greenhouse that is heated during winter (Zittelli et al., 2013). The produced biomass is directed to the cosmetic, food, or pharmaceutical markets, however several bottlenecks of low surface/volume ratio,

biofouling, and the need for a very large number of units for large-scale production are limiting the application of this low-cost system (Zittelli et al., 2013).

Thin layer photobioreactor is among the first large-scale research facilities for mass microalgae production (Šetlík et al., 1970). This system is now restricted to cultures of *Scenedesmus* and *Chlorella*, owing to their high growth rates, and good resistance to a large range of temperatures and to shear stress. The main advantage of these systems is the use of well-mixed, dense microalgae culture (10 g L^{-1}) in a relatively thin layer (5 cm) on a plane surface fitted with transversal baffles which offers optimal light absorption, good mixing, and efficient gas exchange (Zittelli et al., 2013).

A pilot-scale system composed of ten 2.8 m^3 horizontal serpentine photobioreactor units is used for production of lutein-rich biomass of *Scenedesmus almeriensis* (Fernández-Sevilla et al., 2010). Each unit occupies a surface area of about 50 m^2 consisting of 20 m long with 9 cm diameter Plexiglas tubes running in a fence-like structure. The system is fully automated, including medium preparation and culture suspension circulation. The annual average lutein productivity is 2.2 kg ha^{-1} d^{-1}, with potential oil production of about 16 t ha^{-1} yr^{-1}. The biomass production cost in this plant was estimated to be around €25 kg^{-1} (Fernández-Sevilla et al., 2010).

I.2.5. Potential utilizations of *Scenedesmus* biomass for human nutrition

Scenedesmus is recognized among the thousands of microalgae forms to be amenable for food technology (Prasad & Gupta, 2007). The valuable biochemical contents of this strains make it more prevalent in food supplements and nutraceuticals. Indeed, the genus has been shown to accumulate ~40% of dry weight in protein or until 50% in lipids under nitrogen starvation (Table I.2; Zhang et al., 2019). In addition, *Scenedesmus* contain carotenoid pigments, vitamins, polysaccharides and polyphenols

that are used in various fields as health food and human nutrition (Bulut, Akın, et al., 2019). Hence, *Scenedesmus* have long been used for treatment of a wide range of diseases and pathological conditions such as Malnutrition, cancer, and tumours (Borowitzka, 2013).

The main factor that can contribute to the nutritional value of *Scenedesmus* species biomass is biochemical composition, which is intrinsically linked to environmental, physicochemical and nutritional conditions (light, temperature, and pH), nutrient availability, and presence of other microorganisms such as coliforms (Abdel-Raouf et al., 2012).

Table I.2. Gross biochemical composition of different species of Scenedesmus

Biochemical composition (%)	***Scenedesmus sp.*** [a]	***Scenedesmus dimorphus*** [b]	***Scenedesmus obliquus*** [b]	***Scenedesmus quadricauda*** [b]
Proteins	47-56	8-18	50-56	47
Carbohydrates	10-17	21-52	10-17	
Lipids	2-14	16-40	12-14	1.9
Nucleic acids	3-6		3-6	

Adapted from: a (Borowitzka, 2013) , b (Guedes et al., 2015)

Scenedesmus biomass may also be considered as a multicomponent system, which is generally more effective to be exploited in food and human nutrition (Gouveia et al., 2008). Among *Scenedesmus* bioactives, carotenoids and phenolics represent potent antioxidants usually extracted from algal biomass with potential applications in food, nutraceuticals, and pharmaceutical formulations (Goiris et al., 2012). On the other hand, *Scenedesmus* has also been proposed as potential feedstocks for lipids especially polyunsaturated fatty acids (PUFA) production for human consumption, having

significant positive effects on heart disease prevention, anti-inflammatory activity, brain development and vision health (Borowitzka, 2013). These presumed effects may advantage the introduction of antioxidants and lipids as food ingredient or as food supplements in replacement to synthetic ones.

In this study, *Scenedesmus sp.* was studied for its lipids and antioxidants content, therefore, relatively more details are addressed in the following parts of this chapter. The term bioactives or biocompounds will refer exclusively to lipids, carotenoids and polyphenols.

I.3. Selected microalgae bioactives for food interest

I.3.1. Microalgal carotenoids

Carotenoids are red, orange, and yellow pigments produced by all photosynthetic organisms like plants and algae, also by some non-photosynthetic bacteria and fungi (Varela et al., 2015). Carotenoids are considered as exogenous chemicals for Animals and Humans since they are incapable to synthetize them (Moran & Jarvik, 2010), so they should be supplied from food or diet. They are lipid-soluble substances that are absorbed with fats and enter the circulation in the blood bound to different lipoproteins (Christaki et al., 2015; Lordan et al., 2011). Globally, more than 700 carotenoids exist, and an annual bioproduction of 100 million tonnes makes carotenoids among the most wide spread and largest groups of pigments in nature (Ngamwonglumlert et al., 2020). Chemically, they occurred mainly for two forms, :*i*) carotenes which are hydrocarbon chains such as α-carotene, β-carotene and lycopene, and *ii*) their oxygenated derivatives named xanthophylls such as lutein and astaxanthin (Mulders et al., 2014; Priyadarshani & Rath, 2012). The basic carotenoid structure is lycopene, a C40 conjugated polyene chain built

up of eight C_5-isoprene units and consisting of conjugated double bonds to which is attributed two isomers cis and trans (Novoveská et al., 2019).

The polyene chains of carotenoids are responsible for the pigmentation of carotenoids and their ability to absorb photons in visible wavelengths (León et al., 2007). Carotenoids are labile compounds with high sensitivity to light, oxygen, high pressure, heat or potentially other chemical components, a trait that restrict their storage and handling capacities (Liu et al., 2014).

I.3.1.1. Occurrence and role in green algae

Microalgae accumulate between 8–14 % of carotenoids in their biomass depending on the species and the culture conditions (Mulders et al., 2014; Priyadarshani & Rath, 2012). Generally, all carotenoids produced by higher plants are also synthesized by green microalgae (ex. β-carotene, lutein, violaxanthin, zeaxanthin, antheraxanthin and neoxanthin); however, specific green algae species possess additional carotenoids such as loroxanthin, astaxanthin and canthaxanthin. Many green algae synthetize α- and β-carotene as major carotenes, while violaxanthin, astaxanthin, canthaxanthin, neoxanthin and lutein represent major xanthophylls. Zeaxanthin, loroxanthin and echinenone are however little found in species of this class (Takaichi, 2011).

From physiological point of view, these compounds are classified depending on their functions in oxygenic photosynthesis into primary and secondary carotenoids. Primary carotenoids are accumulated in the chloroplast (*i.e*, lutein) where they play an effective role in light harvesting by absorbing visible light and transferring energy to the chlorophyll, resulting in singlet-excited chlorophyll (Latowski et al., 2014). Therefore, they are considered as important structural and functional components of the photosynthetic apparatus of the cell and essential for cellular survival.

Conversely, secondary carotenoids are not suitable for photosynthesis (ex. astaxanthin, canthaxanthin …) and are accumulated in large quantities outside the chloroplast or in lipid bodies in response to stress conditions, where they exert a protective function against oxidative damage (Guedes et al., 2011). Such trend makes them very useful to preserve cells from reactive radicals, inhibit lipid peroxidation and to enhance the photosynthetic apparatus stability and functionality. Similarly, other positive effects may be ascribed to secondary carotenoids, like promoting cell membranes integrity and fluidity under high temperature or high light conditions (Guo et al., 2016).

I.3.1.2. Commercial and functional values of algal carotenoids

In microalgae, the main carotenoids of commercial interest are β-carotene followed by astaxanthin and lutein, making up almost half of the global carotenoid market (Figure I.6; Gong & Bassi, 2016).

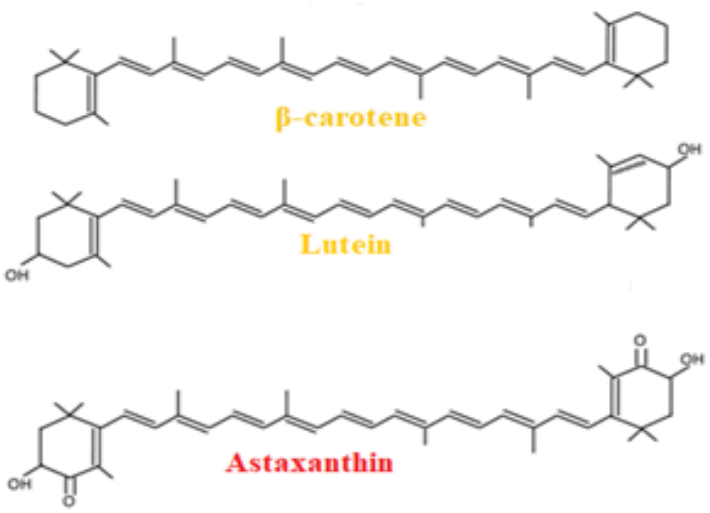

Figure I.6. Chemical structure of common commercialized carotenoids found in microalgae (Gong & Bassi, 2016).

I.3.1.2.1. β-carotene

The orange pigment β-carotene from the Chlorophyceae *Dunaliella salina* was the first carotenoid from microalgae to be commercialized in the 1980s in Australia, and the United States and expanded later to other countries including India and China (Borowitzka, 2013). Natural β-carotene is a mixture of all-trans and 9-cis isomers, which is rarely obtained in synthetic carotenoids (Dewapriya & Kim, 2014). Although it is in competition with the less-expensive synthetic form, it is often preferred because it is considered to have anticancer activity (Borowitzka, 2013). More than 90% of commercialized β-carotene is produced synthetically, however, its bioavailability is lower compared to natural β-carotene (Paniagua-Michel, 2015).

Actually, the annual production of *Dunaliella salina* reaches 1200 tonnes of biomass accumulating up to 12% of β-carotene on a dry weight basis along with smaller amounts of α-carotene, lutein, and lycopene (Spolaore et al., 2006; Taucher & Baer, 2016). Moreover, this halotolerant microalga is considered as the richest natural source of β-carotene when subjected to stress conditions such as high light intensity or nutrient starvation (over 10 %) (Emeish, 2012). The current price output of this natural product on a pure basis is valued around US $1500 per kilogram, which can vary depending on the marketing and commercial demand for the product (Gellenbeck, 2012).

Obviously, β-carotene is known for its antioxidant potential to protect the cells from damaging free radicals and to prevent the body from the risk of heart disease (Oren, 2005). It contributes also to improve the immune system and may have a preventative role in eye diseases like night blindness and cataract as a provitamin A (Dufossé et al., 2005).

I.3.1.2.2. Astaxanthin

Astaxanthin is a red-orange pigment responsible of the red-pinkish dye of aquatic fish and shrimps representing 74-98% of their total pigments (Lordan et al., 2011). The annual world market of this pigment is estimated at 200 million $, with 95% consuming synthetically derived astaxanthin (Paniagua-Michel, 2015). Ultimately, it has received extensive attention because of its strong antioxidant and natural colorant potential with high market value (2500 to 10,000 $ per kg of astaxanthin; Leu & Boussiba, 2014). The commercial production of astaxanthin is achieved by the microalgae *Haematococcus pluvialis* that can accumulate approximately 4% of astaxanthin inside their extra plastidial lipid bodies on a wet weight basis (Wayama et al., 2013). Approximately, 300 tons of *Haematococcus* biomass have been produced annually as a natural source of astaxanthin (Brennan & Owende, 2010) with production costs of 718 $ per kilogram of biomass (Li et al., 2011). According to its high stability, this secondary xanthophyll is a powerful biological antioxidant that occurs in nature, protecting membranous phospholipids and other lipids against peroxidation (Mata et al., 2010). In addition to its strongest antioxidant effect (several-fold stronger than vitamin E and β-carotene), other health benefits are also ascribed to astaxanthin such as anti-aging immunomodulatory and anti-inflammatory effects (Li et al., 2011).

I.3.1.2.3. Lutein

Lutein is a yellow primary carotenoid found in microalgae species, such as *Scenedesmus* (4.5-5.5 mg/g) and *Chlorella* (4.6 mg/g) (Plaza et al., 2009). Despite its strong antioxidant aspect and increased importance in the nutraceutical market, the commercial production of lutein remains in its infancy and set up only to pilot-scale facilitations of lutein-rich cells of *Scenedesmus sp*. and *Muriellopsis sp*. (Yaakob et al.,

2014). These species in addition to others such as *Chlorella zofingensis*, *Chlorella protothecoides* may be considered as potential lutein producers able to accumulate significant contents between 0.5–1.2 % dry weight (Cezare-Gomes et al., 2019; Chan et al., 2013).

Lutein produced from microalgae is recognized as safe for human consumption exhibiting beneficial properties for human health by preventing the development of cataracts and also preventing blindness or decrease in vision caused by age-related macular degeneration (Chiu & Taylor, 2007; Yaakob et al., 2014). Moreover it may protect people from cardiovascular diseases (Dwyer et al., 2001) and some cancers (Ho et al., 2014). It is also used as a natural colorant in the feed industry, in drugs, and in cosmetics (De Jesus Raposo et al., 2015). Global lutein market in 2016 accounted for 249.7 million $ and is expected to increase by 6.3% per year (the fastest projected growth in individual carotenoid sales), to reach 357.7 million $ in 2022 (MarketsandMarkets, 2020).

I.3.1.3. Advantages of using microalgae for carotenoids production

Currently, natural carotenoids produced from microalgae tend to be more desirable by consumers over synthetic ones due to increasing health consciousness, awareness, and ill-effects of the synthetic products. Mass production of natural carotenoids is already successfully achieved from Chlorophyceae strains, where greater amounts of β-carotene, astaxanthin, canthaxanthin, lutein were extensively harvested from *Dunaliella salina*, *Haematococcus pluvialis*, and some *Chlorella* species, respectively (Kyriakopoulou et al., 2015; Prommuak et al., 2013; Zhang et al., 2016). Microalgae have the potential to grow fast and everywhere, even in extreme conditions without competing with plants for land and water footprint. Their cultivation for carotenoids production can be carried continuously all-over the year in minimal

requirements of farming operations which may avoid carotenoids degradation related to long-term storage and excessive processing. Compared to higher plants, microalgae are viewed as the best potential reservoir for such compounds displaying voluminous contents of lutein and β-carotene for example several times higher than land plants (Ho et al., 2014; Kyriakopoulou et al., 2015). Moreover, astaxanthin is more specifically provided by microalgae since this potent active substance is seldom found elsewhere (Gong & Bassi, 2016).

Despite these perquisites, many drawbacks of high cost and low production (at most 10 % of carotenoids per dry biomass) of algal carotenoids compared to synthetic production process have limited their commercial extension to satisfy market demands in human consumption.

I.3.2. Polyphenols produced by microalgae

I.3.2.1. Definition and chemical properties

Polyphenols constitute an important class of natural antioxidants widely distributed in fruits, vegetables, cereals, plants and algae. These secondary metabolites define aromatic benzene ring compounds possessing one or more aromatic rings with one or more hydroxyl groups, including their functional derivatives (Mekinić et al., 2019). They are divided chemically according to the number of phenol rings they contain into several classes, such as phenolic acids, flavonoids, isoflavonoids, tannins, stilbenes and lignans (Haminiuk et al., 2012). Mainly, phenolic acids and flavonoids are the most abundant polyphenols in the diet accounting for one third and two thirds of the total intake respectively (Machu et al., 2015). Phenolic acids and flavonoids are usually present in their free form (Figure I.7) or bounded to sugar molecules such as lignins and other components where they form complex structures of large size and high molecular weight

(Haminiuk et al., 2012; Machu et al., 2015). In general, as being polar compounds, polyphenols solubilize very easily in highly polar solvents such as water, alcohols and acetone, however their solubility depends greatly on the degree of polymerisation (Mekinić et al., 2019).

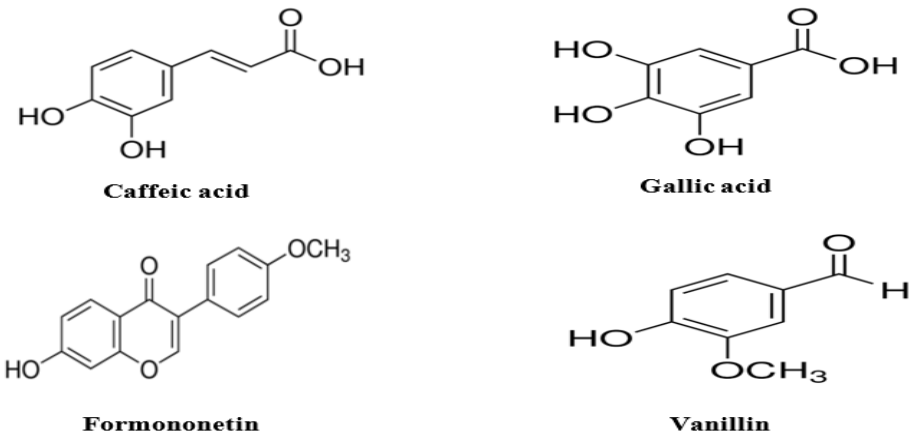

Figure I.7: Chemical structures of some phenolics in their free form including phenolic acids and flavonoids (Haminiuk et al., 2012).

I.3.2.2. Occurrence and role in algae

Commonly, phenolic compounds have been associated with plants, although their description in algae still lacking information and remain focused in macroalgae rather in microalgae. The existing data on algal phenolics include phlorotannins such as fucols, phlorethols, fucophlorethols, fuhalols and halogenated and sulfited phlorotannins found in brown algae, in addition to rutin, hesperidin, morin, caffeic acid, catechol, catechin and epigallocatechin gallate detected in some red macroalgae (Ibañez & Cifuentes, 2013). In microalgae, polyphenols have so far received little attention (Goiris et al., 2015), although only few reports on green microalgae were published bringing insight the potential of the studied species for polyphenols production (Kováčik et al., 2010; López et al., 2015; Zakaria et al., 2017). More recently, Goiris et al., (2012) indicated that the antioxidant potential of microalgae is not only determined by its carotenoid content but also other

components, including phenolics, are important contributors to overall antioxidant activity. Also, the presence of simple phenols (Klejdus et al., 2009; Onofrejová et al., 2010), as well as flavonoids in microalgae has been acknowledged, albeit at low levels (Goiris et al., 2014; Klejdus et al., 2010). Microalgae can further produce some remarkable polyphenolic antioxidant molecules such as BHT, the well-known food additive (E321), which was found in the chlorophyte Botryococcus (Babu & Wu, 2008).

Besides being secondary metabolites, algal phenolics may play a protective role of algal cells against oxidative damage initiated when the algae are exposed to biotic stress such as harmful UV radiation, nutrient availability, salinity and temperature (Montero et al., 2018). In macroalgae, phenolic compounds represent a structural components of cell wall as the case of phlorotannins in brown algae and also are involved in algal reproduction as well as in chemical defense against herbivores, bacteria, and fouling organisms (Mekinić et al., 2019).

I.3.3. Microalgal lipids

Microalgae have received increasing research interest owing to their ability to synthesize lipids not only for biodiesel production but also for functional food (Hoffmann et al., 2010; Ma et al., 2014). Lipids are important structural and functional parts of microalgae since they play roles as energy storage molecules (nonpolar lipids) or in formation of biological membranes (polar lipids) and both can account for as high as 70% of dry weight in some microalgae species (Guedes et al., 2015).

Storage lipids or neutral lipids are present mainly in form of triacylglycerides (TAGs) formed by SFA and MUFA (Figure I.8) and forming intracellular lipid droplets in the cytosol and/or in the chloroplast (Barreira et al., 2015). TAGs can be transesterified to biodiesel, that can serve as energy feedstock (Trentacoste et al., 2013). Some

microalgae also produce large amounts of lipids in the form of TAGs including *Chlorella*, *Scenedesmus* and *Dunaliella*, which the production varies with the species and culture conditions (Sharma et al., 2012). Storage lipids (TAGs) tend to accumulate in the algal cells under specific environmental stress conditions where they serve as protective mechanism to for example phosphate or nitrogen limitation/starvation and high light intensity (Minhas et al., 2016) .

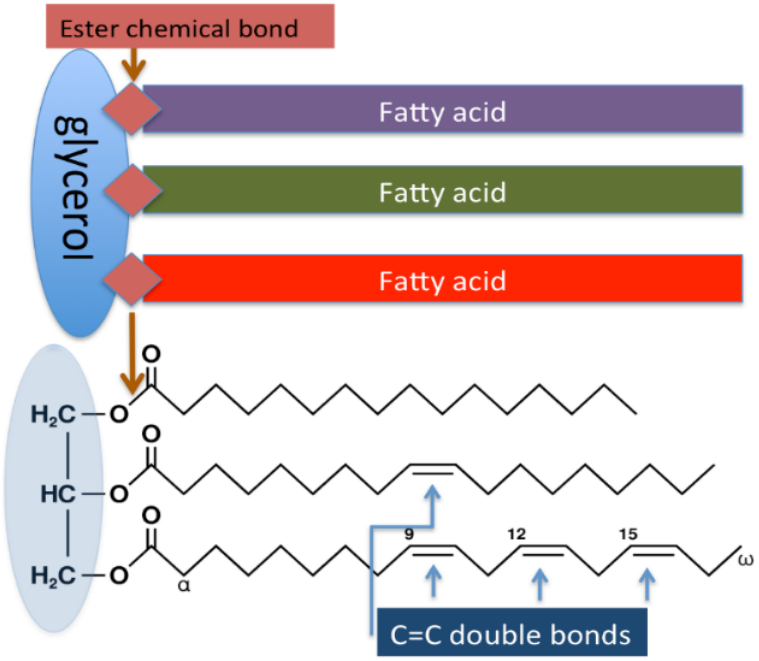

Figure I.8. Structural formula of a triacylglycerol (TAG)

Structural lipids on other hand are essential component of cell membrane having human applications (Sharma et al., 2018). The major polar lipids in microalgae are present in the form of glycerol-based phospholipids and glycolipids that are known to contain high levels of polyunsaturated fatty acids (PUFA) (Barreira et al., 2015). They are involved in membrane functionality and fluidity enabling various metabolic processes in response to environmental changes and even may act as important intermediates in cell signalling pathways (Sharma et al., 2012).

The most studied lipid compounds in algae are fatty acids which the distribution varies across microalgae strains, with C16, C18 and C20 being the majority (Chen et al., 2017). They are classified into saturated, monounsaturated and polyunsaturated fatty acids (PUFAs). The synthesis of fatty acids by algal cells is regulated by various means, such as overexpressing the genes that regulate the enzymes responsible for lipid content, increasing accessibility of the precursor molecule, such as acetyl CoA, and using inhibitors that down-regulate the catabolism of FAs to change the FA profiling through regulatory enzymes such as desaturases (Hannon et al., 2010).

PUFAs are important bioactive substances, consisting of two principal families: ω-3 and ω-6 series, with their first double bond at carbon atom number 3 and 6, respectively (Li et al., 2014). ALA (α-linolenic acid *ω*-3 C18:3), EPA (eicosapentaenoic acid, *ω*-3 C20:5), DHA (docosahexaenoic acid, *ω*-3 C22:6) and ARA (arachidonic acid, *ω*-6 C20:4) are PUFAs claimed to perform many vital functions in biological membranes and as precursors of a variety of lipid regulators of cellular metabolism (Borowitzka, 2013). They have a wide range of beneficial effects including improved heart health and reduced inflammation, thus they may decrease the risks of several diseases such as cardiovascular disorders, cancer, asthma, arthritis, and skin disorders, depression, schizophrenia and Alzheimer's and Parkinson's (Sharma & Sharma, 2017). These Multiple health benefits associated especially with omega-3 FAs have increased the demand for these ingredients in nutraceuticals and functional food industries. Thus, the demand for *ω*-3 FAs in 2020 is estimated to 241 thousand metric tons with a value of 4.96 billion $ (Market watch, 2020).

Overall, microalgae could be considered as good sources of fatty acids especially polyunsaturated fatty acid (PUFA) which the content and profile depends on the species,

environmental factors such as water temperature and irradiation (Ibañez & Cifuentes, 2013).

I.4. Solvent extraction of selected bioactives from microalgal biomass

Despite microalgae are commercially used to produce food additives and nutraceuticals, they are not commonly used directly as food to any significant extent (Chisti, 2020). Commonly, microalgae could be used as extracted biomass regardless the fact that their bioactive compounds are more stable in their natural matrix than in the extracts (Christaki et al., 2015). The main parameters driving selection of an extraction technology are biochemical characteristics of extracted molecules, scalability, rapidity, reproducibility, extraction yield, selectivity, and protection of extracted molecules against chemical transformation, dimension, cost and easiness. It is also necessary to maintenance the biological activities and integrity of the biomolecule extracted (Ventura et al., 2017).

The extraction of microalgae biomass represents a challenge due to the composition and the presence of a thick and hard cell wall. Therefore, the first step in bioactives recovery is the cell wall disruption followed by the use of organic solvents to attend the target fraction of the microalgae (Sánchez & Morales, 2015).

Cell disruption is usually performed on concentrated cell preparations (50–200 kg/m^3 dry weight) in order to efficiently extract materials from inside the algal cells with reduced cost and energy consumption (Greenwell et al., 2010). Various methods have been established for cell disruption such as mortar grinding, bead-milling, high hydrostatic pressure (HHP°), microwaves (MV), ultrasonication and/or autoclave, enzymatic lysis and Pulsed Electric Fields (PEF) (Aouir et al., 2015; Grimi et al., 2014). For instance, Cerón et al., (2008) reported that cell disruption is necessary and found out that bead mill disruption was the best option among the tested treatments to extract lutein

from *Scenedesmus almeriensis.* In addition, for solvent-based extractions, an appropriate solvent should be inexpensive, nontoxic, volatile, and highly selective to efficiently recover the target molecule from the biomass (Natarajan et al., 2015). Furthermore, the nature of the bioactive components should be considered in order to achieve good extraction efficiency. Indeed, lipophilicity or hydrophilicity affects the phytochemical solubility in solvent extraction, and conversely, solvent polarity also has an impact on the extraction efficiency (Goiris et al., 2015). For example, chloroform, methanol, n-hexane, propan-2-ol, are some of the solvents being evaluated in lipid extraction (Bermúdez Menéndez et al., 2014), while, water, organic solvent or liquefied gas, or combinations of them are used for antioxidant recovery (Guedes et al., 2013).

The most common solvent-based extractions include maceration (soaking), percolation, counter current extraction, pressurized liquid extraction, and Soxhlet are widespread technologies described to extract bioactives. These methods are reproducible and allow a rapid chemicals extraction. However, they may usually imply the use of large amounts of solvents with the risk of thermal molecular interest denaturation or transformation (Ventura et al., 2017).

Microalgal lipids extraction is usually performed with a mixture of chloroform-methanol or chloroform-methanol-water, with reference to the first conventional lipids extraction method described by Bligh and Dyer, (1959). Although, chloroform solvent due to its environmental and health risks tend to be replaced by hexane, which is supposed to be less harmful (Kim et al., 2012). Furthermore, non polar antioxidants such as carotenoids are efficiently extracted with reduced polarity solvents as acetone or hexane. For polar antioxidants such as phenolics are generally extracted using water, alcohols or a mixture water/alcohols (ex. Water/ethanol) (Assunção et al., 2017).

Combining solvent extraction with innovative and emerging methods seems to be an alternative in order to increase bioactive compounds yields extraction by saving solvent consumption and extraction times. Therefore, microwave, ultrasonic and PEF-assisted extractions have been proposed as efficient and rapid processes to extract antioxidants and lipids from microalgae with equivalent or higher extraction yields as well as low to moderate costs and negligible added toxicity (Aouir et al., 2015; Balasubramanian et al., 2011; Pasquet et al., 2011). Indeed, PEF-assisted extraction technique has been reported to be suitable for large scale bioactives recovery because it retained most of the target molecules in a suspended phase, thus reducing the use of solvent for downstream processing (Aouir et al., 2015; Sheng et al., 2012).

The use of enzymes, such as snailase (a mixture of more than 30 enzymes), cellulases and trypsine could be of interest for enhancing pigments and lipids extractability in microalgae, however digestion process costs remain to be less desired (Horst et al., 2012; Liang et al., 2012).

Bio-solvents, recognized as environmentally safer such as terpenes d-limonene, α-pinene, and p-cymene have also demonstrated to be effective as they allowed the elimination of water from the microalgae biomass, lipids extraction, and total terpene solvent recycling (Tanzi et al., 2013).

I.4.1. Supramolecular solvents in separation and extraction process

I.4.1.1. Fundamental aspects

Supramolecular solvents (SUPRAS) are nanostructured liquids generated from a spontaneous process of self-assembly and coacervation (Ballesteros-Gómez & Rubio, 2012). The phenomenon was described for the first time by the colloidal scientists Bungenberg de Jong and Kruyt in 1929, before being introduced in analytical chemistry

couple of years later as cloud point (CP) extraction process by Watanabe and Tanaka (Watanabe & Tanaka, 1978). Ultimately, supramolecular solvents were ascribed to nanostructured solvents with specific properties in order to differentiate them from ionic liquids and other organic solvents (Ballesteros-Gómez et al., 2009).

Colloidal systems are partitioned by mean of coacervation mechanism into two liquid phases, a new colloid-rich liquid phase in equilibrium with a bulk solution, which contains the amphiphile at the critical aggregation concentration (Ballesteros-Gómez et al., 2010). SUPRAS can be easily tailored by monitoring the initial synthesis conditions (environment for self-assembly and type of amphiphiles) to fulfil specific physicochemical properties of cleaning-up/extraction for analytical applications. Thus, the target analyte properties and the complexity of the matrix should be carefully considered (Ballesteros-Gómez et al., 2019).

Due to their amphiphilic character, SUPRAS show two different polarity regions providing several types of interaction and wide polarity range for the solubilization / extraction of analytes. They also offer high concentration of binding sites and high surface area due to their discontinuous character which makes them excellent extraction materials for a lot of different compounds (Ballesteros-Gómez et al., 2010; Caballo et al., 2017).

SUPRAS act as restricted access materials (RAM) that preconcentrate a target analyte in a biological matrix up to 500 times the initial concentration (typically 100-500), which permit to exclude interferences and making its assessment more accurate. Therefore, SUPRAS are considered as green sample treatment stuff (Caballo et al., 2017).

I.4.1.2. Synthesis of SUPRAS

As mentioned above, SUPRAS are spontaneously produced by self-assembly and coacervation of colloidal suspensions of amphiphilic three-dimensional aggregates

(normal or inverted micelles or vesicles) (Ballesteros-Gómez et al., 2019; Caballo et al., 2017). These processes are triggered by an external stimuli (pH, temperature change, addition of salt or of a poor solvent for the amphiphile) that diminishes the repulsion among the polar groups of the amphiphiles. This leads to the growth of the aggregates which will associate as clusters of individual droplets and finally assemble together via non-covalent bonds (ion–ion, ion–dipole, dipole–dipole, hydrogen bonding, π–π and cation–π) and reorganize into a new phase called SUPRAS or coacervate. Coacervate droplets, which stay in equilibrium with the bulk solution at the critical aggregation concentration, continue to keep their initial integrity and characteristics in the SUPRAS (Figure I.9; Ballesteros-Gómez et al., 2019).

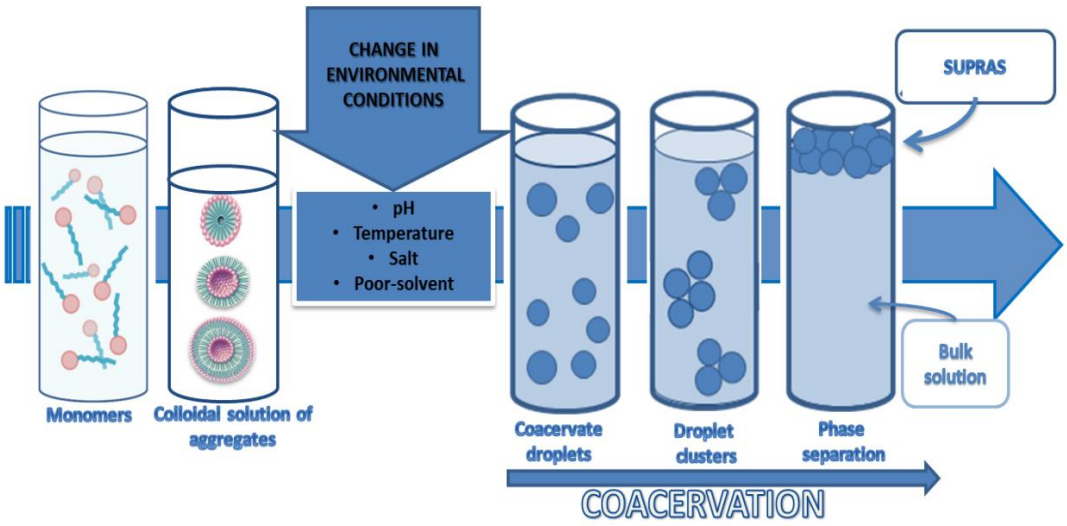

Figure I.9. General scheme of supramolecular solvents synthesis induced by self-assembly and coacervation.

I.4.1.3. Coacervation induction strategies

Coacervation is a spontaneous and reversible process that lead to the formation of three main ordered structures (*i.e.* micelles, vesicles and reverse micelles). Thereby, in aqueous colloidal solutions micelles and vesicles are preferentially formed, while in

nonaqueous colloidal solutions reverse micelles are preferred (Figure I.10; Caballo et al., 2017).

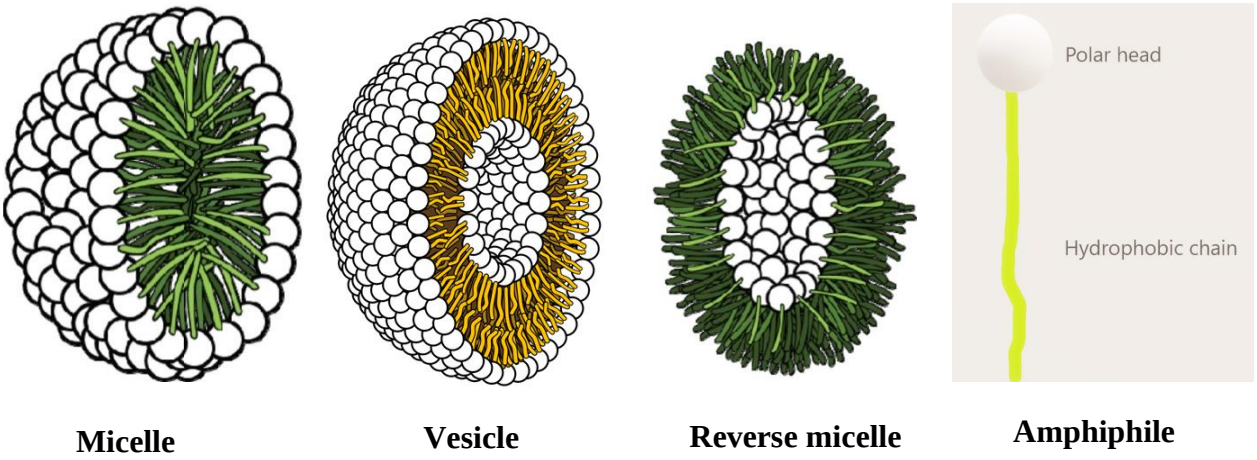

Figure I.10. Main Ordered structures occurring in colloidal solution.

Coacervation depends on the nature of the amphiphile especially the size of the polar head group and the length of the lipophilic chain, as well as the environmental conditions involved in the self-assembly like the nature of solvent forming the colloidal solution (Caballo et al., 2017). The formation of coacervate droplets is promoted by two main strategies depending on the nature of colloidal systems *i.e*. ionic or nonionic systems. Obviously, nonionic surfactants are effortless to form aggregates due to the absence of the strong repulsive electrostatic interactions that occur between the charged groups of ionic surfactants. Therefore, coacervation in nonionic colloidal systems is achieved by lowering the number of solvent molecules through the modification of the temperature of the amphiphilic solution or by the addition of a poor solvent for the amphiphilic molecules. In this way, the polar heads are reduced in size, and interactions among the neighbor micelles are facilitated. These interactions cause micellar growth and promote SUPRAS formation (Accioni et al., 2019).

Regarding ionic amphiphiles, coacervation is initiated via the neutralization of the charge by the addition of coacervating agents (ex. inorganic or organic salts or amphiphilic counterions) or by changing the pH of the solution.

Temperature is an effective coacervating agent for nonionic, zwitterionic, and mixtures of nonionic and nonionic/ionic systems. The temperature which induces turbidity and aggregation in colloidal solutions is called cloud point (CP). CP values depend on the chemical structure and concentration of the surfactant; they are inversely proportional to the hydrocarbon chain length and directly proportional to the number of oxyethylene groups of the surfactant (Samaddar & Sen, 2014). CP is also related to the ionic strength of the solution and the presence of organic compounds, in fact CP values are inversely proportional to the amount and the type of electrolyte employed to enhance the process (e.g., PO_4^{-3}> SO_4^{-2} >Br^-) and are affected positively by the addition of nonpolar organic compounds which are solubilized in the micellar core, but negatively by polar compounds, which are solubilized in the surface of the micelle (Samaddar & Sen, 2014).

Solvent-induced coacervation is usually the most proposed technique for the synthesis of SUPRAS from nonionic carboxylic acids and alkanols (Ballesteros-Gómez & Rubio, 2012; Ruiz et al., 2007). In this procedure, a poor solvent for the amphiphile (*e.g.*, tetrahydrofuran, ethanol, dioxane, methanol, etc.) is added to the colloidal solution (water is primarily used as the coacervating agent) provided that the colloidal solution solvent and the one used as coacervating agent must be miscible. The SUPRAS is produced at a relative proportion of water and organic solvent that depends on the dielectric constant of the latter and the hydrocarbon chain of the amphiphile. Water-induced SUPRAS are not affected by changes in temperature or by the presence of

electrolytes. All their outstanding properties depend on the initial synthesis composition of the properly selected ternary mixture (Caballo et al., 2017).

Acid-induced coacervation is commonly used to produce SUPRAS from colloidal solutions of ionizable amphiphiles (e.g., alkyl sulphates, sulfonates and sulfoccinates) (Casero et al., 1999). Very high acid concentrations are used (3-4M) for the coacervation depending on the structure and the pH of the solution formed (pH must be below the pKa of the surfactant) as well as the concentration of surfactant used (Jia et al., 2007).

I.4.1.4. Potential of SUPRAS for extraction purposes

Due to their discontinuous character, SUPRAS offer excellent microenvironments of different polarity that are very suitable to solubilise target compounds. Therefore, various binding sites with ionic, dipole-dipole, hydrogen bonding and dispersive interactions are efficiently implied in the SUPRAS multimodal extraction mechanism (Ballesteros-Gómez et al., 2010). In addition, the concentration of amphiphile in SUPRAS (0.1-1 mg μL^{-1}) provide a great capacity for the solubilisation of high amount of solute even using small volumes of solvent.

Regardless the amphiphile introduced in SUPRAS, the solubility of non-polar compounds can be estimated by octanol/water constants or outstandingly by the ability to form co-aggregates in the case of amphiphilic compounds. However, the amphiphile chain length and the CP should be considered in the extraction of polar compounds to obtain quantitative yields (Caballo et al., 2017). Thereby, SUPRAS are capable to solubilise efficiently non-polar analytes through dispersive, dipole-dipole and dipole-induced dipole interactions; while polar analytes are extracted trough ionic interactions, hydrogen bonding, π-cation interactions, and π-π interactions. Moreover, other polar and hydrophobic interactions like non-ionic-non-ionic and anionic-cationic are observed in

the mixed aggregates of amphiphilic compounds dispersed by SUPRAS (Ballesteros-Gómez et al., 2010).

The ability of SUPRAS to concentrate the targeted solutes is affected by diverse parameters mainly the volume of SUPRAS and the environment for coacervation. In general, the volume of SUPRAS depends linearly on the concentration of amphiphile used for coacervation, thus for concentrations below 4% high concentration factors (typically, 100-500) can be achieved (Ballesteros-Gómez & Rubio, 2012). Also, the environmental sphere including the coacervation agent may promotes SUPRAS formation and influences in the final solvent composition. For instance, the addition of salts decreases the CP, but increase salting-out in non-ionic micelles. Similarly, the volume of solvent formed in water-induced SUPRAS is exponentially dependent on the percentage of organic solvent in the synthetic solution (Caballo et al., 2013).

I.4.1.5. Formation models of SUPRAS

Regarding the nature of sample, two formats of SUPRAS synthesis can be considered: *in situ* synthesis for liquid samples or *ex situ* synthesis for solid ones. In the former, SUPRAS are generated and the solute is extracted simultaneously in sole step. Therefore, SUPRAS are spontaneously obtained by self-assembly and coacervation of the amphiphile solution added directly to the matrix under proper environmental conditions (Figure I.11).

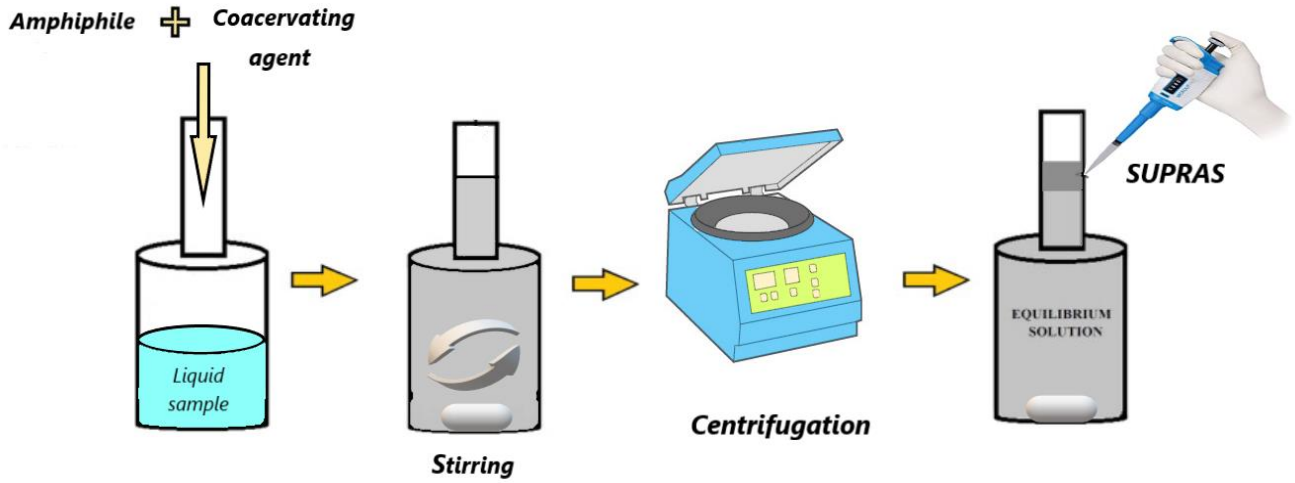

Figure I.11. *In situ* SUPRAS formation and extraction of liquid sample

In this approach, small amounts of amphiphile (0.1-2% w/v) are mixed by stirring (to enhance solute extraction) with a determined volume of sample (10-100 mL), then after phase separation (enhanced by centrifugation) SUPRAS are removed totally or just an aliquot, to be analyzed directly or after proper dilution with organic solvent (for dense SUPRAS). Typically, low density ionic surfactants-, carboxylic acid and alkanol-based SUPRAS are formed in the upper layer, and easily separated from the equilibrium solution (Caballo et al., 2017).

Concerning solid samples extraction, SUPRAS synthesis can be achieved by *in situ* or *ex situ* procedure depending on the solid matrix. By mean of the *in situ* format, the extraction is done in exactly the same way as explicated above for liquid samples. Accordingly, three phases appeared: a solid residue in the bottom consisting of the insoluble components of the sample, an aqueous equilibrium solution and the SUPRAS extract on the top. Thereby, nonpolar compounds insoluble in water can be efficiently extracted by the solvent present in the SUPRAS phase; however polar compounds may be distributed between the SUPRAS and the equilibrium solution which may alter their extraction efficiency (López-Jiménez et al., 2010). Such insight could be improved by the *ex situ* extraction procedure that require a prior synthesis of SUPRAS which is

subsequently added to the sample (Figure I.12). By the way, enough SUPRAS is synthesized (sufficient for 20–30 samples) and polar analytes are more efficiently recovered (Noelia Caballero-Casero et al., 2018).

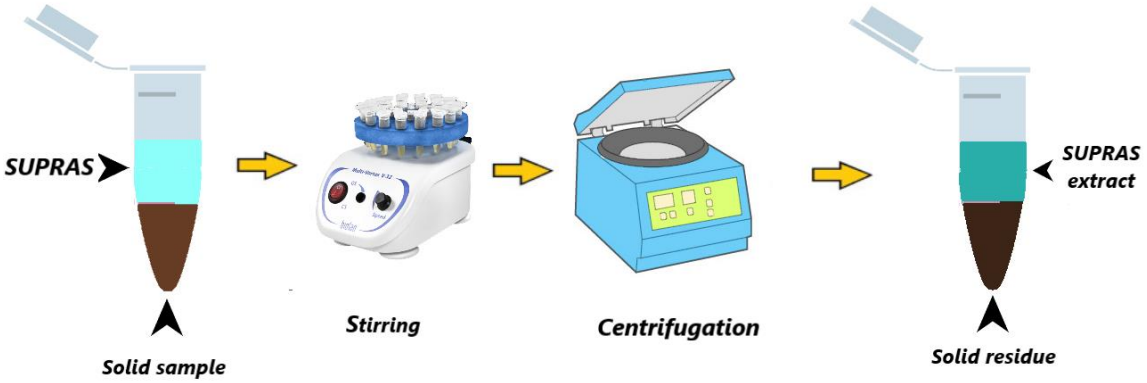

Figure I.12. Ex situ SUPRAS formation and extraction of solid sample

Additionally, to these main SUPRAS formats, other updates consisting of miniaturization and automation procedures were developed to enhance SUPRAS adaptability for the extraction of different matrices with an appreciable efficiency. Therefore single-drop microextraction (SDME) and SUPRAS of a nonionic surfactant in flow injection analysis (FIA) were successfully used for the determination of chlorophenols in environmental waters (López-Jiménez et al., 2008) and polycyclic aromatic hydrocarbons (PAHs) by LC (Li et al., 2008) respectively.

I.4.1.6. Application of SUPRAS for extraction of bioactives in food matrices

Recently, the suitability of SUPRAS for the quantitative recovery of antioxidants from food samples including microalgae and coffee by-products have been successfully tested (Salatti-Dorado et al., 2019; Torres-Valenzuela et al., 2019). Water-induced SUPRAS, formed by carboxylic acids or alkanols, have been by far the most used for the extraction purposes. This type of SUPRAS is characterized by the high concentration of amphiphiles and water they contain, which provides multiple binding interactions including hydrogen bonds and polar interactions. Thereby, better extraction of polar and

moderate polar compounds can be achieved. The applications of these SUPRAS have focused on the extraction of carotenoids and polyphenols from dry food matrices, where appreciable yields were obtained (Salatti-Dorado et al., 2019; Torres-Valenzuela et al., 2020).

I.5. Separation, identification and quantification of target bioactives

Chromatographic methods are nowadays the most suitable for the separation and purification of many phytochemicals. The main considered separation approaches for antioxidants and lipids are high performance liquid chromatography and gas chromatography, respectively.

I.5.1. Carotenoids and polyphenols profiling

The recent developed method for the separation of these phytochemical antioxidants employs C18 silica reverse column in combination with DAD and MS detection. However, for more complex samples, a C30-column seems to have better separation and selectivity than the conventional C18 material (Montero et al., 2018). Target compounds are eluted by solvent system circulating in the column with specified gradient and flow rate. Mass detection is achieved through instruments accommodated with ionization techniques mainly electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) which are recognized as the two most widely used ionization methods for antioxidant phytochemicals (Soares et al., 2019). APCI and ESI can be operated under both positive and negative ion modes. Sensitivity and selectivity of detection is critical for the identification of antioxidant phytochemicals due to complexity and diversity of structures. Therefore, additional structural information for the identification of antioxidant phytochemicals may be provided by successive

fragmentation of the molecule of interest using tandem mass spectrometry, *i.e*. two (MS–MS) or more (MS*n*) mass analyzers coupled in series (Van Breemen et al., 2012).

I.5.2. Fatty acids profiling

Analysis of fatty acids to characterize the lipidic fraction is commonly carried out by gas chromatography (GC). Owing to their low volatility, fatty acids in the extract cannot be analyzed directly in a gas chromatographer, so they are converted into low molecular weight Fatty Acid Methyl Esters (FAME) by hydrolysis and/or transesterification using either chemical or enzymatic catalysts (Takisawa et al., 2013).

FAMEs are separated into columns with polar phases such as polyethylenglycol (PEG) or cyanopropylsiloxane. Elution velocity of each methyl ester depends mainly on its volatility, which depends on the length of the carbon chain and the number and position of the molecule´s unsaturations. In general, FAMEs with less number of carbons elute faster, and those with double bonds elute slower than saturated FAMEs. Peaks can be identified based on the retention time, confirmed by mass spectrometry data (Linares et al., 2015).

According to the whole information given above, algae seem to be very useful organisms that can be applied for food and nutraceutical. *Scenedesmus* may be considered as food and nutraceutically important green microalgae and thus it is cultivated in biomass to produce large amount of valuable compounds. Among, *Scenedesmus* phytochemicals, fatty acids, carotenoids and polyphenols are potential antioxidant and food supplement with human health benefits. Their extraction, identification and quantification from the whole biomass in addition to the assessment of their bioactivity seem to be powerful approach toward their valorisation in food sector.

Chapter II: Development of green supramolecular solvent for the extraction of antioxidants from *Scenedesmus sp*.

II.1. Introduction

The development and application of novel solvents in replacement of organic solvents in analytical and industrial extraction processes, with advantageous physicochemical properties and minimal environmental and toxicological impacts, represents a challenge to which many researchers are devoting efforts. In this sense, supramolecular solvents (SUPRAS) design and synthesis is performed as environmentally friendly solvents for antioxidants recovery that should show characteristics with fulfilment of specific functions.

The extraction of antioxidants was investigated through the recovery of carotenoids and polyphenols, also optimal conditions were further assessed. These compounds are of capital interest in the pharmaceutical, cosmetic and food industries, thereby the use of SUPRAS seems to be very encouraging to preserve their safety and bioactivity.

The microalgae *Scenedesmus sp*. is rich source of natural antioxidants including carotenoids and polyphenols that are synthesised to prevent cell damage caused by oxidative stress (Ishaq et al., 2016). However, their recovery in the same time from the cellular matrix is often prone to several limitations especially for cell disintegration, solvent choice and further purification which make its efficiency process-dependant. Traditionally, chemical cell disintegration by conventional solvents such as methanol, ethanol, acetonitrile, acetone and hexane is most used for intracellular antioxidant release. Bulut et al., (2019) investigated the potential of four solvents with different polarities namely ethanol/water (3:1 v/v), ethyl acetate, hexane, and water to extract simultaneously carotenoids and polyphenols from the dry biomass of *Scenedesmus sp.* This was carried

out by sonication and shaking for more than one time and finally the solvents were evaporated. At last, neither higher antioxidants content nor strong antioxidant activities were reached suggesting that these were significantly affected by solvent polarity. Moreover, very low antioxidant activities were observed in the hexane and water extracts, also carotenoids were not detected in the latter.

Overall, the efficiency of organic solvents for simultaneous extraction of both types of antioxidants should be discussed for several reasons: *i*) the processes led to little extraction yields; *ii*) they are solvent and time-consuming; *iii*) deserve high-energy inputs especially for cell disruption and solvent evaporation, *iiii*) and they are also ecologically troublesome since that most of solvents used for extraction are toxic or even generate pollutant residues.

Recently, it has been demonstrated the capability of SUPRAS to efficiently extract, encapsulate and stabilize astaxanthin from *Haematococcus pluvialis* biomass to produce astaxanthin-rich SUPRAS oleoresins suitable for food industry applications (Salatti-Dorado et al., 2019). A high extraction yield (96 ± 7%) was obtained, including all forms of astaxanthin (free, monoesters and diesters). Thus, strong antioxidant activities and remarkable time stability were reported for SUPRAS nanostructured lipid carriers.

Similarly, the suitability of SUPRAS for the recovery of polyphenols and alkaloids from spent coffee grounds was also recently demonstrated (Torres-Valenzuela, Ballesteros-Gómez, Sanin, et al., 2019).

In order to overcome the limitations of using conventional solvent extractions, SUPRAS extraction of antioxidants were adopted in response to the following main objectives:

- Saving time, energy and algal material by one step rapid and simple microextraction procedure.
- Optimizing extraction yields by enhancing SUPRAS efficiency for both types of antioxidants.
- Achieving green and cost-effective process by selecting low-cost and food-authorized ingredients in SUPRAS formulation.

Thereby, we hypnotized that SUPRAS could present a green alternative process for the extraction of antioxidant compounds from the microalgae *Scenedesmus sp*.

II.2. Material and Methods

II.2.1. Chemicals and reagents

All chemicals used in the study were of analytical grade. Octanoic acid, *Folin-Ciocalteu* reagent and sodium carbonate were purchased from Sigma–Aldrich (Steinheim, Germany). The HPLC grade solvents methanol, ethanol and acetone were purchased from Panreac (Barcelona, Spain). Ultrapure deionized water was locally obtained through the Milli-Q purification system (Millipore, Bedford, MA, USA). The Hydrochloric acid used to prepare acidified water was also purchased from Panreac (Barcelona, Spain), whereas, Gallic acid standard was acquired from Sigma–Aldrich (Steinheim,Germany).

II.2.2. Algal material

The *Scenedesmus* strain was isolated from natural algal biofilms samples taken from wastewater treatment plant in El Viso-Villaralto (Cordoba, Spain). The obtained cultures were screened for their ability to grow in commercial fertilizer *Sportsmaster WSF*

Spring & Summer medium (see Appendix 1 for media recipe). The optimum growth rate was no more than 12.34 $g.m^{-2}.d^{-1}$.

After 15 days of cultivation in plastic tubular photobioreactors, the biomass was harvested by centrifugation, then freeze at -20 ºC and finally freeze dried. The algae powder was stored in a refrigerator in dark 500 ml containers until use.

II.2.3. Synthesis of octanoic based SUPRAS

SUPRAS of different compositions were produced by dissolving octanoic acid in ethanol and distilled water (pH ~ 3) as coacervation-inducing agent (poor solvent for the amphiphile). SUPRAS spontaneously is formed in the mixture and separated as a top layer from the bulk solution (named as EqS, equilibrium solution). The volume of the tricomponent synthetic solution was 50 mL, the octanoic acid concentration was 5% v/v, while ethanol and water varied in the ranges of 9.5-36 % and 59-85.5% v/v, respectively. Mixtures were carefully vortexed and shaken (Vortexer, Heathrow Scientific, Vernon Hills, IL, USA) for 30 s at 500 rpm and centrifuged (Mixtasel BLT, Selecta, Cham, Switzerland) for 5 min at 3,500 rpm. SUPRAS and EqS phases were then separately, collected and stored at ~25 °C, for a week in closed containers.

II.2.4. Optimization of antioxidants extraction with SUPRAS

The extraction procedures were done in 2 mL-microtubes Safe-Lock (Eppendorf Iberica, Madrid, Spain) by wetting 10 mg of lyophilized biomass with different SUPRAS volumes (and corresponding EqS) prepared according to the previous methods described in chapter I. Samples were extracted in a glass bead vortexing (3mm glass beads, 2500 rpm, 5 min) for cell wall lysis. The mixtures were then centrifuged at 10000 rpm for 30 min to achieve complete phase separation (solid sample remained at the bottom, the EqS

(if added) in the middle and SUPRAS at the top of the solution. SUPRAS extracts (SE) were collected and further, methanol was added (if needed) before analysis. The full process was conducted under dim light environment.

The experimental optimization of antioxidant extraction conditions from *Scenedesmus sp*. biomass aimed to reduce the consumption of solvent in the eco-friendly separation procedure. Thus, the latter involved the study of three variables; (1) SUPRAS composition, carried out by extracting the microalgae with SUPRAS, synthesized in different ethanol: water ratios (from 9.5 to 36% of ethanol) according to the procedure specified more above; (2) SUPRAS: EqS ratio, studied in SUPRAS range (30–100%) ; SUPRAS and EqS used as extractant and wetting phases, respectively; and (3) total volume of the extractant (0.25 to 5 mL). Total carotenoids and phenolics content in *Scenedesmus sp*. were also calculated by extracting them with acetone and methanol, respectively, according to modified conventional procedures described by Pan et al., (2018) but with higher organic solvent/sample ratio (100 mL per g of sample). The obtained values were taken as reference to calculate SUPRAS extraction yields.

II.2.5. Estimation of total carotenoids and phenolics

For total carotenoid content, SUPRAS extracts were diluted ten times with methanol and centrifuged at 10,000 rpm to remove solids and obtain clear solutions. The absorbances of diluted extract solutions were measured at 665.2, 652.4, and 470 nm (Lambda 25 UV/VIS spectrophotometer, PerkinElmer Inc., Norwalk, CT, USA) which are the major absorption peaks of chlorophylls *a*, *b* and carotenoids respectively. Concentrations were calculated according to equations 2.1, 2.2 and 2.3 (Lichtenthaler, 1987):

$$Chla(mg/L) = 16.72\ A665.2 - 9.16\ A652.4 \quad (2.1)$$

$$Chlb\ (mg/L) = 34.09\ A652.4 - 15.28\ A665.2 \quad (2.2)$$

$$Car\ (mg/L) = (1000\ A470 - 1.63\ Chla - 104.9\ Chlb)/221 \quad (2.3)$$

A variable named "relative pigment content" and referred to the ratio (total carotenoids / total chlorophylls) was calculated to assess the selectivity of the solvent for carotenoids recovery.

The total phenolic yield in SE was determined by colorimetric *Folin-Ciocalteu* method as described by Tanna et al., (2018). First, an aliquot (100 µL) of crude SE was oxidized with 0.1 N *Folin-Ciocalteu* reagent (250 µL); then, the mixture was neutralized with 0,5 mL of 20% (w/v) sodium carbonate solution and incubated in the dark at room temperature. After 90 min reaction time, the absorbance resulting to a blue colour was measured at 725 nm using the aforementioned spectrophotometer (Lambda 25 UV/VIS spectrophotometer, PerkinElmer Inc., Norwalk, CT, USA). Gallic acid was used as standard with distinct concentrations ranging from 0.008 to 1 mg mL^{-1}; the calibration curve (Abs_{725}=1.4188 TP, R^2= 0.996) served to quantify total polyphenols. Results were expressed as milligram of gallic acid equivalent (GAE) per gram dw.

II.2.6. Statistical analysis

Variables were optimized by varying each factor and results are presented as mean ± standard deviation (SD) for three individual extractions. Statistical comparisons were performed with SigmaPlot software v.11.0 (Systat Software Inc., Chicago, USA) using one-way analysis of variance (ANOVA) and Tukey's tests (p-value < 0.05).

II.3. Results and Discussion

II.3.1. Analytical and chemical properties of synthetized SUPRAS

Supramolecular solvents were synthesized from octanoic acid, ethanol and water. Alkyl carboxylic acids have been previously reported to produce SUPRAS in various hydro-organic media, including ethanol, acetone, methanol, acetonitrile, tetrahydrofuran, etc. as water miscible organic solvents (Ruiz et al., 2007), with tetrahydrofuran: water as the most used mixture for analytical purposes (Ballesteros-Gómez et al., 2019). Also, shorter chain amphiphiles usually produce SUPRAS in a wider range of conditions than acids with higher and lower hydrocarbon chain length and thereby, provides stronger hydrogen bonds for better extraction of polar and moderate polar compounds (Ballesteros-Gómez et al., 2019). The formation of SUPRAS is simple and occurs in three steps. First, octanoic acid forms a colloidal suspension of inverted micelles above the critical aggregation concentration in ethanol. Then, under addition of water, which is a poor solvent for octanoic acid and acts here as a coacervation-inducing agent, the supramolecular aggregates become bigger and form oily droplets that associate in clusters of individual droplets. Finally, the low density resulted conglomerates cream and separate as a new liquid phase called SUPRAS. These type of SUPRAS have been described as highly packed inverted hexagonal phases with the carboxylic groups surrounding internal aqueous pools and the hydrocarbon chains dispersed in the organic solvent and arranged in outer layers (see **Figure II.1**) (Ballesteros-Gómez & Rubio, 2012).

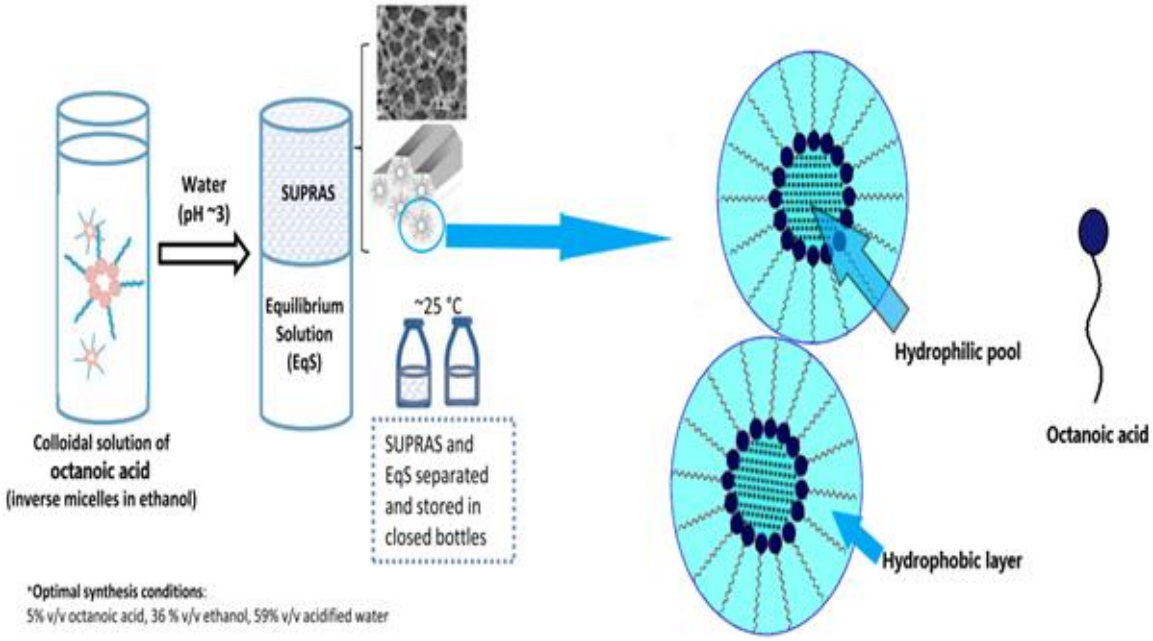

Figure II.1. General description of the synthetised SUPRAS

Due to the fact that only the protonated form of octanoic acid is involved in coacervation (pKa = 4.75), the formation of SUPRAS should be carried out in acidified media (pH < 3). The SUPRAS is immiscible with a hydro-organic equilibrium solution (EqS) that contains the amphiphile at a low critical aggregation concentration. The EqS is often used for extraction in combination with the SUPRAS phase with the aim of wetting the sample. SUPRAS components were selected on the basis of their suitability for further industrial applications (food authorized ingredients) and potential to simultaneously maximize three main binding forces expected to drive the extraction of carotenoids and polyphenols. Therefore, octanoic acid's functional groups (viz. -OH and -COOH) donate hydrogen bonds and polar interactions, while the alkyl chains establish dispersion interactions, so both carotenoids and polyphenols can be solubilized in the hexagonal nanostructures of the SUPRAS by mixed mode mechanisms, which should enhance extraction efficiency. Additionally, octanoic acid, with a saturated hydrocarbon

chain, has been previously proved to protect carotenoids against lipid peroxidation (Salatti-Dorado et al., 2019).

On the other hand, ethanol was selected to produce the colloidal suspension of the amphiphile, due to its great polarity associated to its hydroxyl (OH) group, which should allow hydrogen bonding to take place and thereby attracts polar groups. Also, the ethyl group (C_2H_5) of ethanol is non-polar and may bind non-polar compounds, hence ethanol can dissolve both polar and non-polar molecules. Moreover, ethanol is the second most used solvent after water in industrial and consumer products thanks to its low toxicity among other alcohols which make it suitable for use in food.

Both the composition and size of the aqueous cavities of inverted hexagonal aggregates of octanoic acid based SUPRAS can be tailored as a function of the environment for their formation (*i.e*. the proportion of ethanol in the synthetic solution). The volume of the SUPRAS formed is associated linearly to the amount of the octanoic acid and exponentially to the percentage of ethanol in the ternary mixture. Thus, increased volume of SUPRAS was obtained by increasing the organic solvent/water volume ratios in the synthesis at constant amphiphile concentration. As it is shown in Figure II.2, SUPRAS with progressively lower concentration of octanoic acid and higher concentration of water and ethanol will be obtained as the proportion of ethanol in the synthetic solution increases (the ethanol percentages tested correspond to those in which SUPRAS is formed).

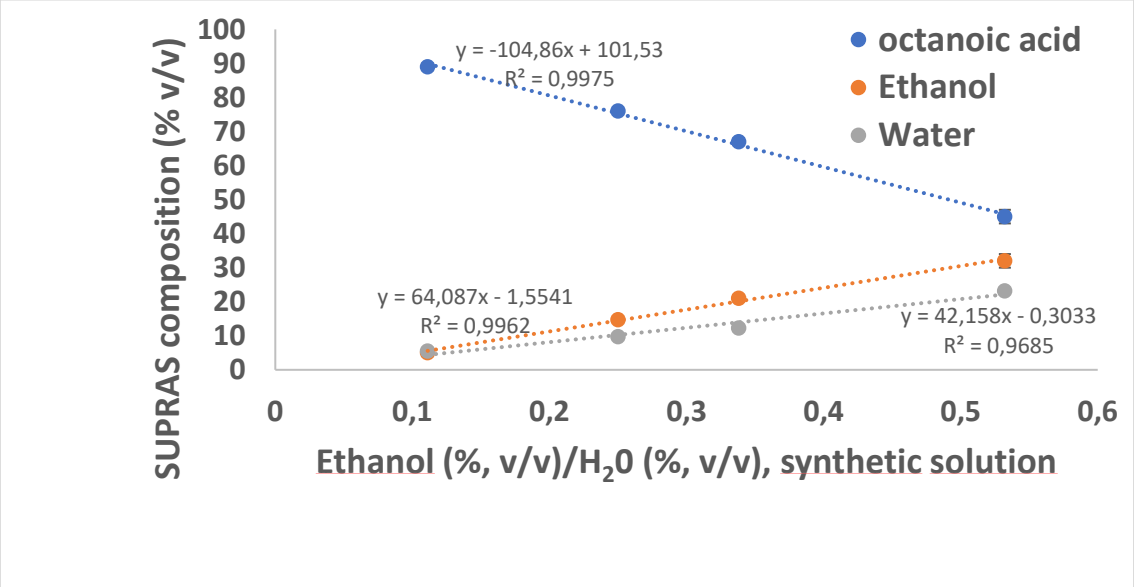

Figure II.2. SUPRAS composition (%, v/v octanoic acid, ethanol and water) as a function of the ratio ethanol/water (%, v/v) in the synthetic solution

On the other hand, the size of the SUPRAS aqueous cavities increases as the percentage of ethanol in the synthetic solution does (Figure II.3) and these vacuoles can size-exclude polar macromolecules (e.g. polysaccharides) (Ballesteros-Gómez & Rubio, 2012).

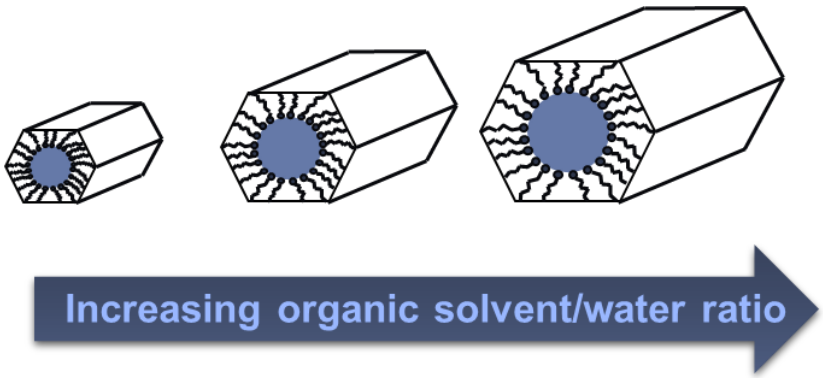

Figure II.3. schematic picture of the increase of the aqueous vacuole size in SUPRAS with the increase of the ethanol percentage in SUPRAS

In addition, proteins are expected to remain in the microalgae residue due to the formation of macromolecular complexes with octanoic acid (Caballero-Casero et al., 2015).

II.3.2. Optimization of SUPRAS based extraction of antioxidants

Optimization of the experimental conditions for extraction of antioxidants from *Scenedesmus sp*. was carried out by studying the influence of SUPRAS composition and SUPRAS:EqS ratio and volume on the recovery of carotenoids and polyphenols. In all the experiments the amount of microalgae was kept constant (*i.e*. 10 mg of dry weight corresponding to around 100 mg of wet weight). The content of carotenoids and polyphenols calculated by extraction with acetone and methanol respectively (Pan et al., 2018), were 1.38 ± 0.05 mg of carotenoids/g dw and 8.6 ± 0.9 mg of gallic acid equivalents (GAE)/g dw. These values were taken as reference for calculation of extraction yields using SUPRAS as the solvent. SUPRAS was produced at different proportions (expressed as volume percentages) of ethanol and water in the ternary mixture (C8: ethanol: water), which permitted to vary both SUPRAS composition and volume. The equilibrium solution generated in SUPRAS formation was also used in the extraction process with the aim of facilitating the dispersion of the dried biomass. Figures II.4. and II.5 represent the influence of SUPRAS composition on the extraction yield for polyphenols (expressed as GAE) and carotenoids, at a SUPRAS: EqS ratio of 30:70 (*i.e*. 0.3 mL of SUPRAS and 0.7 mL of wetting EqS) selected because of previous good results (Salatti-Dorado et al., 2019). These graphs clearly demonstrate that the amount of polyphenols and carotenoids extracted by SUPRAS decreased and increased, respectively, as the percentage of ethanol in the synthetic solution increased. Under these experimental conditions, the maximum content of carotenoids and polyphenols extracted by SUPRAS was 0.58 ± 0.06 mg of carotenoids/g dw and 10.68 ± 1.1 mg GAE/g dw. This means that SUPRAS was able to improve the yield for polyphenols obtained with

methanol, however, the yield for carotenoids was quite low (only around 42% of the content extracted by acetone).

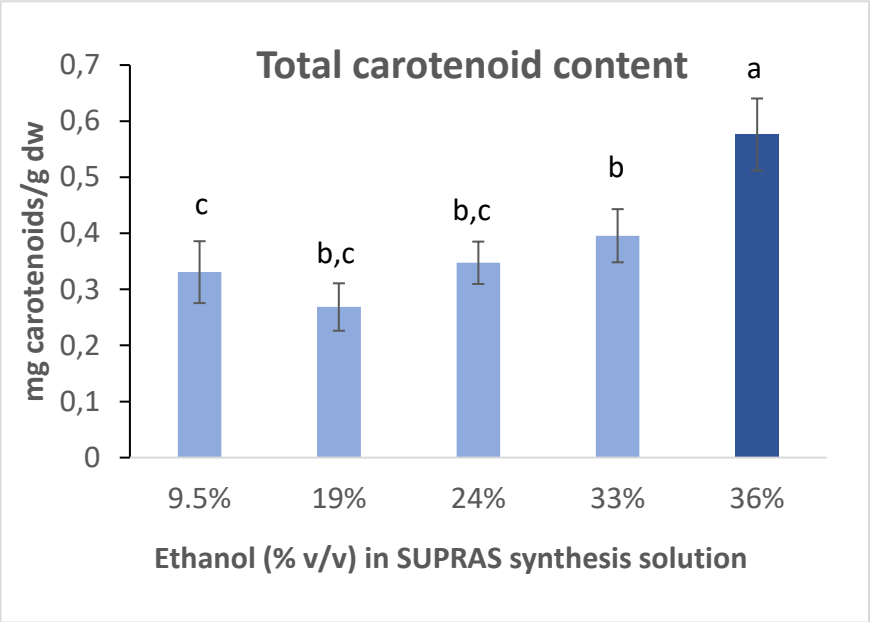

Figure II.4. Extraction yield of carotenoids under different ethanol percentages for SUPRAS synthesis (The letters on the top of the bars indicate the differences between the means as evaluated by the Tukey test; means that do not share a letter are significantly different; a for optimal values; $p < 0.05$)

The maximal extraction yield of carotenoids (*i.e.* 0.58 ± 0.06 mg of carotenoids/g dw) was significantly different for the five synthetic conditions tested, and was achieved at 36% of ethanol (see figure II.4 and Appendix 2A).

The maximal extraction yield of polyphenols (around 10.68 ± 1.1 mg GAE/g dw) was also significantly different for the employed SUPRAS (see Figure II.5 and Appendix 2B). Therefore, the synthetic condition with the lowest percentage of ethanol (*i.e.* 9.5%) presented the optimal conditions.

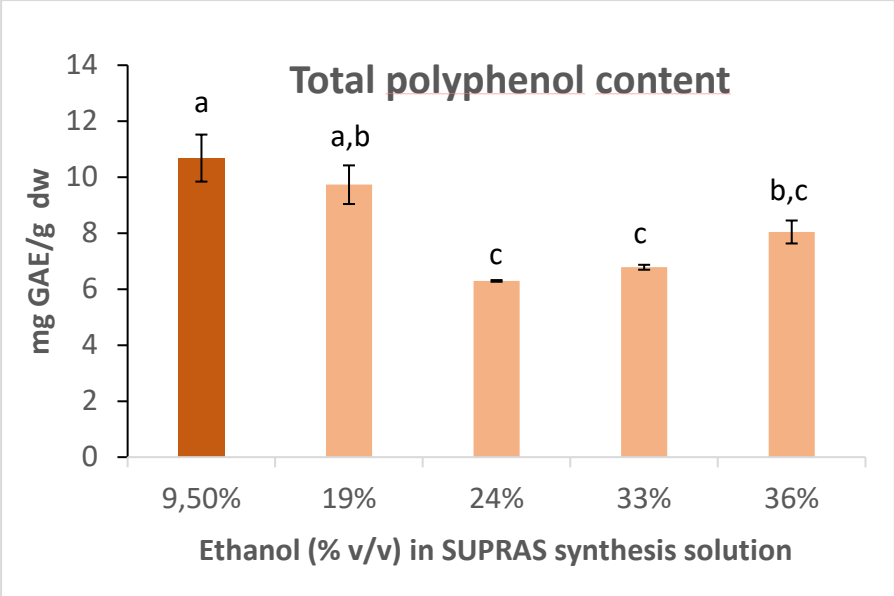

Figure II.5. Extraction yield of polyphenols under different ethanol percentages for SUPRAS synthesis (The letters on the top of the bars indicate the differences between the means as evaluated by the Tukey test; means that do not share a letter are significantly different; a for optimal values; $p < 0.05$)

The different behaviour of polyphenols and carotenoids as a function of SUPRAS composition could be a consequence of both the relative proportion of octanoic acid-ethanol-water in the SUPRAS (see Figure II.2) and the specific environment where they are expected to be solubilized according to available binding interactions. Thus, the length of the hydrocarbon chain of carotenoids such as lutein (*i.e.* the most frequently carotenoid found in *Scenedesmus* (Gilbert-López et al., 2017)) closely resembles the length of the hydrocarbon chain of two octanoic acid molecules that form the SUPRAS inner hydrophobic layers. The lutein molecule ends contain also hydroxyl groups which are hydrogen donors and acceptors, and thereby can interact with the carboxylic groups of octanoic acid oriented towards the water vacuoles of SUPRAS (see Figure II.6). Both

dispersion interactions and hydrogen bonding are suspected to be the responsible forces conducting the extraction.

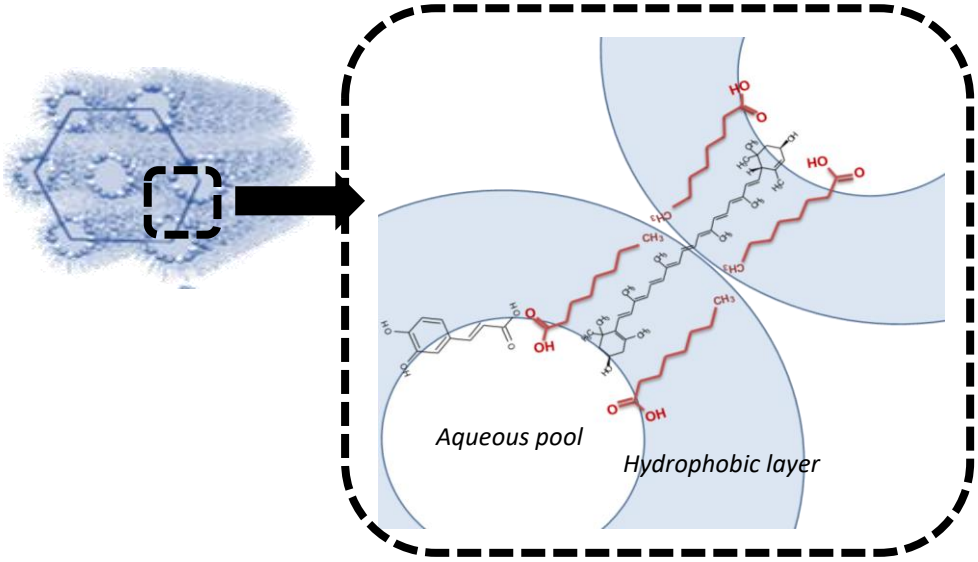

Figure II.6. Distribution of polar and non-polar antioxidants in octanoic acid based SUPRAS nanostructure.

Because of the relatively large size of polar groups in lutein, it is probable that SUPRAS with the highest vacuoles (*i.e.* those synthesized from the highest ethanol concentration in the synthetic solution, see Fig. II.3) give the best environment for lutein solubilisation, this providing the highest extraction yields (Figure II.4). On the contrary, polyphenols are expected to be solubilized near the polar groups of the amphiphile in order to establish hydrogen bonds, polar and dispersion interactions (see Fig. II.6). So, SUPRAS containing a high number of amphiphiles (*e.g.* those synthesized from low ethanol content, Fig. II.2) are expected to give the highest extraction efficiencies for these compounds (see Fig. II.5). Considering that SUPRAS synthesized from 36% ethanol were able to extract more than 90% of the polyphenols solubilized in acetone, and that they gave the best yields for carotenoid extraction, they were selected as a compromise for further optimization experiments. The composition of the resulting SUPRAS phase from

this synthesis mixture was as follows: 45:32:23 for amphiphile: ethanol: water v/v/v. Therefore, according to its high concentration of amphiphile, SUPRAS may contain a huge number of binding sites for bioactives which should enable the efficient extraction of the target compounds even at low SUPRAS/dry biomass ratios.

The influence of the SUPRAS: EqS ratio on the extraction yields obtained for polyphenols and carotenoids was investigated from 30 to 100% SUPRAS (with a total volume of 1 mL of SUPRAS + EqS) in order to know if the EqS was a suitable wetting solution. The results showed in Figure II.7 clearly highlight that SUPRAS (100 % without Eqs) was a better extractant for both types of antioxidants than the mixture SUPRAS: EqS and that it allowed to solubilize up to 10.3 ± 0.3 mg GAE/g dw and 1.04 ± 0.07 mg carotenoids/g dw. This trend could be explained by the fact that small quantities of target compounds especially those with lower polarity are lost in the EqS. The obtained yields kept constant as the SUPRAS volume decreased up to about 0.5 mL for extraction of 10 mg of dw microalga. Lower volumes caused a decrease in recoveries of both carotenoids as a result of incomplete sample dispersion.

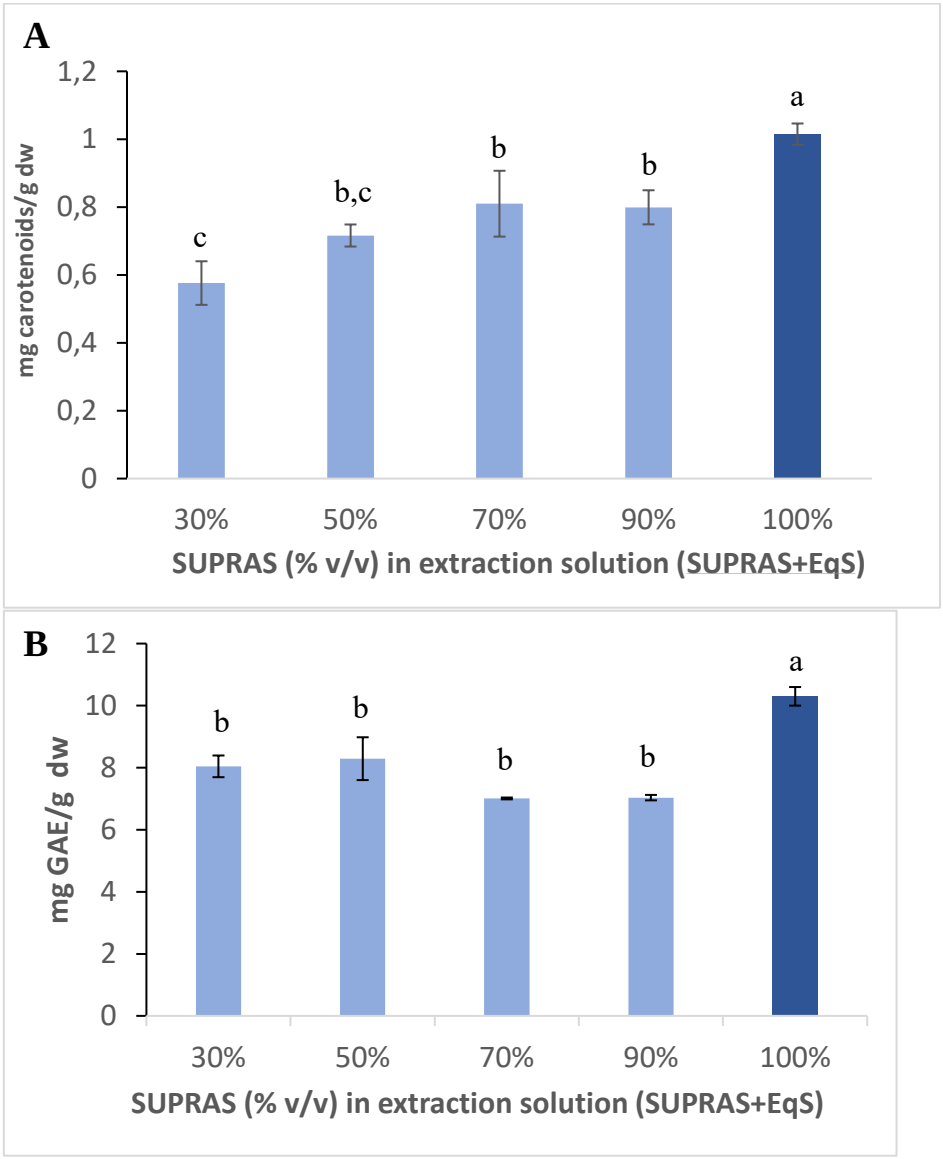

Figure II.7. Yields of polyphenols and carotenoids under different ratios SUPRAS: EqS in the extraction solution (The letters on the top of the bars indicate the differences between the means as evaluated by the Tukey test; means that do not share a letter are significantly different; a for optimal values; $p < 0.05$)

Moreover, significant differences were found in the extraction efficiencies for both antioxidants in the whole interval for SUPRAS: EqS with higher values for 100% SUPRAS, therefore this ratio was selected as optimal (see also Fig. II.7 and Appendix 3).

Overall, significantly cost-effective and energy-saving extraction of carotenoids and polyphenols was achieved in single step by employing low SUPRAS to sample ratios (*i.e*. 1 mL of SUPRAS to extract 10 mg of dry biomass) which may guarantee process sustainability and reproducibility. Unlike, conventional methods operating at higher solvent to sample ratios, high temperature, assisting energy inputs and several repetitions with pre-treatment were reported for the extraction of both antioxidants from microalgae. For instance, methanolic extracts from five microalgae species were prepared by standard solvent extraction of 1 g of lyophilized biomass with 250 mL methanol, carried out three times and finalized by using rotary evaporator and heating at 40 ºC for solvent removing (Foo et al., 2017). The process yielded significant amounts of carotenoids for three algal species, while their polyphenols contents were relatively low except for *Isochrysis galbana* (12.24 ± 1.61 mg GAE. g^{-1} dw of phenolic content). However, the methanolic extracts of the two remaining species exhibited minor quantities of both antioxidants especially the diatom *Phaeodactylum tricornutum* (Foo et al., 2017). In contrast, such process was efficiently employed by Maadane et al., (2015) who reported a two times solid−liquid extraction of dried biomass using 100 mL of three different solvents: ethanol, water or ethanol/water (1/1, v/v) for 3 hours and pre-treatment by ultrasonication for water extracts. Therein, ethanol was the best extractant of both antioxidants for the nine microalgae species studied with exceptionally small amounts for *Chlorella sp*.

II.3.3. Total carotenoids and phenolics contents

Under optimal experimental conditions (room temperature, vortexing for 5 min), SUPRAS extraction provided maximum yields of total carotenoids of 1.07 ± 0.07 mg/g dw of *Scenedesmus sp*. This value was in line with those previously reported for *Scenedesmus obliquus* (0.44 ± 0.06 mg/g dw) (Goiris et al., 2012) and *Scenedesmus sp*.

(1.11 mg/g dw g−1) (Aburai et al., 2013) cultivated under standard culture conditions. When stress conditions for the enhancement of carotenoids production were applied (*e.g*. light, nutrient starvation), the carotenoid content increased (i.e. up to 0.69% dw for *Scenedesmus almeriensis* (Sánchez et al., 2008), and between 0.61 and 2.08 % dw (Přibyl et al., 2015) and 34.2 ± 3.8 mg/g dw (Aburai et al., 2015) for *Scenedesmus sp*., which has fostered the use of different strains of this microalgae for commercial use.

In chemosystematic approach, Paliwal et al., (2016) have studied the carotenoid composition of 57 microalgae strains among them three species of *Scenedesmus sp*. that leaded to fluctuations between 0.53 and 5.57 mg/g dw.

Gilbert-López et al., (2017) investigated the use of supercritical fluid and pressurized liquid extraction as green technologies for the extraction of carotenoids from the microalgae *Scenedesmus obliquus* where record yields of 48.39 mg/g dw and 75.48 mg/g dw were achieved by supercritical carbon dioxide and pressurized ethanol extraction respectively.

It should be underlined that total carotenoids content in SUPRAS extracts could be improved by increasing sample amount and repeating the extraction process several times (data not shown).

Concerning polyphenols, their total content (TP) in SUPRAS extracts under optimal conditions reached a value of 10.3 ± 0.3 mg GAE/g dw. These yields are comparatively close to those reported for *Scenedesmus rubescens* (TP = 10.24 ± 1.06 mg GAE/g dw and 11.34 ± 0.88 mg GAE/g dw for the intracellular water and ethyl acetate extracts, respectively) (Morowvat & Ghasemi, 2016). Lower TP values have been reported for *Scenedesmus obliquus* (1.94 ± 0.16 mg GAE/g dw) in ethanol: water extracts (Goiris et al., 2012), and five *Scenedesmus* strains (from 0.7 to 3.5 mg GAE/g dw) in

methanol: water extracts (Aremu et al., 2014). The phenolic content of *Scenedesmus quadricauda* was increased by cultivation under irradiation with UV-light (up to 22 mg GAE/g dw) (Kováčik et al., 2010).

In a recent research, Bulut et al., (2019) have studied the carotenoid and phenolic contents in four different extracts of *Scenedesmus sp*. prepared from ethanol/water mixture (3:1 v/v), ethyl acetate, hexane, and water. The extraction leaded to low amounts of both antioxidants in the range 0.15 – 0.8 mg/g dw and 1.13 – 5.40 mg GAE/g dw for carotenoids and polyphenols respectively. In fact, ethanol/ water was the best solvent for polyphenols, ethyl acetate extracted maximum carotenoids that were not detected in water extracts.

II.4. Conclusion

Our results indicate that *Scenedesmus* can be a valuable source for both carotenoids and polyphenols. These two fractions were successfully extracted from *Scenedesmus* biomass using SUPRAS technology. The optimized composition of SUPRAS and the use of SUPRAS phase without EqS was responsible for improved recovery of lipophilic and hydrophilic antioxidants which is remarkably dependent on the polarity of solvent. In comparison to traditional organic solvent, SUPRAS extraction was suitable to fulfil an economic green analytical chemistry perspective by extracting interesting antioxidant compounds approved for use as food additives without any damage.

The SUPRAS technology shows great promise for scaling up because of its simplicity, no need of auxiliary energies, high temperature or time-consuming consecutive extractions and evaporation steps. For industrial application, we should keep the same

sample to SUPRAS ratio and we should scale up the process with suitable technology for mixing and for phase separation at high volumes/amounts. It is also worth mentioning that taking into account the high-water content in the SUPRAS synthesis mixture (up to 59% v/v) these solvents have a great potential for the direct extraction of wet samples without compromising the extraction yields, which could be advantageous to simplify the procedure.

Chapter III: LC-MS screening of carotenoids and polyphenols in supramolecular extracts from *Scenedesmus sp*.

III.1. Introduction

The accurate measurement of antioxidants in food even at low levels has been considered as an interesting and challenging task in the food industry. Currently, the most readily applied technique for antioxidant identification and quantification is high performance liquid chromatography (HPLC)-UV/VIS analysis which was extensively used to determine carotenoids and polyphenols in microalgae (Cerón-García et al., 2018; da Silva et al., 2017). Recently, the screening of crude extracts using LC–MS (liquid chromatography–mass spectrometry) techniques has become a powerful tool for chemical identification due to their high specificity and sensitivity, separation efficiency and structural information ability (Acero et al., 2019). Moreover, high-resolution mass detectors have been widely used for qualitative studies of several substances since they show many advantages, mainly the large amount of structure-related information based on the molecular mass and their fragmentation pattern (Ganzera & Sturm, 2018). LC-MS/APCI and LC-MS/ISI techniques were successfully applied for the screening of microalgae where important contributions regarding the antioxidant components present in these microorganisms have been attentively reported (Goiris et al., 2014; Rao et al., 2010). This suggest that LC/MS is more sensitive, specific and selective, making it particularly important for studying antioxidants obtained from microalgae, since these compounds are usually present in trace quantities and are often contaminated with biological matrices (Sivathanu & Palaniswamy, 2012).

Besides, the use of supramolecular solvents in combination with liquid chromatography (LC) coupled to UV-visible detectors (Luque et al., 2012), fluorescence (Moral et al., 2009) and mass spectrometry (MS) (Ballesteros-Gómez & Rubio, 2012) have been effectively approved. The structure and physicochemical properties of the

surfactant are critical for a successful LC separation and detection of the target analytes (Ballesteros-Gómez et al., 2019). Usually, the issued extracts from an analytical extraction with the SUPRAS can be injected directly into the LC or after a proper dilution with an organic solvent depending on the mobile phase used for elution (Caballo et al., 2013).

In this respect, we perform an LC-MS/MS analysis on the previously prepared supramolecular extracts in order to identify by characteristic transitions the major antioxidants in *Scenedesmus sp*. biomass. Particularly, carotenoids were also quantified using authentic available standards.

III.2. Material and Methods

III.2.1. Chemicals

The solvents and chemicals used in this study were of HPLC and analytical grades. Methanol, acetonitrile, acetone, and acetic acid were purchased from Panreac (Barcelona, Spain). Sodium hydroxide (NaOH) pellets were from Merck (Darmstadt, Germany). Ultrapure water was produced in a Millipore (Millipore, Bedford, MA, USA) purification system. Standards of carotenoids (astaxanthin, lutein, canthaxanthin, β-carotene) were purchased from Sigma–Aldrich (Steinheim, Germany).

III.2.2. Saponification of Supramolecular extracts

Prior to LC-MS analysis of carotenoids, and in order to simplify the identification procedure, esterified carotenoids in the supramolecular extracts were transformed in free carotenoids by saponification as described below (Hu et al., 2013). First, supramolecular extracts were diluted five times with methanol, then 1 mL aliquot was mixed with 200

µL of fresh 50 mM NaOH solution prepared in methanol. Finally, the mixture was left for 3 h at room temperature in the dark for complete hydrolysis of ester bonds.

The saponified extracts were filtrated (0.22 µm Nylon tube filter, Corning, NY, USA) to remove possible agglomerates, then stored in the freezer until injection into LC-MS system.

III.2.3. Chromatographic analysis of antioxidants

III.2.3.1. LC-MS/MS system

The antioxidants were analysed by injecting 10 µL of the saponified supramolecular extract into an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) equipped with an on-line degasser, a binary pump and a high-performance auto-sampler. The injected matrix was separated using a reverse-phase C18 column (3 C18-PFP 3.0 mm × 150 mm, 3 µm, ACE, Reading, UK). The system was coupled to an Agilent Technologies 6420 Triple Quadrupole mass spectrometer operated in two different modes (APCI and ESI) according to components to be analysed (Figure III.1). The quadrupole analyser uses the stability of trajectories in an oscillating electric field to separate ions according to their (*m/z*) ratio, also it has a unitary resolution over its entire mass range.

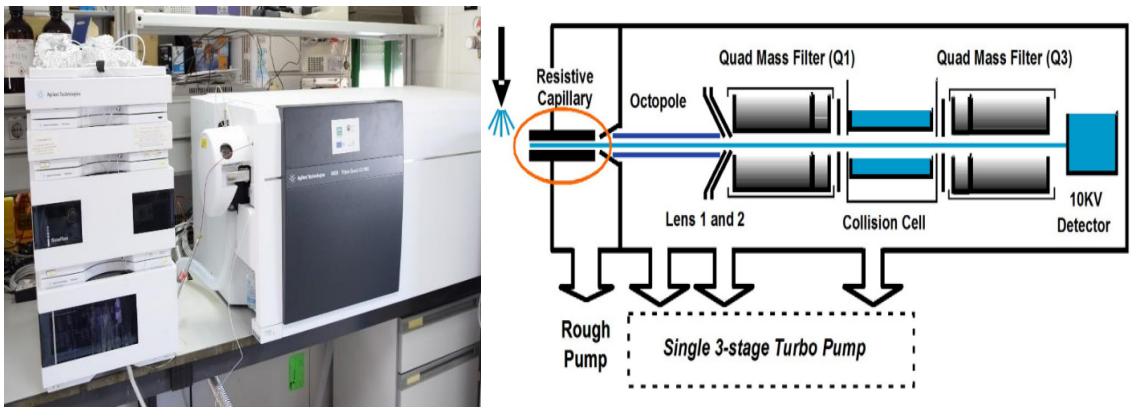

Figure III.1. LC-MS instrument for the identification of antioxidants (left) and design of the 6420 Triple Quad Mass Spectrometer (right)

III.2.3.2. Analytical conditions for LC-MS/MS analysis of antioxidants

The Triple Quadrupole mass spectrometer was operated separately in the parameters set for each type of compound (see table III.1). The binary mobile phase for the separation of carotenoids consisted of solvent A (water: methanol 1:4 v/v) and solvent B (acetone: methanol 1:1 v/v), and the following gradient method was used to separate the pigments: 25% B 0–8 min, 75% B 8–18 min, 90% B 18–23 min, 100% B 25–27 min, 25% B 27–32 min, as recommended by Cerón-García et al., (2018).

The separation of polyphenols was achieved by a mixture of two solvents: water/acetic acid (99/1, v/v) (A) and acetonitrile/methanol (50/50, v/v) (B). The gradient was started with 5% of solvent B, increasing to 30% in 25 min, 40% in 10 min, 48% in 5 min, 70% in 10 min, 100% in 5 min, isocratic at 100% for 5 min, resuming to 5% in 10 min and finishing the run in 12 min.

All the samples were analyzed by MS/MS in the multiple reaction monitoring (MRM) mode to maximize sensitivity.

Table III.1. Operational parameters for LC-MS analysis of carotenoids and polyphenols

Parameters	**Carotenoids**	**Polyphenols**
Ionisation mode	APCI	ESI
Mode charge	positive	negative
Drying gas	N_2	N_2
Drying gas temperature	350 °C	350 °C
Drying gas flow rate	4 L min^{-1}	12 L min^{-1}
Source temperature	400 °C	420 °C
Nebulizer gas pressure	30 psi	40 psi
Capillary voltage	4000 V	4000 V
Fragmentor voltage	30–140 V	30–140 V
Collision energies	1–10 eV	1–10 eV
Fragmentation Energy	1.4 V	1.4 V
Corona current	4 μA	4 μA
Scan range	100 - 1000 m/z	100 - 1000 m/z
Mobile phase flow rate	300 μL min^{-1}	1.0 mL min^{-1}

III.2.3.3. Identification of major carotenoids and polyphenols

The carotenoids were tentatively identified according to their elution order and mass spectrum matched with available standards, and mass spectrum compared to data available in the literature (Soares et al., 2019; Van Breemen et al., 2012).

The identification of polyphenols was based on spectral characteristics and MS/MS transitions as described in several previous studies (Rajauria, 2018; M. Ye et al., 2012).

III.2.3.4. Construction of calibration curves for carotenoids quantitation

The calibration curves were prepared based on external standardization method using the authentic standards dissolved in acetonitrile. Six points analytical curves were

prepared for lutein, astaxanthin, canthaxanthin and β-carotene at concentrations of 0.1, 0.2, 0.5, 0.7, 1.5, and 2.0 mg L^{-1}. Each standard solution was injected in triplicate using the above-described LC-MS program, and the mean concentration for each compound was used. The standard curves were prepared by plotting standard concentration ratio against its area value and linear regressions were also evaluated. The contents of carotenoids determined by LC-MS/MS were expressed as μg/g of freeze-dried biomass.

III.2.3.5. Data acquisition and processing

MassHunter Workstation Software Data Acquisition Version B.07.00 (Agilent Technologies, Palo Alto, CA, USA) was used for control and acquisition of all data. LC-MS/MS data processing was performed in MassHunter Qualitative Analysis (Agilent Technologies, Palo Alto, CA, USA) Software version B.07.00.

At the beginning, a database was created from parent and fragment ions of common carotenoids and polyphenols found in microalgae and reported in the literature. So that was used later as referential for the identification of antioxidants in supramolecular extracts. As a matter of fact, each peak was assigned by comparing its characteristic transitions (precursor ion → product ion) with those of the compound already listed in the database.

For carotenoids standards, peaks areas were calculated by manual integration in the MassHunter Qualitative Analysis software, and their plots and regression equations were performed in Microsoft Excel version 365.

III.3. Results and Discussion

III.3.1. Identification of major carotenoids

Generally, microalgae cells contain primary carotenoids as structural components of the photosystems, and secondary carotenoids that are accumulated under stress conditions (Goiris et al., 2015). The peak identity of supramolecular extracts analysed by LC-MS/QQQ/APCI was assigned based on comparison of the retention times and spectra with reference standards. Also, a list of common carotenoids in microalgae with their LC-MS/MS characteristic transitions was established and pinned to the instrument library with the aim to facilitate the detection an identification procedure (see Appendix 4).

It can be speculated that during the microextraction of algal biomass, esterification of almost xanthophylls prevails by octanoic acid present in SUPRAS. Thus, saponification is habitually considered before chromatography in order to precipitate fatty acids and simplify qualitative and quantitative analysis of carotenoids.

Almost, three main peaks with distinct intensities were detected at relatively higher retention times (see Figure III.2 for total ion chromatogram (TIC) and Table III.2 for spectra data with peak assignments).

One compound was identified as lutein ((3R,3′R,6′R)-β,ε-carotene-3,3′-diol) with the molecular formula is $C_{40}H_{56}O_2$ as determined by APCI-MS m/z ([M + H]-H_2O) which correspond to the removal of a molecule of water. This is consistent with the fact that the loss of a hydroxyl group or a molecule of water is characteristic of the presence of a hydroxyl group in the compound as in the case of xanthophylls (Erdoğan et al., 2015). Also, as depicted from Figure III.2, the peak of lutein was the most representative in the mass spectrum indicating that this compound was the dominant in the supramolecular extract.

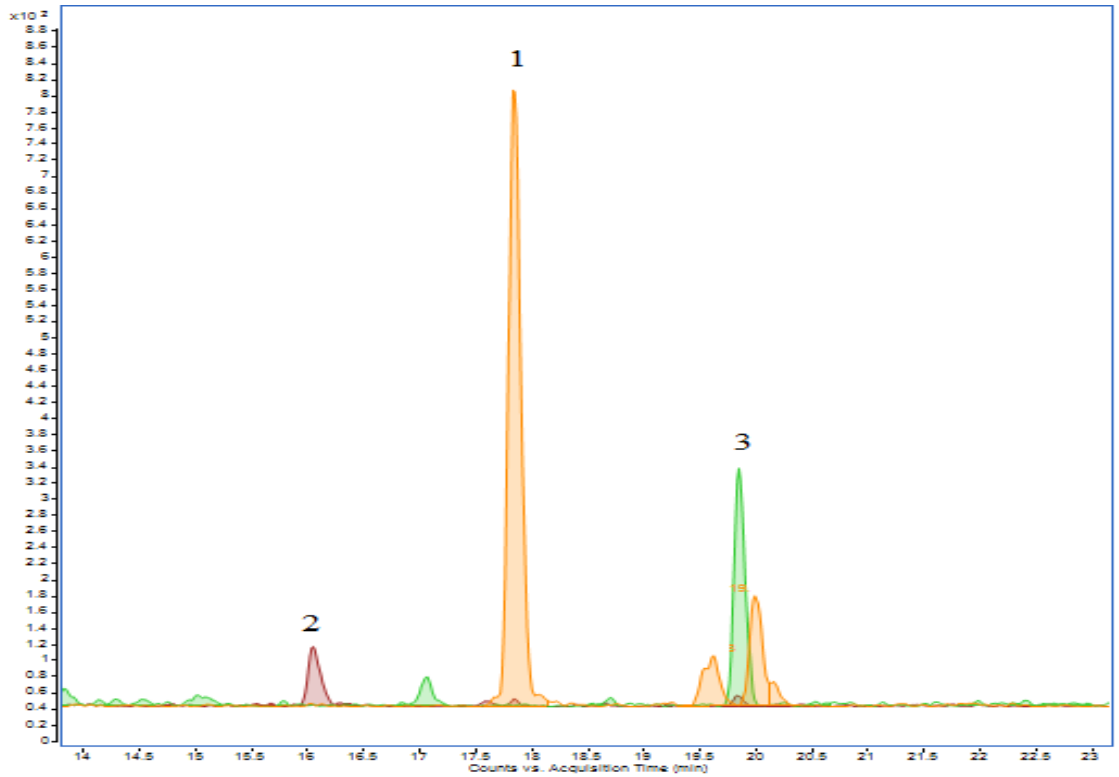

Figure III.2. Total ion chromatogram (TIC) of identified carotenoids in supramolecular extracts of Scenedesmus sp. Peak identification: (1) lutein; (2) astaxanthin; (3) unknown carotenoid resembles to canthaxanthin.

Peak 2 was positively identified as astaxanthin on the basis of comparison of mass spectra with authentic standard, showing protonated molecule at m/z 597.4, which was consistent with the molecular formula of $C_{40}H_{52}O_4$; and fragment at m/z 147.1, corresponding to dehydrated terminal ring with cleavage of the 7,8 carbon–carbon bond (Van Breemen et al., 2012). Contrarily to fact that astaxanthin is known to be the main carotenoid in *Hematococcus* species, its peak was the less abundant in the supramolecular extracts of *Scenedesmus sp.*

Furthermore, as it is shown in Table III.2, a peak at Rt = 19.8 was registered, which produced a molecular ion $[M-H]+$ at m/z 565 similar to the molecule of canthaxanthin ($C_{40}H_{52}O_2$), but neither the retention time nor the relative abundance of the monitored transitions matched the standard canthaxanthin; therefore it was assigned as

unknown carotenoid with a relative abundance comprised between those of lutein and astaxanthin. It is expected that canthaxanthin occurred at high levels in chlorophyceae species in response to stress conditions where it can play a significant protective role (Paliwal et al., 2016).

Table III.2. Tentative identification of carotenoids analysed by LC-MS/MS in supramolecular extracts of Scenedesmus sp.

Compound	RT (min)	Observed parent ion	Most abundant fragment	Peak Area
Astaxanthin	16.048	597.4 $[M+H]^+$	147.1	595
Lutein	17.84	551.4 $[M+H-H_2O]^+$	135.1	6052
Unknown carotenoid[a]	19.846	565.4	109.1	2042

[a]The transition measured for the unknown carotenoid corresponded to a secondary transition of canthaxanthin but neither the retention time nor the relative abundance of the monitored transitions matched canthaxanthin.

III.3.2. Quantification of identified carotenoids

The identified carotenoids in the supramolecular extracts were restrictively quantified using the calibration curve of pure lutein and astaxanthin (see Appendix 5 for standard curves). Since the retention time and the most abundant fragment of the unknown carotenoid did not matched those of canthaxanthin, it couldn't be quantified.

The analysis revealed lutein as the main carotenoid with substantial quantities reaching 1.1 ± 0.1 mg/g, followed by two less abundant peaks, astaxanthin (6.6 ± 2.0 µg/g) and an unidentified carotenoid (see Table III.2). These results confirm the total carotenoid content (up to 1 mg/g) measured spectrophotometrically for the supramolecular extracts (Refer Chapter 2).

Similarly, Minhas et al., (2016) revealed lutein followed by astaxanthin as the major carotenoids in various *Scenedesmus* isolates grown autotrophically and without any known stress, where their intracellular lutein contents varied from 0.26 to 2.9 mg/g dw.

In their study, Aburai et al., (2013) observed that the most abundant carotenoid in *Scenedesmus sp*. cultivated under low light intensities was lutein with a concentration of 0.60 ± 0.05 mg/g, which was doubled when stress light conditions were applied.

Other authors also reported lutein as the main carotenoid in *Scenedesmus obliquus* (Guedes et al., 2011), *Scenedesmus protuberans* (Erdoğan et al., 2015) and *Scenedesmus obliquus* CNW-N (Chan et al., 2013). Lutein content under favoured cultivation conditions varied from 2.17 ± 0.10 mg/g dw to 2.58 ± 0.08 mg/g dw in *Scenedesmus almeriensis* depending on the extraction technique, specie and growing conditions used (Sánchez et al., 2008).

The efficiency of supramolecular solvents to extract lutein in appreciable rates by one step simple procedure was more superior to other green technologies including supercritical fluids technique. Thus, the content of lutein extracted from *Scenedesmus almeriensis* by supercritical carbon dioxide oscillated between 0.0084 and 0.0466mg/g dw for the technical parameters tested, which is highly lower than the supramolecular extracts of *Scenedesmus sp*. reported in our study (Macías-Sánchez et al., 2010).

III.3.3. Identification of major phenolics

In respect to confirm the results of colorimetric assay, polyphenols in the supramolecular extract were analysed by LC-ESI-MS/MS in negative ionisation mode. However, only a qualitative analysis was considered due to the lack of phenolic compounds standards, which affected their determination accurately. As the same as carotenoids, LC-MS/MS data of common polyphenols in microalgae previously

published in some reports (see Appendix 6) were used to support compounds detection and peaks resolution in the Quadrupole mass spectrometer.

Thereby, the following phenolic compounds were tentatively identified based on the MRM transition data reported in the literature: simple phenols (phloroglucinol), simple phenolic acids (caffeic and sinapic acids and a compound that could be either p-hydroxybenzoic acid or salicylic acid (since they share the same MS transitions), a phenolic aldehyde (vanillin), a phenolic diterpene (carnosic acid) and a isoflavone(formononetin). The most intense peak was assigned to caffeic acid in agreement with its parent ion (m/z 179) and MS/MS fragment (135) (relative abundance 35%) (see Table III.3).

Table III.3. Tentative identification of polyphenols in supramolecular extracts of Scenedesmus sp.

Compound	RT (min)	Observed parent ion[M+H]$^-$	Most abundant fragment	Peak Area	Abundance[a]
Carnosic acid	53.368	331	287.1	1084	0.05
Sinapic acid	38.398	223	179	2846	0.12
Caffeic acid	1.346	179	135	8110	**0.35**
Unknown polyphenol[a]	43.841	169	125	4289	0.19
Vanillin	18.671	151	136	2968	0.13
p-hydroxybenzoic acid or salicylic acid	11.89	137	93	2570	0.11
Phloroglucinol	1.885	125	97	464	0.02
Formononetin	52.833	267	252	757	0.03

[a]The transition measured for the unknown polyphenol corresponded to gallic acid but the retention time did not match the authentic standard.

Previous studies suggested that phenolic profiles in microalgae strongly depend on the microalgae species, culture conditions and solvent used for the extraction (Rico et al., 2013). Among the few reports related to the study of microalgae polyphenols profiling, Bulut et al., (2019) demonstrated that a mixture of ethanol/water yielded six different phenolic compounds in *Scenedesmus sp.* (*i.e*. three benzoic acid derivatives (gallic–4-hydroxy benzoic–vanillic acid), two cinnamic acid derivatives (caffeic–chlorogenic acid) and a flavanol (quercetin)), which were increased to eleven by the use of ethyl acetate; however the extraction yield in the latter was almost the moiety of the former with gallic acid as the most abundant compound.

Previously, López et al., (2015) reported ten phenolic compounds in the methanolic extracts of *Dunaliella tertiolecta*, among which gentisic acid, catechin, epicatechin and chlorogenic acid were predominant (López et al., 2015). Zakaria et al., (2017) found that caffeic acid was the abundant phenolic acid in subcritical water extracts of *Chlorella sp*.

More recently, Haoujar et al., (2019) determined the phenolic profiles of four species of microalgae (*Phaeodactylum tricornutum*, *Tetraselmis suecica*, *Nannochloris sp*, and *Nannochloropsis gaditana*) which are composed of four classes of phenolic compounds with caffeic acid represented the most abundant compound (class of hydroxycinnamic acid) in the methanolic extracts of *P. tricornutum* and *T. suecica.*

In another study performed on *Arthrospira platensis*, da Silva et al., (2017) discerned catechin, vanillic, gallic and syringic acids as the major phenolics in the high pressure/temperature extracts. Kováčik et al., (2010) detected nine benzoic acid derivatives (gallic–salicylic acid) and three cinnamic acid derivatives (caffeic–p coumaric acid) in methanolic extracts of *Scenedesmus quadricauda* exposed to UV-A and UV-C illumination.

III.4. Conclusion

This chapter focused on the LC-MS/MS screening of antioxidant metabolites produced by the green microalgae *Scenedesmus sp.* and extracted with optimized supramolecular solvents. As result, mass spectra from both antioxidants were successfully recorded and resolved. The main carotenoid identified in the supramolecular extracts was lutein (1.1 ± 0.1 mg/g) followed by astaxanthin (6.6 ± 2.0 µg/g) and the unidentified carotenoid. This result is in accordance with the total carotenoid content measured for supramolecular extracts.

Moreover, the profile of phenolic compounds revealed the presence of eight main polyphenols including simple phenols, phenolic acids, phenolic aldehyde, phenolic diterpene and isoflavone with the predominance of caffeic acid. It should be emphasized that the mass spectrum from polyphenols cannot be assigned unambiguously without further use of standards.

Overall, the applied LC-MS method allowed an efficient screening of antioxidants in supramolecular extracts, a trait that indicate a strong selectivity of the supramolecular solvents to recover both antioxidants. We expect that our studied extracts regarding their composition can exhibit strong antioxidant effects that will be assessed in the next chapter.

Chapter IV:

In Vitro radical scavenging properties of extracts

IV. Introduction

Antioxidants are precisely defined as a molecule stable enough to donate an electron to a rampaging free radical and neutralize it, thus reducing its capacity to damage. They interact with free radicals and terminate chain reactions by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves (Srdić-Rajić & Konić Ristić, 2016). Carotenoids and polyphenols are probably the most antioxidants being investigated and exploited from natural sources including microalgae. For instance, astaxanthin from *Haematococcus pluvialis* and phlorotannins from brown algae have demonstrated their protective effect against oxidative stress originating from high concentrations of free radicals and other strong oxidizing agents (Li et al., 2011; Mularczyk et al., 2020).

Recently, antioxidant activity of supramolecular extracts has been investigated in different food matrices, including the microalgae *Haematococcus pluvialis* (Salatti-Dorado et al., 2019) and coffee by-products (Torres-Valenzuela et al., 2019), where significant results were registered. Such treat was attributed to the antioxidant-rich composition of supramolecular extracts mainly in the xanthophyll astaxanthin and polyphenolic compounds for the microalgae and spent coffee grounds, respectively. This suggest that in our case, the supramolecular extracts obtained from *Scenedesmus sp*. may exhibit strong antioxidant activity against free radicals, since lutein (and to a lesser extent astaxanthin) and phenolic acids (mainly caffeic acid) are predominantly present.

The colorimetric assays ABTS and DPPH represent the most routinely practiced for assessment of free radical scavenging potential due to their accuracy, simplicity, and time- and cost-effectiveness (Guedes et al., 2013; Mishra et al., 2012). Moreover, they are used to assess both isolated compounds and mixed extracts from complex food

matrices regardless their solubility as being water or lipid soluble antioxidants (Floegel et al., 2011; Pérez-Burillo et al., 2018).

Here we report the scavenging properties of supramolecular extracts *in vitro* against ABTS and DPPH radicals in rapid and accurate approach.

IV.2. Material and Methods

IV.2.1. Chemicals and reagents

All reagents and standards were of analytical grade. Ascorbic acid (99,00%) was obtained from Fluka (Germany). Potassium persulfate (99,00%), 2,2 diphenyl-1-picrylhydrazyl (DPPH), 2,2´-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma–Aldrich (Steinheim, Germany). All the reagents and standard solutions were prepared using HPLC grade methanol obtained from Panreac (Barcelona, Spain).

IV.2.2. Sample and standard curve preparation

The crude supramolecular extracts were used entirely (*i.e*. concentration of 10 mg supramolecular extract mL^{-1}) and after dilution with methanol to give concentrations of 1, 2, 4, 6 and 8 mg supramolecular extract mL^{-1}. The samples were stored at −20 ºC until analysis.

Besides, five points standard curve was prepared for ascorbic acid dissolved in methanol at concentrations of 12.5, 50, 100, 150, 200 µM. These solutions were analysed in the same conditions as the samples and the standard curve was used to estimate the antioxidant concentration in the sample expressed as µM ascorbic acid equivalent per gram of dry biomass (µM $AE.g_{DB}^{-1}$).

IV.2.3. Antioxidant activity of Supramolecular extracts

The radical-scavenging capacities of SE were assessed via the ABTS radical cation ($ABTS^{\bullet+}$) and the DPPH radical ($DPPH^{\bullet}$) assays.

IV.2.3.1. The ABTS assay

This method is commonly called Trolox equivalent antioxidant capacity (TEAC) assay. It measures the ability of antioxidants to scavenge the stable radical cation $ABTS^{\bullet+}$ (2,2′-azinobis (3 ethylbenzothiazoline-6-sulphonic acid)), a blue-green chromophore with maximum absorption at 734 nm which is generated by reacting ABTS with potassium persulfate for few hours.

In this respect, the $ABTS^{\bullet+}$ inhibition by supramolecular extracts was evaluated according to (Guedes et al., 2013) with slight refinements and referred to ascorbic acid equivalent antioxidant capacity (AEAC). Briefly, a 7 mM stock solution of the free radical $ABTS^{\bullet+}$ was prepared by mixing 1/1 (v/v) ABTS solution (7 mM) and potassium persulfate solution (2.45 mM) in methanol. The mixture was first let to react for 16 h in the dark at room temperature, then, diluted with methanol to give an absorbance of 0.7±0.05 at 734 nm (Lambda 25 UV/VIS spectrophotometer, PerkinElmer Inc., Norwalk, CT, USA). One hundred microliters of extract were added to 1.9 mL of diluted ABTS solution and the absorbance of the resulting complex was measured after incubation overnight at room temperature. Diluted SUPRAS in methanol was considered as blank and ascorbic acid was used as standard. The scavenging effect of ABTS radical was calculated using the equation (4.1):

$$SP\ (\%) = [(A\ control - A\ sample) / A\ control] \times 100 \quad (4.1)$$

Where:

SP: is the scavenging percentage.

A sample: is the absorbance of the complex ABTS-SUPRAS extract or ABTS-Standard.

A control: is the absorbance of diluted ABTS solution.

The whole procedure was done under deem light and the antioxidant content of supramolecular extracts was expressed as µM AE.$g_{DB}{}^{-1}$.

IV.2.3.2. The DPPH assay

The DPPH radical scavenging assay is a colorimetric method based on changes in the color of a stable chromogen radical with a deep purple colour, DPPH, which is used as purchased commercially without prior generation. If the DPPH radical is neutralized or reduced, the purple colour converts to yellow with concomitant decrease in absorbance at 517 nm. The discolouration is monitored spectrophotometrically and utilised for the determination of the antioxidant efficacy (Apak et al., 2013).

The DPPH radical scavenging assay was conducted according to (Assunção et al., 2017) with some modifications. Samples of supramolecular extracts were mixed with methanolic DPPH solution (1.9 ml) and incubated overnight at room temperature in the dark. The absorbance was recorded at 517 nm against a blank (Lambda 25 UV/VIS spectrophotometer, PerkinElmer Inc., Norwalk, CT, USA). The scavenging percentage (SP) was calculated by the formula (4.2):

$$SP\ (\%) = [(A0 - AI) / A0] \times 100 \qquad (4.2)$$

Where:

A_I: is the absorbance of methanolic DPPH solution with the presence of all the extract samples and standard.

A_0: is the absorbance of the methanolic DPPH solution at 0.06 mM.

Furthermore, regarding the fact that the antioxidant activity by DPPH scavenging method is often reported as IC50 which is defined as the effective concentration of the

antioxidant necessary to decrease the initial DPPH concentration by 50%; supramolecular extracts concentrations were plotted against their corresponding scavenging percentages and the (IC50) was directly deducted from the plot.

IV.3. Results and Discussion

The profile screening of SUPRAS extracts showed the presence of a variety of antioxidant molecules ranging from polar or moderate polar as phenols to strongly lipophilic compounds as carotenoids. These components have been previously demonstrated to be closely related to the radical scavenging ability of microalgal biomass (da Silva et al., 2017; Goiris et al., 2012). Their antioxidant behaviour in the supramolecular extracts may occur synergically or separately upon distinct mechanisms and in fact, to evaluate it precisely, both ABTS and DPPH, classified as mixed mode electron and hydrogen atom transfer (ET/HAT) assays (Apak et al., 2016) were used.

IV.3.1. Calibration curve for ascorbic acid

The results of ABTS and DPPH radical-scavenging capacities were expressed in ascorbic acid equivalents antioxidant capacity (AAAC), which are almost equal to Trolox equivalents antioxidant capacity (TEAC) for the ABTS assay according to (Apak et al., 2007). Indeed, Trolox was replaced by ascorbic acid because: (1) ascorbic acid is widely used by the food industry; (2) results are at least as reproducible; (3) solution preparation is easier; and (4) the final solution exhibits a higher stability (Guedes et al., 2013).

Scavenging percentages (SP, %) against ABTS and DPPH radicals of ascorbic acid solutions were plotted against ascorbic acid concentrations (AA, mM). Thus, the inhibition percentage increased with the concentration of the standard according to significant linear correlations with SP = 289.95 AA + 5.1552 (R^2 = 0.9846) and SP = 427.62 AA- 15.497 (R^2 = 0.996) for ABTS and DPPH respectively (Appendix 7).

IV.3.2. Antioxidant activity of SE measured by the ABTS assay

The SE showed great performance against ABTS free radicals providing at most concentrated extract (10 mg mL^{-1}) an inhibition of 80.65 ± 2.20 % (Figure VI.1A), which correspond to a concentration of 25.04 ± 0.73 µM AE/g (Figure VI.1B). These values were higher than those obtained by Goiris et al., (2012) from aqueous ethanolic extracts of *Scenedesmus obliquus* (5.87±0.28 $\mu mol_{trolox.eq}.g^{-1}$ dw).

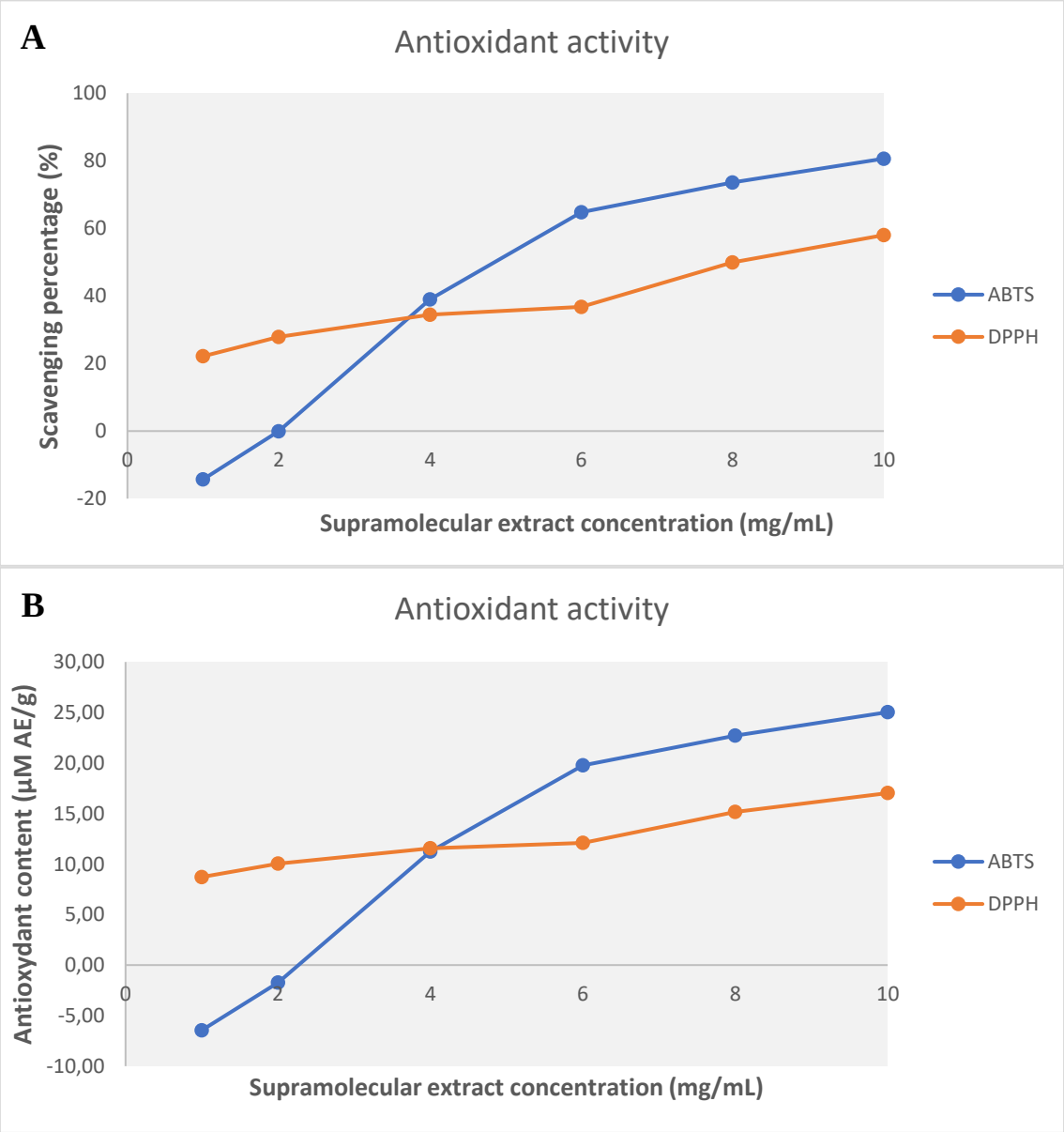

Figure VI.1. Antioxidant capacity of supramolecular extracts: (A) inhibition percentages; (B) antioxidant concentrations.

Moreover, this effect was comprised in the balance 3.34 ± 0.50 to 149.00 ± 46.60 $mg/L_{equivalent\ ascorbic\ acid}$ made up from eleven species of *Scenedesmus* and published by (Guedes et al., 2013). Other studies also investigated the antioxidant capacity against the same synthetic radicals in different compressed fluid extracts of *S. obliquus* and reported values in the range 0.100 - 0.639 TEAC mmol g^{-1} (Gilbert-López et al., 2017).

IV.3.3. Antioxidant activity of SE measured by the DPPH assay

The antioxidant activity of supramolecular extracts reached a maximum of 58% at 10 mg of supramolecular extract mL^{-1} (Figure VI.1A) with a value of 17.02 ± 0.45 µM AE/g (Figure VI.1B). This result fall within the range reported by Aremu et al., (2014) for five strains of *Scenedesmus* with different culture ages (3.1-69.9 % DPPH radical scavenging). In the same way, as demonstrated previously by Morowvat & Ghasemi (2016), *Scenedesmus rubescens* extracts behaved as the most potent DPPH radicals quencher achieving maximum antioxidants concentration of 14.04 ± 0.88 µmol Trolox g^{-1} dw.

Our values are rather high when compared to those of different solvent extracts of *Scenedesmus sp. ME02* where inhibition percentages ranging from 8.40 ± 0.4 to 52.02 ± 2.61 % were reported (Bulut et al., 2019).

In a recent study performed on *Scenedesmus bajacalifornicus* BBKLP-07, maximum radical scavenging effects of 72.42, 71.25 and 71.12% were observed at 50 µg/ml concentrations of ethanol, methanol and aqueous, respectively, whereas chloroform and acetone extracts have shown highest radical scavenging effects of 60.45 and 63.57% at 50 µg/ml concentration but lowest effects of 36.51 and 38.45% were observed at 10 µg/ml concentration respectively (Patil & Kaliwal, 2019).

For IC50 values, as depicted from the linear regression of DPPH assay data (SP = 3,8106X + 18,518, R^2 = 0,9699; Figure 4.2A), a concentration equals to 8.26 mg/mL of supramolecular extract was sufficient to inhibit the moiety of DPPH radicals which was in line with the rates reported for hexane and acetone extracts of the microalgae *Scenedesmus sp*. (IC50 = 6.53 ± 0.19 and 3.56 ± 0.14 mg/mL, respectively) (Custódio et al., 2014). Assunção et al., (2017) have evaluated the DPPH scavenging capacity of 42 strains of microalgae of different genera and the best IC50 observed was 44.27 ± 0.39 mg mL^{-1} from ethanolic extracts of *Characiopsis sp*.

IV.4. Conclusion

The antioxidant activity data indicate that supramolecular extracts were broadly able of scavenging ABTS and DPPH in a concentration-dependant manner. This strong effect agreed with the antioxidant-rich composition of supramolecular extracts since notable amounts of carotenoids and polyphenols were detected. It is noticeable that the inconsistence observed between the two assays may be devoted to nonspecific antioxidant interaction mechanisms and reaction kinetics producing different results. As observed recently, phenolic compounds reveal delayed response in the DPPH radical reaction resulting to lower scavenging values compared with the ABTS assay (Afify et al., 2018).

It is still questionable if these effects were a result of direct or indirect antioxidant effects or more specific interactions with relevant molecular and cellular targets. Besides the fact that the antioxidant capacity of both carotenoids and polyphenols may be related to the structure especially their conjugated systems, the reactivity of these compounds is also affected by the environmental conditions where they are found mainly the solvent. Usually, these compounds are altered or degraded by conventional solvent extraction processes in contrast to SUPRAS where their conservation and stability were greatly

appreciated. The higher antioxidant potential exhibited by the supramolecular solvent extracts make them nutritionally interesting. In addition, their use as natural antioxidant additive in the food industry may constitute a good solution to enhance the stability of especially fatty food against oxidative damage.

Chapter V: Lipid and ω-3 linolenic acid content

V.1. Introduction

There is great evidence that dietary lipids provide several health benefits including reducing coronary artery disease, heart failure, and cardiac arrhythmias (Willett, 2012). Among lipids classes, omega- 3 PUFA represent an important group with three common members DHA C22:6, EPA C20:5 and α-linolenic acid (ALA) C18:3, which their intake is highly encouraged by the World Health Organization (WHO) due to their promising benefits for cardiovascular health (Fleming & Kris-Etherton, 2014).

Microalgae are traditionally considered good sources of fatty acids especially polyunsaturated fatty acids (PUFA) and therefore contain all the genes encoding the enzymes required for their synthesis (Vaezi et al., 2013). Typically, microalgae display FA profiles comprising significant proportions of PUFA which promote their potential use for both human and animal nutrition (Borowitzka, 2013). As matter of fact, the FA profiles of different microalgae species has been extensively studied and reviewed by several authors searching for interesting species with applications in the human nutrition market (Barreira et al., 2015).

Regarding their multipolarity network, SUPRAS may offer a suitable hydrophobic microenvironment to extract lipids from their cellular matrices, therefore we hypothesize that a considerable amount of microalgal lipids will remain in the supramolecular extract previously prepared and analysed for antioxidants. This bring in front the potential of SUPRAS to be used as vehicle for lipid amending within fatty food processing.

In this sense, this chapter addresses the total lipids content and FA profile analysed by (GC-MS) in particular linolenic acid (C18:3) of *Scenedesmus sp*. for functional food and nutraceuticals application.

V.2. Material and Methods

V.2.1. Chemicals and reagents

FAME standards were acquired from Sigma Aldrich (St. Louis, MO, USA). Tetramethylammonium hydroxide (TMAH) solution used for the methylation of fatty acids was purchased from Sigma Aldrich (St. Louis, MO, USA). Other analytical grade solvents (isopropanol, hexane, chloroform, decane and methanol) were provided from Panreac (Barcelona, Spain).

V.2.2. Total lipids content

Total lipids were extracted from lyophilized biomass following a modified method of Bligh, E.G. and Dyer (1959) and quantified gravimetrically. Briefly, 50 mg of dry biomass were weighted in glass centrifuge tubes, then smashed with 2 mL chloroform-methanol (2:1 v/v). After 5 min vortexing (Vortexer, Heathrow Scientific, Vernon Hills, IL, USA), the mixture was sonicated for 30 min (Ultrasonic cleaning bath, Selecta, Barcelona, Spain), then continuously shacked for 30 min at 300 rpm, and centrifuged at 3500 rpm for 10 min. The cycle was repeated 3 times until the pellet become colourless to ensure that all lipids have been recovered. In order to remove cell debris, each sample was filtered (0.45 µm) using a glass syringe and rinsed with extraction solvent. Finally, the pooled extracts were evaporated under a nitrogen flow and the residue was then weighed for lipid quantification basing on the formula (5.1) (Lee et al., 2013):

$$\text{Total lipids (\%)} = \frac{Wr - Wt}{Wb} \times 100 \qquad (5.1)$$

Where *Wb* is the weight of dry algal biomass (g); *Wt* is the weight of empty tube (g), and *Wr* is the weight of glass tube containing the lipids residue (g).

V.2.3. Fatty acid extraction and methylation

Prior to GC analysis, fatty acids were extracted first from the freeze-dried biomass as previously performed (Martins-Noguerol et al., 2020). In summary, a known amount (100 mg) of dried algal biomass was mixed with 1 mL of hexane: isopropanol (2:1, v/v) in the presence of glass beads for 3 cycles of 30 s at 6000 rpm in a Precellys homogenizer (Precellys 24, Ozyme). The organic (upper) phase was carefully harvested, and the solvent was evaporated under a nitrogen steam. Next, the lipid residue was resuspended in chloroform-methanol (1:1 v/v) and subsequently methylated with 5 µL of TMAH solution. After stopping the reaction by adding 50 µL of Decane, and phase separation, the upper phase, containing fatty acid methyl esters (FAME) was removed for chromatographic analysis.

V.2.4. Gas chromatography

The extracted FAME (10 µL) were ultimately injected into an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with a Supelco SP-2380 fused silica capillary column (30 m length, 0.25 mm i.d., 0.20 µm film thickness; Supelco, Bellefonte, PA, USA) and a Quadruple Mass spectrometer using electron impact ionization. The initial oven temperature of 70 °C was gradually increased by 10° C/min until it reached 170 °C. The detector and injector temperatures were set at 200 °C. Hydrogen was used as the carrier gas at 28 cm/s. A hexane injection was used as a blank and the run time for a single sample was 36 min. The most common FAME were identified and quantified by comparing their GC profiles with those of known standards. Fatty acid peaks were further analysed and integrated using a chemstation chromatography software (Agilent Technologies, Germany).

V.3. Results and Discussion

A critical issue in valorising promising algae for alternative ω-3 PUFA feedstock is to combine high lipid content with high target fatty acids production in a sustainable approach.

The study of lipidic fraction was conducted following conventional protocols using organic solvents. Several attempts were made to analyse this fraction in the supramolecular extracts, although, neither total lipid content nor FA profile were estimated precisely due to the high amount of octanoic acid in the SUPRAS which altered considerably the reliability of the result. Anyway, it is analytically admitted that significant part of biomass lipids will obviously remain in the supramolecular extract, and starting from this, the result of conventional methods may be used as reference for SUPRAS.

V.3.1. Total lipid estimation

The results of gravimetric estimation of cellular lipids in *Scenedesmus sp.* showed high content reaching a value of 27.45 ± 0.39 % dw. This result is comprised in the interval reported by (Minhas et al., 2016) for different strains of *Scenedesmus* (13.60 - 36.53 %). Lower values observed by various other researchers (Gilbert-López et al., 2017; Rai & Gupta, 2017) were such that the maximum lipid contents of 13.83 % and 14.83 % were obtained from extracts of *Scenedesmus obliquus* and *S. abundans* respectively.

Our *Scenedesmus sp.* strain also had a small advantage over the reported *Scenedesmus acutus* cultivated in large scale Raceway pond (21.5%) (Eustance et al., 2016).

The total lipid content of the tested alga specie in this study was comparable with the recently reported in same *Scenedesmus* sp. strains cultivated in different wastewaters (Pham et al., 2020; Silambarasan et al., 2021).

Higher total lipid contents achieving values of 44 %, 37% and 36% were calculated in stationary phase harvested cultures of *S. dimorphus*, *S. obliquus* and *S. acuminatus* respectively (Gao et al., 2019; Jin et al., 2020; Tejada Carbajal et al., 2020).

Ye et al., (2020) reported total lipid content for eight *Scenedesmus* species cultivated in simulated wastewater medium ranged from 10% to 16% with *Scenedesmus sp.* HXY2 had the highest lipid content (15.56%), which represents almost the moiety of our finding for *Scenedesmus sp.*

Our result suggests that *Scenedesmus sp*. could be a potential candidate for lipids production since higher contents were detected, such fraction could be amplified by submitting the algae to stressful culture conditions.

For instance, Sánchez-García et al., (2020) investigated the influence of nitrate depletion and resupply on *Scenedesmus obtusiusculus* AT-UAM and observed a doubling of total lipid content from 14% to 30 % in indoor flat panel photobioreactor cultures. The same trend was also remarked in two stage *S. acuminatus* cultivated over 9 days of nitrogen starvation then other 6 days under nitrates supplementation where the maximum lipid content reached 36.46% dw at the end of cultivation (Zhang et al., 2019).

Rai & Gupta, (2017) revealed that with increase in light intensity (from 27 to 40.5 μmol m^{-2} s^{-1}) lipid content increases in *S. abundans* cells (from 29.6% to 48%) but decreases with further increase of light intensity (only 33% under 54 μmol m^{-2} s^{-1}).

V.3.2. Fatty acid profile and linolenic acid detection

Fatty acids (FAs) composition of algal biomass is considered as key criterion that determine its suitability for further use in nutritional applications, even though that fatty acids occurred in microalgal biomass mainly as chains of 16 and 18 carbon length (Chen et al., 2017).

Accordingly, as shown in Table V.1, the lipidic fraction of *Scenedesmus sp.* is composed of saturated fatty acids (SFA) (21.4%), monounsaturated fatty acids (MUFA) (13.51%) and polyunsaturated fatty acids (PUFA) (65.09%), primarily consisting of C14-C22. The PUFA class was dominated by linolenic acid (C18:3n3) followed by linoleic acid (C18:2n6) with an occurrence of 48,21 % and 8.76 % respectively. Basically, high levels of n-3 PUFAs such as ALA, EPA and DHA are strongly advantaged in human nutrition. The prominent MUFA identified in the lipid extract was oleic acid (C18:1) (8.11%), while the highest SFA percentage was observed for palmitic acid (C16:0) (14.35%). Other fatty acids were detectable in small amounts ranging from 0.11−3.04% (see Table V.1).

Such high content of linolenic acid is to our knowledge, the best recorded for *Scenedesmus* species except strain *Scenedesmus obliquus* where the moiety of fatty acids fraction (50.91%) consisted of linolenic acid (Hlaing et al., 2020).

The linolenic acid content of *Scenedesmus sp.* was reported to be from 3.2 to 19.2% of total lipids under variable nitrogen sources (Zhu et al., 2019).

The percentage of linolenic acid varied between 2.05 and 5.96% and even not detected, as previously reported for five strains of *Scenedesmus sp.* (Ye et al., 2020).

The FA profile of our microalgae biomass corroborated that of *Scenedesmus obliquus* harvested and dried using different methodologies, with linolenic acid (C18:3) as major fatty acid (de Oliveira et al., 2020). Conversely, this fatty acid was minorly

detected in microwave treated and untreated biomass as highlighted in recent study for *Scenedesmus sp.* (max 1%) (Sivaramakrishnan et al., 2020).

According to Mercado et al., (2020), the PUFA content of *Scenedesmus sp.* cultivated in industrial dairy wastewater was 44.29% with linolenic acid content was the dominant (23.79%). However, An et al., (2020) reported that the content of linolenic acid was slightly affected by growth condition of *Scenedesmus obliquus* where smallest values not exceeding 1.1% were observed. The FA profile of *Scenedesmus sp*. cultivated heterotrophically under different carbon sources was composed mostly of oleic acid and palmitic acid, while linolenic acid was totally not reported (Arora et al., 2020).

Table V.1. Fatty acid composition of *Scenedesmus sp*. (identified with available standards)

Fatty acids	% total fatty acids
14:0	0,11
16:0	14,35
16:1(1)	0,85
16:1	0,77
17:0	1,92
16:2	2,40
18:0	3,04
16:3	2,99
18:1	8,11
18:1a	0,96
18:2	8,76
20:0	0,00
18:3	**48,21**
20:1	2,82
20:2	2,74
22:0	1,98
SFA	21,40
MUFA	13,51
PUFA	**65,09**
SFA/UFA (IA)	**0,27**

It seemed that our *Scenedesmus sp*. contained higher linolenic content than most strains in the same genus.

The diversity of the contents of linolenic acid in *Scenedesmus* strains may be related to the strain and environment dependency of fatty acid profiles in microalgae (Barreira et al., 2015).

Besides, low value of the ratio SFA/UFA which is also called index of atherogenicity (IA) were calculated in the lipidic fraction of *Scenedesmus sp.* reaching a value of 0.27 (Table V.1). This index is usually used to assess the lipid quality of foods through the prediction of their cardiovascular-related health benefits. Since SFA are considered proatherogenic and UFA anti-atherogenic, a low IA ratio is desirable (Garaffo et al., 2011). Our IA value is considerably lower than those of Palm oil (0.81) but higher than those of olive oil (0.14) and sunflower oil (0.12) (Sajjadi et al., 2016). Moreover, this result was comparable to other animal sources of n-3 PUFA such as raw mackerel (0.28), lamb (1.00), beef meat (0.70-0.74) and raw tuna (0.69-0.76) (Garaffo et al., 2011). This fact may advantage *Scenedesmus sp*. as healthier biomass with great potential for development as a nutritional supplement. Its use for linolenic acid production seems to be a sustainable tool to reduce the dependence of this FA intake on traditional crops without competing for arable land.

V.4. Conclusion

The current study investigated on the one hand the potential of *Scenedesmus sp*. for the production of valuable ALA with health benefits and on the other hand the ability of SUPRAS to concentrate indirectly the lipidic fraction. The latter was extracted from the whole biomass using previously established protocol and the total lipids content was evaluated. In addition, FA were profiled by GC-MS to determine the most abundant

components. As result, high total lipids content was quantified in the biomass indicating the good performance of the microalgae in lipid accumulation. This fraction was composed dominantly of ALA with top-level surmounting other strains of the same genus. Quality assessment of FA fraction was achieved through IA index, hence low value was calculated allowing further development as a source of health food supplements. Such trend makes *Scenedesmus sp*. a promising and sustainable feedstock for nutritional and food applications. Also, the hypothesis of linolenic acid abundance in the supramolecular solvent network is strongly approved which make SUPRAS a potential ω3 fatty acid enrichment material.

Conclusions and future considerations

The results of this study demonstrate the feasibility of *Scenedesmus sp.* strain as feedstock for the co-production of lipids and antioxidants. The profile screening of algal extracts showed the presence of a variety of antioxidant molecules ranging from polar or moderate polar as phenols to strongly lipophilic compounds as carotenoids, in addition to high concentration of polyunsaturated fatty acids, particularly ALA. Large scale utilization of these natural bioactives for nutraceutical or food industry is mainly associated with the availability of a renewable source with sustainable production process. Thus, in the context of developing sustainable and economically feasible algae-based bioactives extraction and characterization:

Here, an easily prepared, green and cost-effective supramolecular solvent enabling high recovery of antioxidants from microalgal biomass (*Scenedesmus sp*.) was developed and optimized. The process led to substantial extraction yields of antioxidants reaching 1.04 mg/g for total carotenoids and 10.29 mg/g for total phenolics. The obtained results provide advancement in the harvesting of antioxidants from algal cells comparing to conventional solvents. Shorter extraction time and higher net extraction efficiency are major achievements in this study by the use of supramolecular solvent.

The latter represent nanostructured liquid made up from well-defined proportions of octanoic acid, ethanol, and water with 5/36/59 % v/v/v respectively was the best condition for extraction. The optimal supramolecular solvent evolved efficient simultaneous extraction of both antioxidants regardless their polarity in single step procedure. The process offers also the ability to be easily scalable and implemented in the food industry since the separation of target compounds was accessible in simple eco-friendly manner, which can offset the cost of algal antioxidants production, making this source more commercially viable.

The antioxidant mix in the supramolecular extract was identified by LC-Triple Quadrupole mass spectrometry, which revealed lutein as the main carotenoid (about 1mg/g) and phenolic acids as the major phenolics (about 50%). Such insight brings in front the interest of applying supramolecular extracts in nutraceutical or food formulations. Although, some discrepancies related to equipment sensitivity and standards availability have impacted the reliability and validity of the analysis making the qualitative and quantitative estimation of both antioxidants significantly restricted. Anyway, the LC-MS profile may explain the extent by which supramolecular solvents can extract simultaneously lipophilic and hydrophilic antioxidants.

Supramolecular extracts produced from *Scenedesmus sp.* biomass exhibited strong antioxidant activity against ABTS and DPPH radicals (80.65 and 52 % respectively) demonstrating that are abundant sources of antioxidant, especially polyphenols. Lutein and polyphenols omnipresent in the supramolecular extracts are known to possess pronounced free radical scavenging ability due to their polarity and number of conjugated double bounds, and thereby may exert beneficial effects on oxidative stress. By the way, supramolecular extracts were shown to have a promising future as antioxidants.

Moreover, during this study, lipid content and fatty acid composition were determined through conventional solvent extraction followed by gravimetric quantification and GC-MS profiling. The extract produced high lipid quantity of 27.45 % and showed a significant richness in PUFA mainly ALA. This essential fatty acid was found in highest amount reaching a record value of 48.21 % which bring in front the appreciable nutritional value of *Scenedesmus sp*. with beneficial effects on human health. Such trend may advantage the use of supramolecular extracts as supplement of ω-3 fatty acids in replacement to depleted conventional sources.

On the whole, variable molecules of interest with different polarities being linolenic acid, carotenoids and polyphenols were harvested from *Scenedesmus sp.* using solvent processes at superior rates, that provide an attractive tool toward sustainable, economic and functional valorisation of algal biomass for food purposes.

However, there are several challenges to be addressed:

The large-scale production of bioactives from microalgae is only possible with the selection of microalgae with high biomass and excellent productivities. Cultivation of microalgae under stress conditions may resulted in an increased percentage of secondary metabolites among which lipids and antioxidants in the algal cells.

Although the extraction methods explored in this study were optimised and performed using dry biomass, it is necessary to explore extraction of wet biomass, since biomass drying is an expensive process.

The optimisation of supramolecular solvents for the extraction of lipids seems to be of great importance in biorefinery approach since it is not economically interesting to extract lipids with a lipid (*i.e*. octanoic acid).

The development of enzyme and pulsed electric fields assisted supramolecular solvents extraction and the use of other natural components in the supramolecular matrix such as terpenes and organic-nanoclays could maintain the biocompatibility of the process and enhance extraction efficiency.

Supramolecular solvents appear to potentially extract useful antioxidants, including carotenoids and polyphenols, although the full characterization of these molecules still remain to be amply considered.

In addition, with the aim of assessing the potential of supramolecular solvents for antioxidants recovery, these supramolecular extracts could be further investigated with other *in vitro* and *in vivo* antioxidant capacity tests.

Finally, the studied method could be investigated for the extraction of other valuable compounds from microalgae (such as carbohydrates and proteins) in a single step procedure.

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Appendices

Appendix 1.

A. Composition of *Sportsmaster WSF Spring & Summer* and nutrient concentration in solution of 37.5 g/L

			Concentrate (37.5 g/L) g/L	MW g/mol	Final concentration mg/L		
28	**%**	**N**	10.5003	15	92.40	6.16	mM
21.4	**%**	**Ureic N**	8.0252	60.06	70.62	1.18	mM
5.6	**%**	**Nitrate**	2.1001	62.00	18.48	0.30	mM
1	**%**	**Ammonium**	0.3750	18.04	3.30	0.18	mM
5	**%**	**Phosphorus pentoxide (P_2O_5) 2.2 % P**	1.8750	141.95	16.50	0.12	mM
19	**%**	**K_2O 15.8 % K**	7.1252	94.2	62.70	0.67	mM
0.020	**%**	**Boron**	0.0075	10.81	0.07	6.11	µM
0.016	**%**	**Copper EDTA**	0.0060	63.55	0.05	0.83	µM
0.130	**%**	**Iron DTPA**	0.0488	55.85	0.43	7.68	µM
0.060	**%**	**Manganese EDTA**	0.0225	54.94	0.20	3.60	µM
0.010	**%**	**Molybdenum**	0.0038	95.95	0.03	0.34	µM
0.016	**%**	**Zinc EDTA**	0.0060	65.38	0.05	0.81	µM

B. Growth medium composition

	mL per L growth medium
Stock solution of *Sportsmaster WSF* fertilizer 37.5 g/L	8.8
Magnesium sulfate solution 20 g/L $MgSO_4.7H_2O$	1

Appendix 2.

A. One-way ANOVA report of Figure's II.4 data

Source	DF	SS	MS	F-Value	P-Value
Factor	4	0,167	0,0417	23,810	<0,05
Error	10	0,0175	0,00175		
Total	14	0,184			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (p = <0,05).

B. One-way ANOVA reports corresponding to Figure II.5

Source	DF	SS	MS	F-Value	P-Value
Factor	4	42,337	10,584	20,097	<0,05
Error	10	5,267	0,527		
Total	14	47,603			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (p = <0,05).

Appendix 3.

A. One-way ANOVA report of Figure's II.7A data

Source	DF	SS	MS	F-Value	P-Value
Factor	4	0,340	0,0850	25,342	<0,05
Error	10	0,0335	0,00335		
Total	14	0,373			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (p = <0,05).

B. One-way ANOVA reports corresponding to Figure II.7B

Source	DF	SS	MS	F-Value	P-Value
Factor	4	21,728	5,432	69,815	<0,05
Error	10	0,778	0,0778		
Total	14	22,507			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (p = <0,05).

Appendix 4.

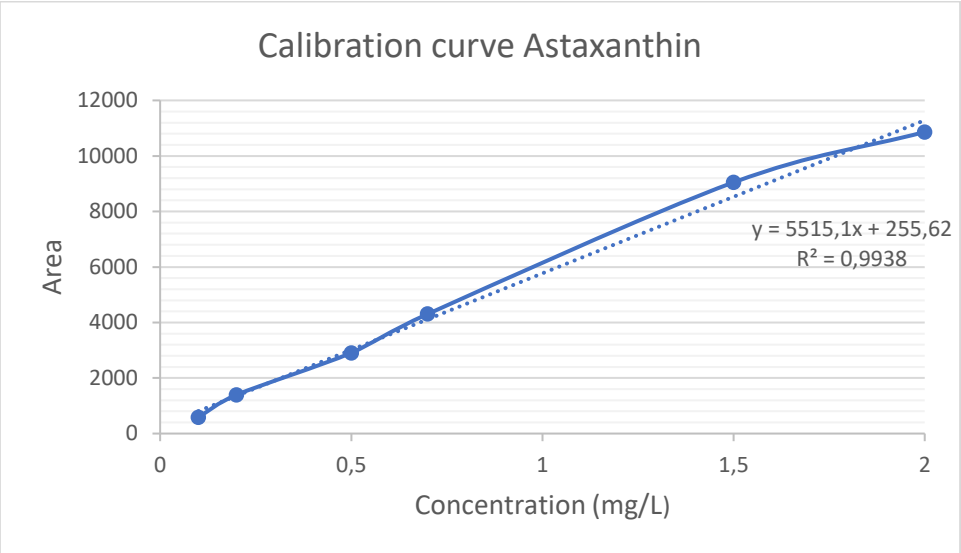
List of LC-MS/MS characteristic transitions for the identification of carotenoids

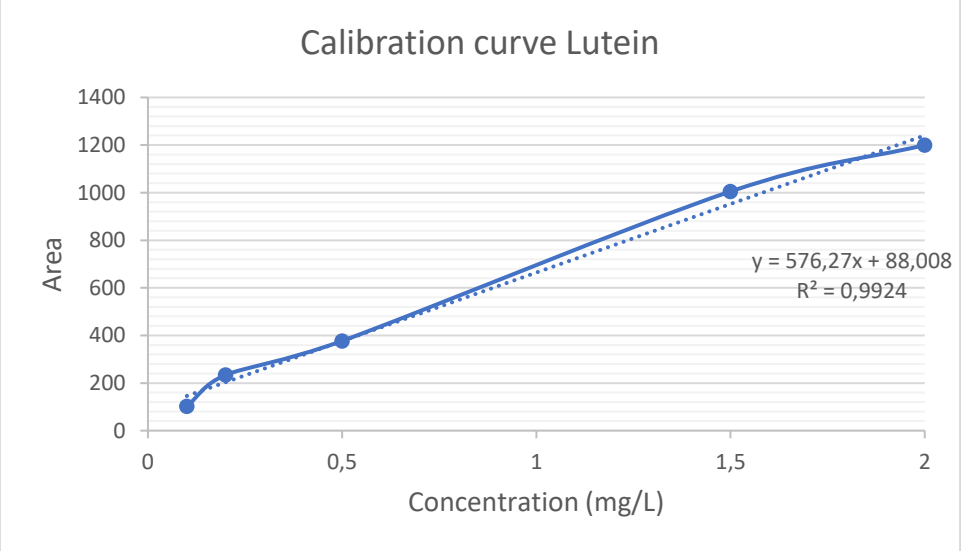
Compound	Parent ion (m/z)	Main fragments	Sub-class and formula	Reference
Neoxanthin	601.8 ($[M+H]^+$)	583.5 ; 565.5 ; 491.4	Xanthophyll $C_{40}H_{56}O_4$	(Gilbert-López et al., 2017)
Violaxanthin	601.5 ($[M+H]^+$)	583.5 ; 565.6 ; 491.5	Xanthophyll $C_{40}H_{56}O_4$	
Astaxanthin	597.4 ($[M+H]^+$)	147.1 ; 173.1 ; 201.1 ;285.2 ; 379.3 ; 579.4	Xanthophyll $C_{40}H_{52}O_4$	(Van Breemen et al., 2012)
Lutein	551.4 ($[M+H-H_2O]^+$)	477.4 ; 119.1 ; 135.1 ;175.2 ; 416.3; 430.3 ; 459.4 ; 495.4	Xanthophyll $C_{40}H_{56}O_2$	
Zeaxanthin	569.4 ($[M+H]^+$)	551.4 ; 477.4 ; 489.4 ; 119.1 ; 135.1 ; 175.2 ; 416.3 ; 459.4	Xanthophyll $C_{40}H_{56}O_2$	
Cryptoxanthin	553.4 ($[M+H]^+$)	535.4 ; 473.4 ; 461.4 ; 119.1 ; 135.1 ; 177.2 ; 400.3	Xanthophyll $C_{40}H_{56}O$	
Echinenone	551.4 ($[M+H]^+$)	471.4 ; 459.4 133.1 ; 203.1 ; 255.2 ; 495.4 ; 536.4	Xanthophyll $C_{40}H_{54}O$	
β-Apo-8`-carotenal	417.3 ($[M+H]^+$)	399.3 ; 325.3 ; 95.1 ; 119.1 ; 157.1 ; 293.2 ; 338.3 ; 361.3 ; 389.3	Oxocarotenoid $C_{30}H_{40}O$	
α-Carotene	537.4 ($[M+H]^+$)	457.4 ; 123.1 ; 137.1 ; 177.2 ; 413.3	Carotene $C_{40}H_{56}$	
β-Carotene	537.4 ($[M+H]^+$)	445.4 ; 457.4 ; 137.1 ; 177.2 ; 269.2 ; 400.3 ;	Carotene $C_{40}H_{56}$	
Canthaxanthin[a]	**565.4 ($[M+H]^+$)**	427.4 ; 413.0 ; 363.4 ; 361.1 ; 459.2 ; **109.1**	Xanthophyll $C_{40}H_{52}O_2$	(Soares et al., 2019)
Fucoxanthin	659.5 ($[M+H]^+$)	567.2 ; 581.6 ; 641.6	Xanthophyll $C_{42}H_{58}O_6$	

[a]Unknown compound showing the transition of Canthaxanthin in bold; retention time and most abundant fragments did not match those of Canthaxanthin.

Appendix 5.

Calibration curves for astaxanthin and lutein standards.





Appendix 6.

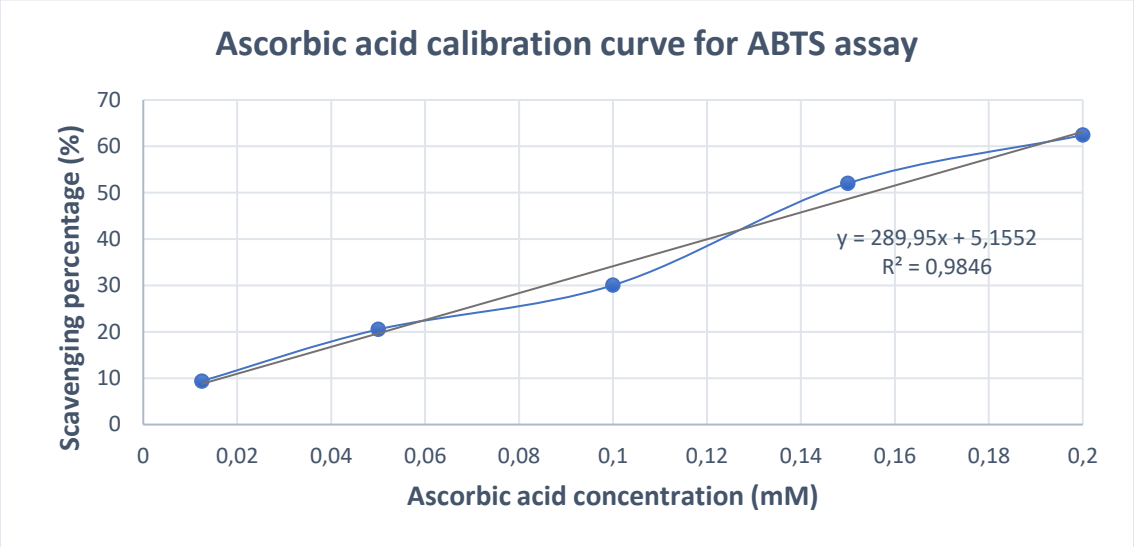
MS characteristic transitions of polyphenols obtained from SUPRAS extracts of *Scendesmus sp.* as reported in the literature (only identified compounds showed).

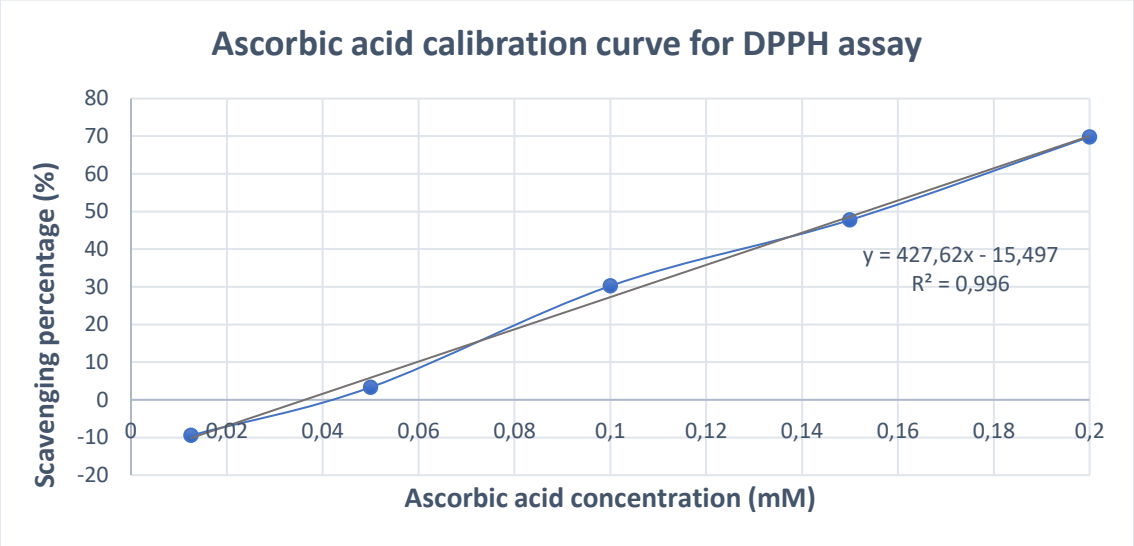
Compound	λ max (nm)	Parent ion [M-H]$^-$ (m/z)	Main fragments	Sub-class	Reference
Carnosic acid	285	331	287.1, 244	Phenolic diterpene	(Rajauria et al., 2016)
Sinapic acid	322	223	179	Hydroxycinnamic acid	(Klejdus et al., 2017)
Vanillin	348	151	136	Phenolic aldehyde	
p-hydroxybenzoic acid	282, 312	137	93	hydroxybenzoic acid derivative	
Caffeic acid	248, 319	179	135	Hydroxycinnamic acid	(Rajauria, 2018)
Gallic acid[a]	214, 270	169.1	125	Triydroxybenzoic acid	
Phloroglucinol	229, 267	125.1	97	Simple phenol	
Formononetin	262	267	252	Isoflavone	(Ye et al., 2012)

[a]not identified, but an unknown compound with different retention time than gallic acid was measured with this MS/MS transition

Appendix 7.

Calibration curves of Ascorbic acid for ABTS and DPPH assays





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