EFFECTS OF VITAMIN E ANALOGUES ON THE GROWTH AND LIPID PROFILE OF *CHLAMYDOMONAS REINHARDTII*

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Universidad de Oviedo.

Europen Bioenergy Research Institute.

University of Aston.

Samuel Vega Estévez.

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RESUMEN.

Ante la creciente preocupación por las emisiones contaminantes al medio ambiente surge como principal alternativa a los combustibles fósiles el uso de nuevas fuentes no contaminantes como el biodiesel. Las microalgas son uno de los biorecursos que más importancia está captando en los estudios de producción de biodiesel, ya que se consiguen altos niveles de producción en mucho menor espacio que con plantas terrestres. Sin embargo la producción de biodiesel a partir de microalgas sigue sin ser rentable y nuevas técnicas tienes que ser exploradas. El uso de antioxidantes para evitar la degradación de los lípidos parece prometedor. Los efectos de análogos de la Vitamina E sobre el crecimiento, perfil oxidativo, perfil lipídico y comportamiento termoquímico de *Chlamydomonas reinhardtii* fueron investigados en este estudio. Ninguno de las diferentes conformaciones de la Vitamina E estimuló el crecimiento, aunque no fueron observados efectos adversos a concentraciones bajas. Únicamente el análogo Trolox hidroxílico produjo un aumento de la población. Trolox carboxílico y Esculetina produjeron inhibiciones en el crecimiento. δ-tocopherol mostró la mayor actividad antioxidante, mientras que las menores fueron los casos de α-tocopherol y 10 μM de Trolox hidroxílico. El perfil lipídico de *C. reinhardtii* para los diferentes tratamiento no sufrió variaciones con respecto a los controles, y tampoco se observaron diferencias en el comportamiento termoquímico. La única diferencia significativa observada fue la disminución del porcentaje de lípidos en peso húmedo con el tratamiento de 10 μM de Trolox hidroxílico.

Debido a la falta de efectos sobre el crecimiento y sobre el perfil lipídico de *C. reinhardtii*, se puede concluir que el uso de las diferentes conformaciones del tocopherol no resulta ventajosa a la hora de producir biomasa apta para biodiesel. Únicamente podría ser ventajoso el uso de 100 μM de Trolox hidroxílico, ya que estimula el crecimiento y no disminuye el contenido lipídico.

ABSTRACT.

Facing the growing concern about the contaminant emissions to the environment, biodiesel appears as one of the main alternatives to fossil fuels. Microalgae are one of the bioresources that is gathering more attention, since high production levels can be achieved in smaller areas than with terrestrial plants. However, biodiesel production from microalgae is not profitable yet, and new techniques need to be explored. The use of antioxidants to avoid the degradation of the lipids seems promising. The effects of Vitamin E analogues over the growth, oxidative and lipid profile and thermochemical behaviour of *Chlamydomonas reinhardtii* have been evaluated in this research project. None of the different conformations of Vitamin E produced growth stimulation, although no adverse effects were seen at low concentrations. Only hydroxylic Trolox stimulated the growth. On the other hand carboxylic Trolox and Esculetin inhibited it. δ-tocopherol showed the highest antioxidant activity, while the lowest was observed in α-tocopherol and 10 μM hydroxylic Trolox . The lipid profile of *C. reinhardtii* for the different treatments did not suffer any variations with respect to controls, and no differences were observed in the thermochemical behaviour neither. The only significative difference was observed in the reduction of the percentage of lipids in wet weight for 10 μM hydroxylic Trolox.

As a conclusion, owing to the lack of effects over the growth and the lipid profile of *C. reinhardtii*, the uso of the different conformations of tocopherol is not advetageous to produce biomass for biodiesel production. Only 100 μM hydroxylic Trolox coudl be benefitial, since it enhances the growth.

FIGURES LIST.

TABLES LIST.

ABBEVIATION LIST.

α-LeA: α-linoleic acid.

μ: Growth rate (hours^{-1}).

AAE: Acyl-ACP esterase.

ABA: Abscisic acid.

ACCase: Acetyl-CoA carboxylase.

ACP: Acyl carrier protein.

C: Chloroplast.

CKs: Cytokinins.

DAG: Diacylglyceride.

DAGT: Acyl-ACP by dyacylglicerol acyltransferase.

DG: Diradylglycerolipids.

DGDG: Digalactosyldiacylglicerol.

DGTS: Diacylglyceryltrimethylhomo-Ser.

DH: Dehydratase.

Dt ($t_{1/2}$): Duplication time (hours).

EPA: Environmental Protection Agency.

ER: Endoplasmic reticulum.

ER: Enoyl reductase.

Esculetin: 6,7-dihydroxycoumarin

ET: Ethylene.

FAME: Fatty acid methyl ester.

FAS: Fatty acid sinthase.

GA: Gibberelins.

GC/MS: Gas chromatography/mass spectrometry

GPAT: glycerol-3-phosphate acyltransferase

HTL: Hydrothermal liquefaction.

IAA: Indolacetic acid.

IM: Inner membrane.

IMS: Inter membrane space.

JA: Jasmonic acid.

KASIII: β-ketoacyl-ACP-synthase III.

LB: Lipid body.

LC_{50}: Median lethal concentration.

LCAS: Long-chain acyl-CoA synthase.

LOX: Lypoxygenases.

LPAT: LysoPA acyltransferase.

LysoPA: Lysophosphatidic acid.

M: Mitochondria.

MAG: Monoacylgliceride.

MCT: Malonyl-CoA:ACP transacylase.

MDA: Malondialdehydes.

MeJA: Methyl-jasmonate.

MGDG: : Monogalactosyldiacylglycerol.

N: Nucleus.

NOAEC: No-Observed-Adverse-Effect Concentration.

OM: Outer membrane.

PA: Phosphatidic acid.

PAP: PA phosphatase.

PDAT: Phospholipid:diacylglycerol transferase.

PE: Phosphatidylethanolamine.

PI: Glycerophosphoinositols.

PSII: Photosystem II

PUFA: Polyunsaturated fatty acids.

Py: Pyrolysis.

Py-GC-MS: Pyrolysis-Gas chromatography-Mass spectrometry.

ROS: Reactive oxygen species.

RT: Retention time.

TAG: Triacylglyceride.

TAP: Tris-Acetate-Phosphate.

TBA: Thiobarbituric acid.

TBARS: Thiobarbituric acid-reactive substances.

TCA: Trichloroacetic acid.

TE: Thioesterase.

TLC: Thin layer chromatography.

Trolox-COOH: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

Trolox-OH: 2-(hydroxymethyl)-2,3,7,8-tetramethyl-6-chromanol

VLE: Vegetative lysis enzyme.

OUTLINE

1. INTRODUCTION

1.1 Biofuels: social needs.

One of the main concerns in the current society is the growing need of reduction of the pollutant emissions to the atmosphere. The global warming is worldwide accepted and is also considered as a threat, not just for human beings, but also for the survival of a great amount of animal and plant species (Oven et al., 2012). Furthermore, the world is going to face a huge energetic crisis in the next years, worsen by the progressive depletion of the fossil fuels. Therefore, the research in new renewable energy sources is an urgent need (Rawat et al., 2011).

Biofuels and biodiesel can be found among the main alternatives to fossil fuels, which currently are the most used energy sources (Armin, 2009). As biodiesel are understood all the biofuels equivalents to diesel, which are obtained from renewable biological materials, that normally require certain processes to be transformed into diesel. Specifically, they are defined as the monoalkyl esters of long-chain fatty acids (C_{14}-C_{24}) derived from the chemical reaction (named transesterification) of renewable feedstocks, such as vegetable oils, or animal fats (Ahmad et al., 2011).

Historically, biofuels have been classified according to their origin. For the production of first generation biofuels, edible oils are used as main feedstock, such as sunflower, rapeseed or palm oil, whereas for second generation fuels, non-edible oils are a promising feedstock (Lam and Lee, 2012). The importance of the biofuels as a new source of energy lies on all the advantages they have when compared to fossil fuels:

a) It is an energy source easy to distribute.
b) It is easily biodegradable (Khan et al., 2009).
c) Aromatics products or other chemical substances are not released to the environment. It is not flammable, and the levels of particulate matter in the air are reduced. Thus, its toxicity is reduced (Atabani et al., 2012).
d) The production can be easily increased in a shorter amount of time.
e) The oxygen content is higher, so the combustions are better than in the petrol-based fuels.

f) Economically, has been probed as favourable for the rural economies, and moreover, reduces the dependence on the foreign crude in non-producer countries (Ahmad et al., 2011).

However, despite all the advantages they present, their use has a lot of detractors who are based on the economic problems and famines which are produced by the destination of part of the world sources for feed to the production of biodiesel. Moreover, other disadvantages have been attributed to biodiesel, such as the corrosive damage they can produce in vehicles or the higher price compared to fossil fuels (Atabani et al., 2012).

1.2 Microalgae as a biorefinery of products.

Facing the search of new sources of biofuels without alimentary and ethical implications, microalgae have come up as an important alternative, since new developments in screening and culture techniques, genetic engineering and microalgal biotechnology have been achieved. This huge interest is not only based on their potential use to produce biofuels, but also on their wide fields of application, from the biomass production for food and feed to valuable products for ecological or pharmaceutical applications (Pulz and Gross, 2004). Hence, microalgae and other microorganisms which are able to synthesise oils are now considered as the producers of third generation biofuels (Lam and Lee, 2012).

Among algae, microalgae are defined as photosynthetic microorganisms which have the capability to grow and live in extreme conditions because of their unicellular or simple multicellular structure (Mata et al., 2010).

Currently, the systematic classification of algae is based primarily on their pigment components, and according to this, 10 divisions can be found. The largest groups are Chlorophyceae (green algae), Rodophyceae (red algae), Phaeophyceae (brown algae), Pyrrophyceae (dinoflagellates), Chrysophyceae (golden-brown algae) and Bacillariophyceae (diatoms) (Harwood and Guschina, 2009), but also many species of Cyanophyceae (cyanobacteria), Prasinophyceae and Eustigmatophyceae (pico-plankton) and Xanthophyceae (yellow-green algae) have been described (Hu et al., 2008). The only example of prokaryotic microalgae are cyanobacteria, and the rest of them are eukaryotic cells (Figure 1)(Mata et al., 2010).

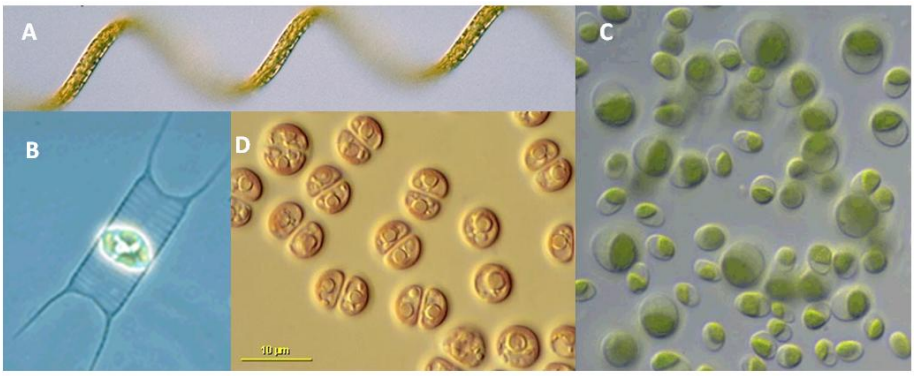


Figure 1. Microscopic images of different microalgae. A: *Arthrospira sp* (Cyanophyceae); B: *Acantotheras sp* (Bacillariophyceae); C: *Chlorella sp* (Chlorophyceae); D: *Rhodosorus marinus* (Rhodophyceae). Source: A, B and C: (https://www.rbgsyd.nsw.gov.au/science/Plant_Diversity_Research/australian_freshwater_algae/algpic The Royal Botanic Gardens & Domain Trust; D: https://ncma.bigelow.org/products/algae/all-algae/ National Center for Marine Algae and Microbiota. Date: 26/05/2015.

The biotechnology of macroalgae and microalgae are both included in the biotechnology of algae, and are closely related. The first report referring to macroalgal use dealt with *Porphyra*, which has been collected since the year 530, and has been cultivated since 1640 (Pulz and Gross, 2004), and from that moment on, the industry of algae started its evolution.

During this evolution, the development of visual techniques lead to the visualization and discovery of microalge, and from that moment, different commercial applications have been developed. Microalgae can produce certain bioactive compounds such as antioxidants, antibiotics or toxins that may be used in pharmaceutical industries, or pigments, which are interesting for cosmetic purposes. Moreover, some microalgae strains have high content of proteins, vitamins, polysaccharides, fatty acids, sterols and lipids, and it makes them interesting as nutrient supplements for consumption, and in the case of lipids, they can be used for the production of biodiesel, and the leftover biomass after the lipids extraction can be converted to other types of biofuels such as biomethane, bioethanol and biohydrogen. But the production of biofuels is not the their only application in solving environmental problems, in addition to this, microalgae can be used to absorb contaminants, and organic and inorganic compounds from wastewaters, producing its bioremediation, or even fixing carbon dioxide released from power plants by photosynthesis and producing nutrients of interest at a minimal cost (Harun et al., 2010)(Koller et al., 2014).

This important characteristic of microalgae, and other organisms, to be used for several applications and to produce a huge amount of components of

interest has led to the development of a new field of study in applied science: biorefineries. Biorefinery is defined as a process of integrated utilization of all the components of the biomass to try to enhance the economics of the process. In the case of producing alternative energies from microalgae, lipids are suitable for the production of biodiesel and bio-oil, whereas polysaccharides are appropriate for bioethanol production. Moreover, the residual biomass can be used to be converted into bio-oils by thermal conversion strategies, so microalgae biomass can be completely used for an integrated production of various liquid biofuels (Lee et al., 2015).

Therefore, thermal conversion strategies have been targeted to try to reduce the high costs of biofuels from microalgae. The main two approaches in thermal conversion are pyrolysis (Py) and hydrothermal liquefaction (HTL).

Py refers to a process by which biomass is thermally degraded at moderate temperatures (350-700ºC) in absence of oxygen and water until the production of solid, liquid and gaseous products, which can be important sources of energy or chemicals. Py can be divided in the different subgroups depending on the operation conditions: slow, fast and flash Py. The main difference between the three types are the heating rate range, the required size of the particles and type of products produced (Table 1)

	Slow Py	Fast Py	Flash Py
Heating rate range (ºC/s)	0.1-1	1-200	>1000
Required size of particles (mm)	5-50	<1	0.2
Types of products produced	Solids, liquids and gases	Liquids or gases	Mostly liquids

Table 1. Main differences between slow, fast and flash Py.

Although the lasts years flash Py seems to be a viable technique for future replacement of fossil fuels with biomass derived liquid fuels mainly because a high biomass-to liquid conversion ratio (95.5%) can be achieved, there are technical challenges to be solved. First, because Py oils are acidic, unstable, viscous and contain solids and chemically dissolved water, and these characteristics are not desired on fuels (and therefore, oils produced by Py require upgrading hydrogenation, catalytic cracking to lower the content of oxygen and removing

alkalis) and also because biomass needs to be dried and this is process is highly energy demanding (Lee et al., 2015)(Marcilla et al., 2013).

1.3 Chlamydomonas species and its biotechnological use.

Among the most important algae species in for industry and research purposes, *Arthrospira*, *Dunaliella*, *Chlorella* strains, as well as the model organism for research *Chlamydomonas*, are highlighted.

Chlamydomonas species belong to The Chlorophytes (or green algae). This division of algae is characterised because they have diverged from plants and their close relatives, but also from animals, Fungi and amoebozoa, dyatoms and oomycetes. These relatively close relationships has permitted *Chlamydomonas* to maintain many similarities that can be traced in plants (the presence of chloroplasts) or in animals (the existence of flagella)(Blaby et al., 2014)(Grossman et al., 2007)(Merchant et al., 2007)(Blaby et al., 2014).

Historically, the species of *Chlamydomonas* have been defined based solely on morphological characters as unicellular chlorophyte algae with two anterior flagella, a basal chloroplast surrounding one or more pyrenoids and a distinct cell wall (Figure 2).

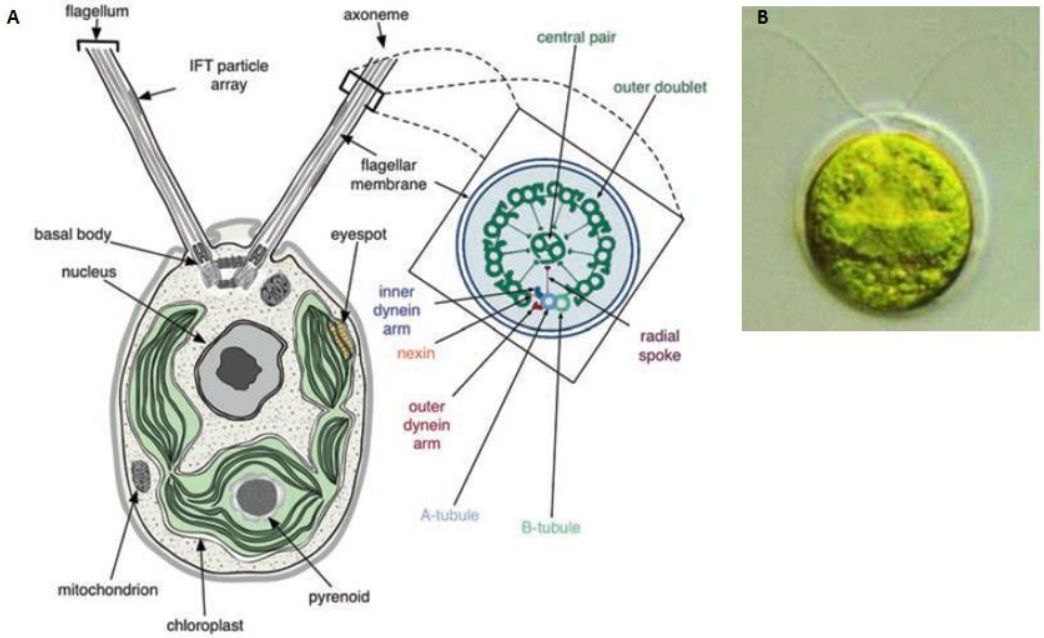


Figure 2. A) Transmission electron micrograph scheme of a *Chlamydomona* cell, showing the anterior flagella rooted in two basal bodies, with intraflagellar transport particle arrays (IFT) between the axoneme and flagella membrane, the basal cup-shaped chloroplast and pyrenoid within it, central nucleus and other organelles. In the right, a transversal cross section of the flagellar axenome shows the typical structure of the flagella in nine outer doublets and the central pair (9+2) microtubules (Merchant et al., 2007). B) Microscope image of *Chlamydomonas reinhardtii*.

Although more than 400 species of *Chlamydomonas* have been already identified, not all of them have significant roles as laboratory organisms. Among the important species in research, *Chlamydomonas reinhardtii* has emerged as the predominant species in laboratory experiments.

The wild-type *C. reinhardtii* is a unicellular, haploid (with a genetically fixed mating type *mt+* or *mt-*) and soil-dwelling organism which averages about 10 µm in diameter (although there is a significant variation through the cell cycle) with multiple mitochondria, two anterior flagella for motility and mating, a singular cell wall (consisting primarily of hydroxyproline-rich glicoproteins and cellulose-free) and a single cup-shaped chloroplast which occupies the basal two thirds of the cells and is in charge of the photosynthetic apparatus and the critical metabolic pathways (Figure 2)(Harris, 2001)(Merchant et al., 2007). Within the chloroplasts, a distinctive body in which CO_2 fixation and the dark reactions of photosynthesis are produced can be found, the pyrenoid (Harris, 2001).

They are also characterise because of the presence of unique features in its cell cycle. Under optimal conditions, cells divide asexually, through mitosis. However, sometimes the mother cells do not divide in just 2 daughter cells, but in more, potentially 2^n, which will be retained inside the mother cell wall (sporangia) and released simultaneously after the secretion of a vegetative lysis enzyme (VLE) which digests the cell wall of the mother cell, freeing the daughter cells (Figure 3, A)(Harris, 2009)(Cross and Umen, 2015).

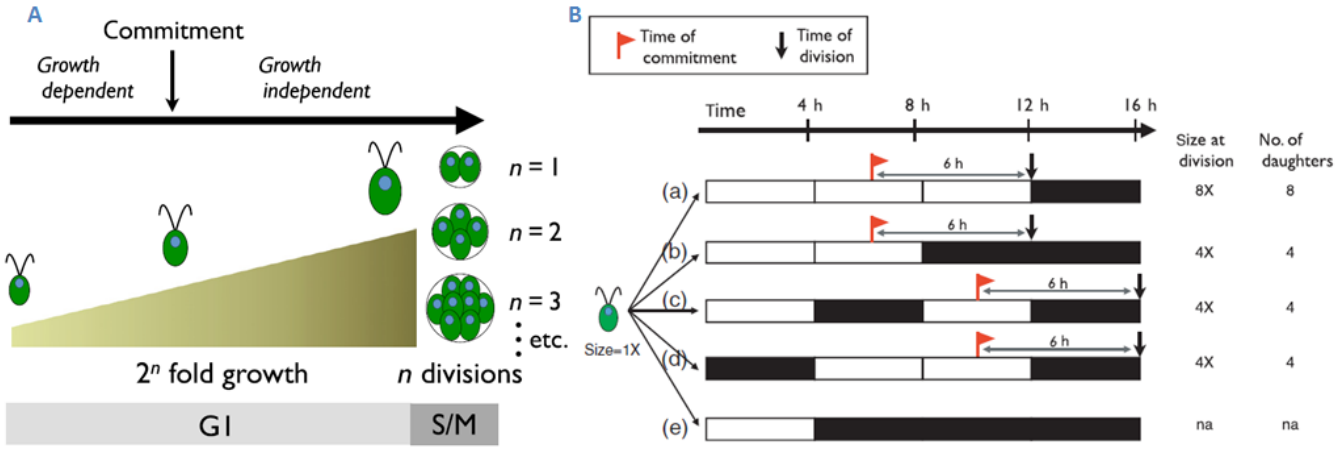


Figure 3. A) Schematic representation of C. reinhardtii multiple-fission cell cycle. Cell cycle is divided into two periods separated by the commitment point. Cell cycle progression before it is dependent in the growth and on cells reaching a minimum size, whereas after the commitment point there is not dependence on the growth anymore. During G1, cells grow and can reach sizes many-fold larger than their starting size. The cell division

number (n) is determined by the size of the mother, and typically ranges from one to three divisions to produce two, four or eight daughters. B) Experimental result of timing of commitment and cell division. The cells divide 6 hours after the commitment point during dark conditions (Cross and Umen, 2015).

For this multiple cells division, cells must be able to undergo more than one mass doubling during the G1 phase, and therefore supress the cell division temporarily, even although they have reached the minimum size to divide. This type of division is normally produced when the conditions of illumination are optimal (Figure 3, B)(Cross and Umen, 2015). However, when the growth conditions are not optimal (for instance lack of illumination, or nitrogen), cells of both mating type *mt+* and *mt-* differentiate into sexually competent gametes to undertake the sexual division (Harris, 2007).

Another important aspect which enhances the use of *C. reinhardtii* in research is that the genome sequence has been completely studied and published, so all nuclear, chloroplastic and mitochondrial genome are available (Blaby et al., 2014)(Hanikenne, 2003).

1.4 Biofuels from microalgae: the importance of lipids.

The importance of the microalgae for biodiesel production lies in the capacity of some microalgae to accumulate lipids up to 60% of their biomass, although, this ability is normally related with growth under stress factors that can compromise biomass productivity (Blatti et al., 2013).

Algal lipids are normally divided in two groups, the polar and the non-polar lipids. The main non-polar lipids are acylglicerols, sterols free (or non-esterified) fatty acids, hydrocarbons, wax and steryl esters, whereas in polar lipids phosphoglycerides and glycosylglycerides are highlighted (Guschina and Harwood, 2013).

Among all the lipid-like compounds microalgae synthesise, glycerolipids are the most abundant and best described. Glycerolipids are characterised by a glycerol backbone with one, two, or three fatty acids groups attached, which can be divided in two main classes according to their function in the cells, being storage or membrane lipids. Membrane lipids contain two fatty acyl groups and the third position is occupied by a polar side-group, whereas the storage lipids have three fatty acyl groups attached to the glycerol, and are known as triacylglycerides, or TAGs (Klok et al., 2014). The different types of lipids can be used to produce

biodiesel, hydrogen or even ethanol, but the produced energy potentials are obviously higher with storage lipids, such as TAGs or hydrocarbons (Gimpel et al., 2013). Hence, oils that are rich in storage lipids are highly desirable in a biofuel context because of their high fuel yield (Murarka *et al*., 2008)(Yazdani and Gonzalez, 2007).

However, the levels of accumulation of storage lipids are highly dependent on the conditions in which the algae are cultured. Normally, when algal growth slows down and there is no need for the synthesis of new membrane compounds, the cells divert fatty acids into TAG synthesis until the conditions for the growth are optimal again, although there are conditions which benefit the production, such as light intensity, nitrogen starvation, salinity, CO_2 concentration, etc. The problem of the modifications in the growth conditions is that the change in the lipids profile will alter the physical properties of membranes, which could lead to the unimpaired functioning in important physiological processes, for instance photosynthesis, respiration and membrane transport (Guschima and Harwood, 2013).

1.5 TAGs from algae: what is already known about them?

Algal TAGs are generally characterised because they are formed by three molecules of saturated or monounsaturated fatty acids which are used in the esterification of a molecule of glycerol. However, some species sometimes contain long chain polyunsaturated fatty acids (PUFAs) which are important when their levels are low, since the PUFAs of PUFA-rich TAG can be used. The presence of these three molecules of fatty acids in TAG determines the fuel yield, since they can all be used for transesterification, whereas in the case of diacylglycerols (DAGs) and monoacylglycerols (MAGs) just two or one molecules respectively can be used, while the remaining components cannot be converted to fuel feedstock (Guschima and Harwood, 2013).

However, despite the importance of the TAG in the production of biofuels, the biosynthesis pathway has not been profoundly studied, although it has been theoretically deduced according to homologies with bacteria and plants, where they have been already characterised (Blatti et al., 2013). In these bioinformatic analyses, a huge similarity in both the key regulatory steps and the import of the fatty acids to the endoplasmic reticulum between plants and algae has been

described (Khozin-Goldberg and Cohen, 2011). Currently two locations for the novo lipid biosynthesis are assigned, being the chloroplast (prokaryotic pathway) and the endoplasmic reticulum (ER)(eukaryotic pathway)(Figure 4)(Klok et al., 2014).

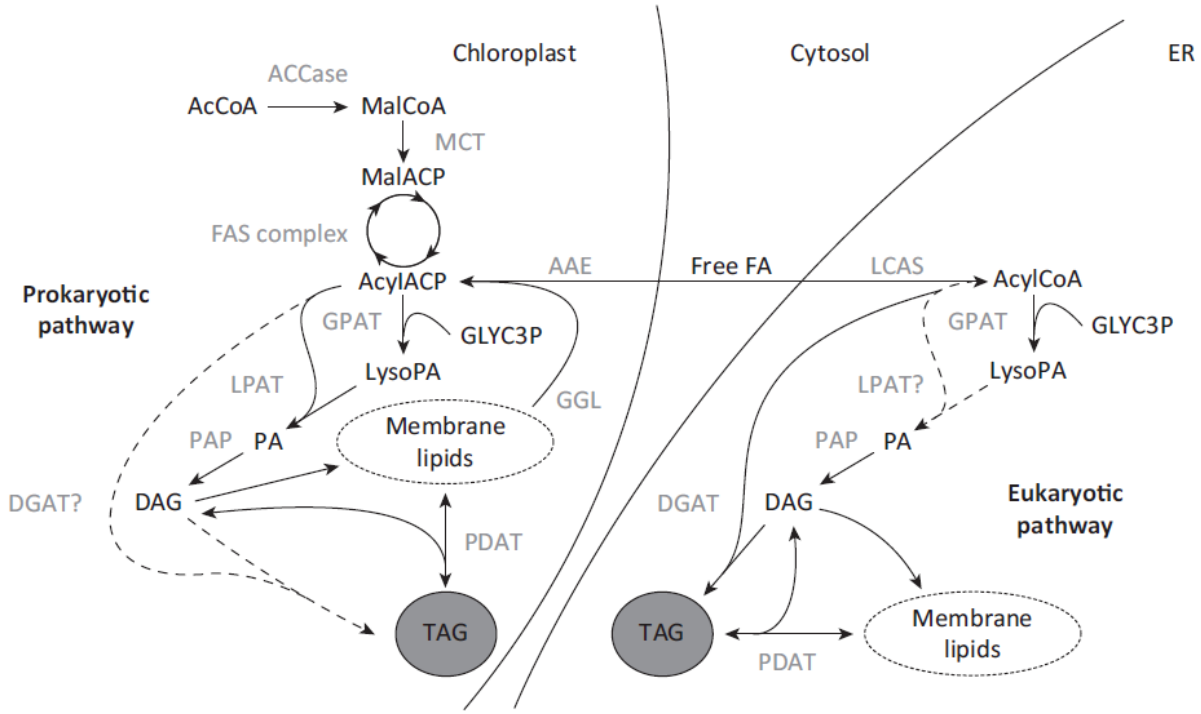


Figure 4. Schematic representation of TAGs biosynthesis in microalgae in the different subcellular locations proposed. Enzymes are coloured in grey whereas the substrates in black. Broken lines and '?' indicates uncertainty about the occurrence of these enzymes in the organelles. Abbreviations described in the text (Klok et al., 2014).

In algal chloroplast, fatty acids are biosynthesised by a type II fatty acid sinthase (FAS), which catalysis the elongation of a growing fatty acid by two carbon units, in its different domains, in an iterative pathway. The process begins as the acetyl-CoA carboxylase (ACCase) carries out the carboxylation of acetyl-CoA to form malonyl-CoA, which is transferred onto an acyl carrier protein (ACP) that acts as a metabolic scaffold during fatty acid biosynthesis. This transference is regulated by malonyl-CoA:ACP transacylase (MCT). Then, FAS acts to extend the malonyl-ACP through its different domains. First, β-ketoacyl-ACP-synthase III (KASIII), the initiating ketosynthase domain, condenses acetyl-CoA with malonyl-ACP to form a β-ketone extended 2-carbon unit on acyl-ACP, which is then reduced by ketoreductse (KR), dehydratese (DH) and enoyl reductase (ER) ezymes. The cycle repeats seven more times, and when the fatty acid reaches the destined length, it is removed from the ACP group by an acyl-ACP esterase (AAE) to produce fatty acids, and they can be maintained in the chloroplast, or transferred to the

cytosol. In the case they remain in the cytosol, fatty acids are transferred to glycerol-3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) to produce lysophosphatidic acid (LysoPA), and then, LysoPA acyltransferase (LPAT) adds an acyl chain to yield phosphatidic acid (PA) which is subsequently dephosphorylated by PA phosphatase (PAP) to yield DAG. After that, DAGs can be used for their incorporation into membrane lipids, or to form TAG via two different routes. In the first route, the fatty acyl group can be transferred from acyl-ACP by dyacylglicerol acyltransferase (DAGT) in the final step of the Kennedy pathway (which entails the three acylations of glycerol-3-phosphate). In the second, fatty acyl donors from membrane lipids are used for the production of TAG, either using phospholipid:diacylglycerol transferase (PDAT), or by means of specific lipase activity on membrane lipids, which liberates the fatty acids. On the other hand, in the case they are transferred to the cytosol they diffuse into the envelope of the chloroplast and are esterified with Coenzyme A by CoA ligase enzyme, and the fatty acyl-CoA departs from the chloroplast for the incorporation to the cellular lipids (Blatti et al., 2013)(Klok et al., 2014). These free fatty acids are used for feeding the production of TAGs in the ER, so they are transported through the membranes and then attached to CoA by long-chain acyl-CoA synthase (LCAS). From here, the same sequence of reactions is followed as in the plastid, using acyl-CoA as a substrate instead of acyl-ACP (Figure 4).

However, the presence of the enzymes from the eukaryotic pathway in some microalgae has not been confirmed yet, although some studies have shown that plastidic lipids in *C. reinhardtii* are only synthesised in the chloroplast, whereas the extraplastidic lipids are exclusively produced in the ER, so a strict compartmentalization is observed. On the contrary, in plants, both pathways provide lipids for both plastidic and extraplastidic membranes (Klok et al., 2014).

TAG synthesis is recognised as a means for storage of carbon and energy in many organisms, including microalgae. When cell proliferation is limited by adverse growth conditions, TAG synthesis allows continuous capturing of energy and carbon, which can be then employed when favourable conditions are restored. Under normal conditions, NADPH is employed for cell proliferation, but when the conditions are adverse, there is an excess of NADPH, which can produce a

dangerous over-reduction of the photosynthetic electron transport chain, and this is prevented by the synthesis of highly reduced compounds, such as TAG.

In addition, in adverse growth conditions, chloroplast size and structure are severely reduced, but microalgae can restore them thanks to the accumulation of certain levels of depository TAG in TAG-filled lipid bodies (LBs). However, the dynamics of LBs are much complex, since other biomolecules have been found, such as photosynthetic pigments or proteins, which do not have obvious connection with TAG synthesis. Hence, presence of TAGs have made LBs garner attention for biofuel production (Kok et al., 2014)(Liu and Benning, 2013).

1.6 Biotechnology to improve the production of biofuels.

However, wildtype microalgae normally do not have innate metabolic pathways optimised for industrial fatty acid production, moreover, the fatty acids produced are not of ideal length and saturation for biodiesel properties (Blatti et al., 2013). In addition, some species of algae accumulate starch as a storage metabolite, maintaining the lipid content relatively stable, so an efficient approach could be the shift of the carbon flux from starch accumulation to TAG biosynthesis (Klok et al., 2014)(Guschima and Harwood, 2013).

Hence, genetic engineering the fatty acid biosynthesis seems a promising strategy to try to increase the lipid yield and quality in algae, especially in cyanobacteria, since they easier to manipulate genetically because of their prokaryotic origin (Figure 5)(Blatti et al., 2013)(Klok et al., 2014).

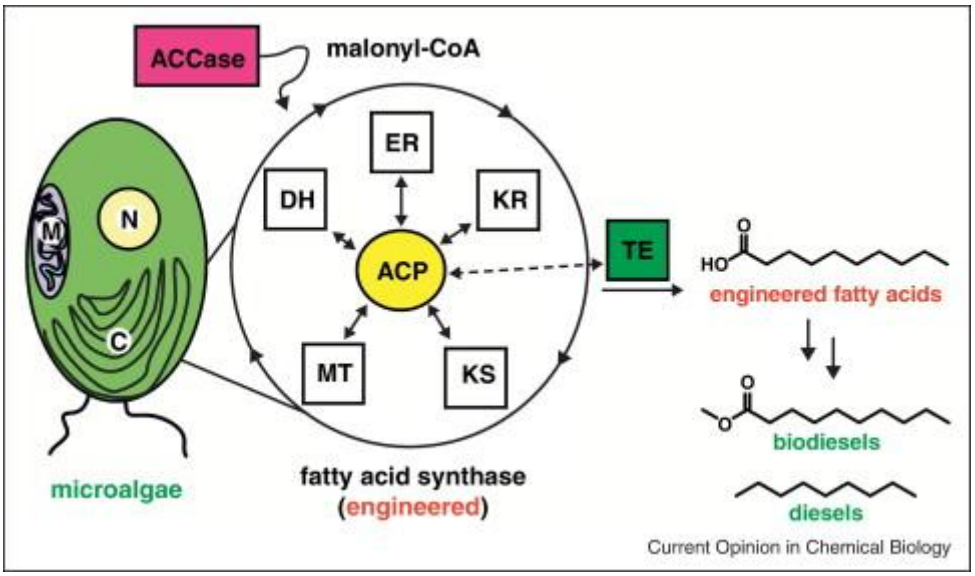


Figure 5. Possible targets to improve the quality of biodiesel in microalgae. After the carboxylation of acetyl-CoA to form malonyl-coA via (ACCase) it attaches to the acyl carrier protein (ACP)and an iterative cycle of chain elongation commences by the action of ketosynthase (KS), ketoreductase (KR), dehydratase (DH) and

enoylreductase (ER) domains and each turn results in an addition of two carbons to the growing fatty acid. Once it reaches a mature lenght a thioesterase (TE) domain catalyses the hydrolysis of the fatty acid and, hence, its release from ACP. Engineering TEs helps to modify the length of the fatty acid chain up to the desired size, so this is a main target in the production and improvement of the biodiesel quality. Abbreviations: C: Chloroplast; M: Mitochondria; N: Nucleus. (Blatty et al., 2013).

In addition, there are intracellular factors which may affect the growth of the cells and other important characteristics of the culture. In the case of higher plants, phytohormones have an important regulatory role in many aspects, such as germination, seed development, vegetative development, and also in growth and biomass production. However, the biosynthesis pathways and roles of the phytohormones in microalgae are not completely understood, so more research in the topic could lead to the use of phytohormones to enhance the biomass production and the accumulation of compounds of interest simultaneously by chemical or genetic manipulation of the enzymes involved in phytohormone metabolism (Figure 6) (Lu and Xu, 2015).

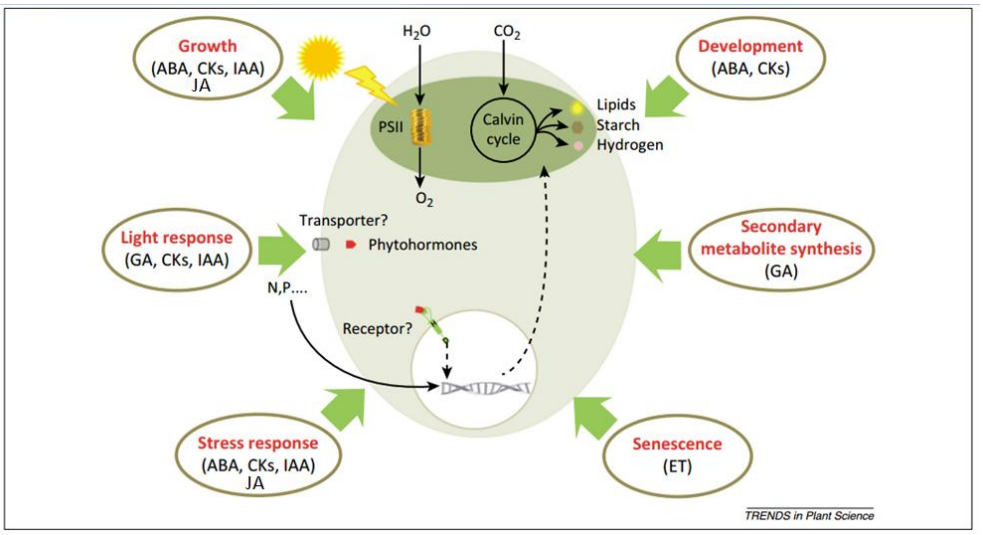


Figure 6. Effect of the endogenous phytohormones on microalgae and desirable objectives to improve economically important traits in microalgae. Abbreviations: ABA: Abscisic acid; CKs: Cytokinins; ET: Ethylene; GA: Gibberelins; IAA: Indolacetic acid; JA: Jasmonic acid; PSII: Photosystem II (Adapted from Lu and Xu, 2015).

Among all the phytohormones, plants produce, jasmonic acid (JA)(and other jasmonates), which are particularly interesting because of their roles in diverse developmental processes, such as seed germination, gravitropism, fertility, embryo development, and especially important, in root and plant growth and in the responses to biotic and abiotic stress (Kazan, 2015), and these functions can be carried out since jasmonates and their metabolites, like other lipid-derived

molecules, are able to act as secondary messengers in cell signaling when stress conditions appear (Waternack, 2007).

1.7 Oxidation products as signaling factors.

Under stress conditions, reactive oxygen species (ROS) are normally formed which cause oxidative damage, since they cause the oxidation of proteins, nucleic acids and lipids. For this reason, cells have different mechanisms to avoid the oxidation of the biomolecules that are mainly based in antioxidants. These antioxidants can be different types of enzymes that degrade the ROS, or even other compounds such as methyl-jasmonate (MeJA) which trigger the induction of antioxidants (Dar et al., 2015).

Jasmonates are cyclopentanones, derived from fatty acids through oxidation reactions, which belong to the family collectively called as oxylipns, that are produced via the oxidative metabolism of polyunsaturated fatty acids, and include jasmonate and their metabolites, such as JA, MeJA, or JA amino acid conjugates (Dar et al., 2015).

Oxylipins are synthesised from α-linoleic acid (18:3) (α-LeA), which is released from the chloroplast membrane by lipase activity, and in this moment, lypoxygenases (LOXs) will form hydroperoxides. Hence, LOXs represent a key step in the oxylipins pathway, so understanding the role of the enzyme in the synthesis will be important to avoid the oxidation of the lipids.

Up to now, these enzymes have been classified depending on the positional specificity of the oxidation over the fatty acid, so their name is derived from the carbon number in which they act (5-LOX in carbon 5, 8-LOX in carbon 8, etc.). However, nowadays, the main classification of the different types of LOXs is made according to their subcellular locations: extraplastidial LOXs, which are designated as type 1-LOX, or plastidial LOXs, which have been classified as type 2-LOX (Figure 7)(Andreou and Feussner, 2009)

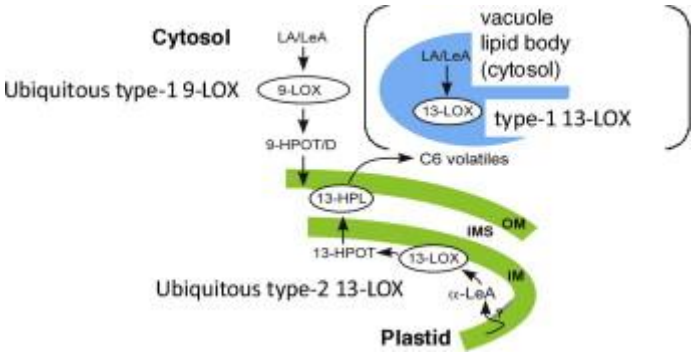


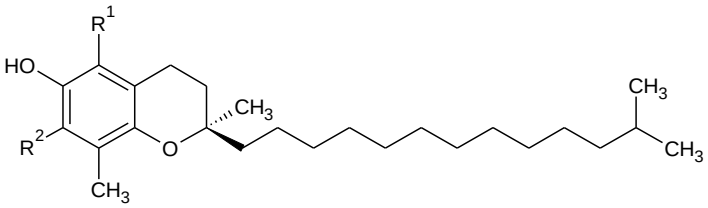
Figure 7. Intracellular locations of the LOX pathways reactions. Abbreviations: OM: Outer membrane of the chloroplast envelope; IM: Inner membrane of the chloroplast envelope; IMS: inter membrane space.

Since the JA is produced under stress conditions in algae, and microalgae are normally grown in stress conditions to improve the yield of different compounds of interest, the oxidation of lipids by JA synthesis via LOX pathway could be activated. All genes encoding enzymes of JA biosynthesis are JA-inducible, and the promoters are more active under JA treatment, thus, a positive feedback regulation has been proposed (Wasternack, 2007). Hence, even though the stress conditions disappear, the LOX enzyme encoding genes can still being transcribed, and the lipids fractions of the algae affected by the oxidation, which is a disadvantage for biofuels production. The silencing of the LOX enzymes does not seem possible owing to the different roles both jasmonic acid and LOXs has in the cells, so different antioxidant compounds have to be found in order to find a solution for the possible oxidation of the lipids.

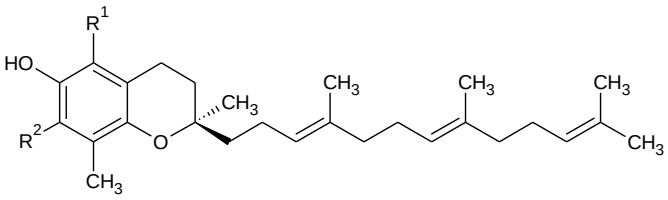
1.8 Antioxidants for the protection of lipids.

Among the components which are largely known as protectors of the degradation of lipids, vitamin E was proposed since the 50's when Tappel and colleagues described the chemical prevention of the oxidation of lipids (Zingg, 2007). The role of vitamin E has been extensively studied in humans and animals, and later the research has been focused in their function in plants, where they are synthesised. The group of vitamins E is integrated by tocopherols and tocotrienols, which are collectively known as tocochromanols. Tocochromanols are lipid-soluble molecules which consist in a chromanol ring system and a polyprenyl side chain, which is saturated in tocopherols and 3-fold unsaturated in tocotrienols. Both subgroups of the tocochromanols can be classified into different homologues (α-, β-, γ-, and δ-forms) depending on the number and position of methyl groups at the chromanol ring (Figure 8). This structural differences may imply certain variations

the biochemical properties and functions of the analogues (Falk and Munné-Bosch, 2010).



Tocopherol



Tocotrienols

R_1	R_2	
CH_3	CH_3	α
CH_3	H	β
H	CH_3	γ
H	H	δ

Figure 8. Tocochromanols and different α, β, γ and δ-variants according to the possible variations in radical groups (Adapted from Zingg, 2007).

Between the tocochromanols, tocopherols are particularly interesting since they can be exclusively synthesised in all photosynthetic organisms, including higher plants, algae, and specially in seeds, whereas tochotrienols are not (Zingg, 2007).

However, the complex structure α-tocopherol complicates their use, and this is the main reason why other vitamin E analogues have been studied. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylyc acid)(Figure 9) is a water-soluble analogue of α-tocopherol which has been the centre of the attention the lasts years due to their chemical properties as a O_2 scavenger, which give some advantages.

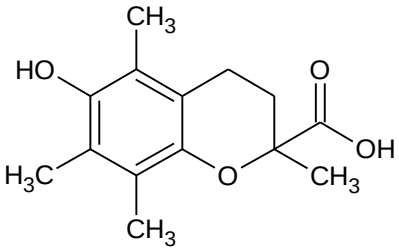


Figure 9. Chemical structure of Trolox (Oehlke et al 2011).

First, Trolox offers higher mobility and scavenging activity than α-tocopherol due to the simplest structure, since the last has very limited mobility owing to the 16 carbons length chain. Second, Trolox can be easily dissolved in neutral and basic pH-adjusted solutions since it has two dissociable protons, with p*K* values of 3.89 for the carboxylic group and 11.92 for the hydroxyl group, which affect its solubility (Arellano et al., 2011). Moreover, there is a profound knowledge of the mechanisms of scavenging, and also, some of the products of the oxidation reactions may act as antioxidants themselves, which could give even more protection (Oehlke et al., 2011).

1.9 Objectives.

Aston University has been committed to the research and development of bioenergy technologies and solutions since the 1970s. In 2008 the bioenergy research and facilities were combined to form the European Bioenergy Research Institute (EBRI), which from that moment on delivers world-class bioenergy research. The algae lab from EBRI is focused in the stimulation of the biomass growth in order to improve the biofuel production process.

The aims of this study will be:

a) Determination of the effect of Vitamin E analogues in the growth of the cell wall mutant *C. reinhardtii*.

b) Estimation of the different toxic profiles of the Vitamin E analogues.

c) Evaluation of the different antioxidant ability of the Vitamin E analogues over lipids enzymatic oxidation in cell wall mutant *C. reinhardtii*.

d) Comparison of the lipid profile of the cell wall mutant *C. reinhardtii* when it growths under different vitamin E analogues.

e) Study of the possible differences in the thermical behaviour of cell wall mutanta *C.* reinhardii grown under different treatment with vitamin E analoges.

2. MATERIALS AND METHODS.

2.1 *Chlamydomonas* culture strain and growth media preparation.

The cell wall mutant *Chlamydomonas reinhardtii* (CC-406) strain used in the experiments was obtained from the Chlamydomonas Resource Centre University of Minnesota (USA).

Once arrived, in order to culture the algae strain Tris-Acetate-Phosphate (TAP) medium prepared according to Gorman and Levine method (Gorman and Levine, 1965) was used. To culture the algae in a solid medium, 1.3% purified agar was added to the TAP liquid media and autoclaved, and then the solution was distributed in Petri dishes (20 ml per dish) in the laminar flow cabinet to prevent from contamination, and were left until solidification.

2.2 Evaluation of the effects of Vitamin-E analogues on growth.

2.2.1 Growth conditions and biomass determination.

The cell-wall mutant strain of *C. reinhardtii* (CC-406) was maintained on TAP agar medium at 25°C under photoperiod 12:12. A single colony was used to make a starter culture in a 250 ml Erlenmeyer flask containing 40 ml of liquid TAP medium. The starter culture was grown under continuous light at 25°C on a incubator shaker (Incubator Shaker Series Innova®4) at 200 rpm for 5 days. Then, the algae were sub-cultured using 2 ml of the starter culture and inoculating 5 new 250 ml Erlenmeyer flasks containing 40 ml liquid TAP medium and were grown in the same conditions to be used for further experiments.

After that 1.8 ml of algae were mixed with 0.2 ml of the chemical compounds to be tested, these being three vitamin E analogues (α-tocopherol, γ-tocopherol and δ-tocopherol), two chemical forms of the analogue of the vitamin E Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, or carboxylic-Trolox (Tr-COOH), and its reduced form 2-(hydroxymethyl)-2,3,7,8-tetramethyl-6-chromanol, or alcoholic-Trolox (Tr-OH)) and a LOX inhibitor ((6,7-dihydroxycoumarin) or Esculetin) (Figure 10).

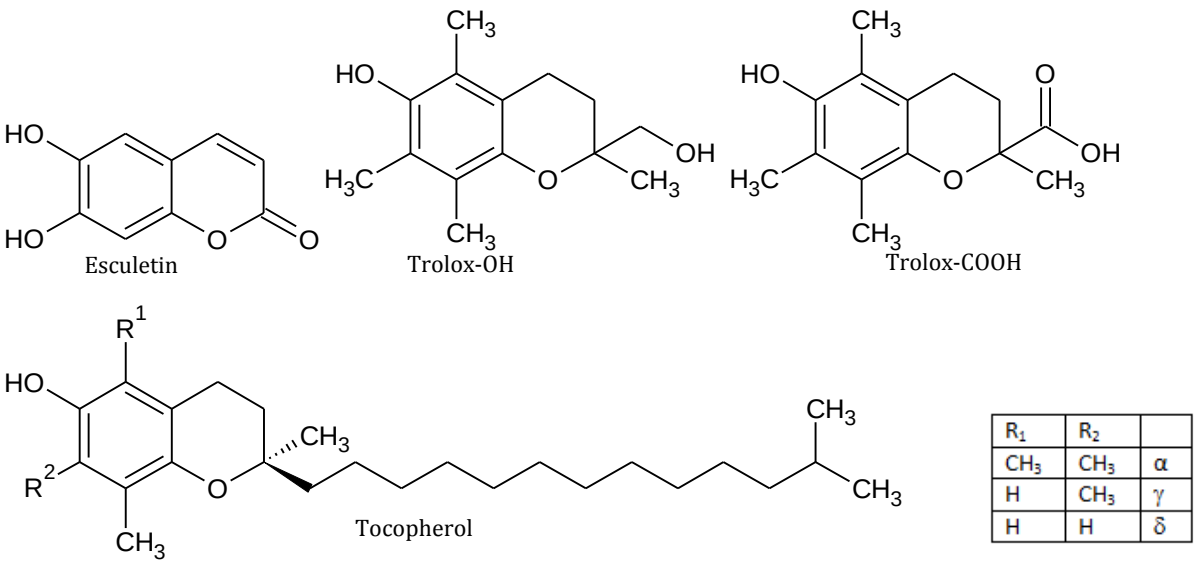


Figure 10. Compounds tested in the different experiments.

Concentrations tested were 10 and 100 µM, and 1, 2, 5 and 10 mM. For control and each different concentration of the compound, three replicates were prepared and the cells were counted daily, up to 144 hours with a 100 µm depth Neubauer improved bright line hemocytometer by transmitted light under a 220-240V optical microscope(Fischer scientific® 0.5A 50Hz 12V/20W). The number of cells per ml was calculated according to the volume of the hemocytometer following the formula 1.

$$\frac{Number\ of\ cells}{Volume\ of\ the\ hemocytometer} = \frac{Nº\ of\ cells}{0.1\ mm^3} = Nº\ of\ cells \cdot 10^4 = \frac{Nº\ of\ cells}{ml} \qquad (1)$$

2.2.2 Kinetic parameters.

Once the concentration of cells in the samples was known, to evaluate the growth of the cells in the different conditions, two common kinetic parameters were evaluated: growth rate and population doubling time (Andersen, 2005). These parameters were calculated according to the kinetics first proposed by Guillard in 1973 (Guillard, 1973), who explained that the growth of the cells followed the next equation:

$$\frac{dN}{dt} = \mu \cdot N \qquad (2)$$

Where N is the number of cells per ml, μ the growth rate for the population (expressed in hours^{-1}) and t the time of culture (expressed in hours), so the variation of the cells in time will depend on the number of cells and the growth rate of them. Thus, to calculate the growth rate of the cells and the population doubling time the derivative of the formula 2 was calculated:

$$\frac{dN}{dt} = \mu \cdot N \rightarrow \frac{dN}{N} = \mu \cdot dt \rightarrow \int_{N_0}^{N} \frac{dN}{N} = \mu \cdot \int_{t_0}^{t} dt \rightarrow \ln\left(\frac{N}{N_0}\right) = \mu \cdot (t - t_0) \rightarrow \frac{N}{N_0} = e^{\mu(t-t_0)}$$

$$N = N_0 \cdot e^{\mu(t-t_0)} \quad (3)$$

Where N_0 is the number of cells per ml at the beginning of the interval, and t_0 the time when the interval started. Solving the equation 3 to obtain the value of μ:

$$N = N_0 \cdot e^{\mu(t-t_0)} \rightarrow \frac{N}{N_0} = e^{\mu(t-t_0)} \rightarrow \ln\left(\frac{N}{N_0}\right) = \mu \cdot (t - t_0) \rightarrow \mu = \frac{\ln(N) - \ln(N_0)}{(t-t_0)}$$

$$\mu = \frac{\ln(N) - \ln(N_0)}{\Delta t} \quad (4)$$

After the growth rate was calculated, the population doubling time (dt, $t_{1/2}$) was also estimated. In the moment of the dt, the number of cells was the double than at the beginning, so:

$$N = 2N_0$$

$$N = N_0 \cdot e^{\mu \cdot t_{1/2}}$$

$$2N_0 = N_0 \cdot e^{\mu(t-t_0)} \rightarrow \frac{2N_0}{N_0} = e^{\mu(t-t_0)} \rightarrow 2 = e^{\mu \cdot t_{1/2}} \rightarrow \ln(2) = \mu \cdot t_{1/2}$$

$$t_{1/2} = \frac{\ln(2)}{\mu} \quad (5)$$

2.2.3 Toxicological analyses.

For the determination of the toxicological effects of the compounds in growth, the median lethal concentration (LC_{50}) and the No-Observed-Adverse-Effect Concentration (NOAEC) were calculated according to recommendations for acute toxicology analysis by the US Environmental Protection Agency (EPA) (EPA, 2002). LC_{50} is referring to the concentration of a compound which is able to kill the fifty percent of the test organisms, whereas NOAEC is the highest concentration at

which survival is not significantly different from the control. LC_{50} was determined by the graphical method representing the percentage of growth inhibition versus the logarithmic concentration of the compound. In the case of NOAEC the hypothesis testing for a multi-concentration acute toxicity test.

2.3 Evaluation of the effects of Vitamin-E analogues on lipid profile.

2.3.1 Growth conditions and algae harvesting.

To study the lipid profile the starter culture of *C. reinhardtii* was used to inoculate TAP medium. After that, 198 ml of the inoculated media were transferred to 2 L Erlenmeyer flasks and 2 ml of the desired compound and concentration were added. Only compounds which showed stimulation or non significant differences with respect to controls were studied. Then the cell were grown for 5 days until the end of the exponential growth phase in an incubator shaker (Incubator Shaker Series Innova®4) under continues illumination with florescent lamps at 25ºC, and continuous shaking speed at 200 rpm. After the 5 days, the cells were then harvested by centrifugation at 5000 G and 25ºC for 10 minutes in a Beckman J2-MC centrifuge. In order to make the most of the biomass cultured, pellets were resuspended in TAP medium and centrifuged in a 5424 eppendorf centrifuge at 5000G for 10 minutes were also performed.

2.3.2 Lipid Extraction.

Total lipids were extracted from whole cells by a modified Bligh and Dyer method (Jackson et al., 1998) based on the use of organic solvents mixture (non polar and polar). First 0.2 g of *C. reinhardtii* cells were transferred into a collector column, and homogenised using 1 ml of TAP medium. After that, the cells were transferred to a Pyrex tube and were acidified with 1 ml of acetic acid 0.15M, and 7.5 of chloroform/methanol mix (1:2 v/v) were also added, and this was followed by the addition of 2 ml of chloroform and 2 ml of distilled water. After the separation of the different layers, the chloroform layer (at the bottom) containing the lipids was separated into a new Pyrex tube and finally a N_2 gas atmosphere was

created in the tube to avoid the oxidation of lipids, and were stored at -20ºC until needed.

2.3.3 Total Lipid Determination.

For the total lipid determination gravimetric analyses were carried out. For the determination 0.1 g of *C. reinhardtii* biomass was transferred into a previously weighted pyrex tube and the lipids were extracted by the modified Bligh and Dyer method previously explained in section 2.3.3. Once the lipids were extracted, they were separated and weighted to determine the wet weight of the lipids with respect to the total biomass.

2.3.4 Gas chromatography/mass spectrometry (GC-MS) analyses.

For the identification of lipids on GC-MS two different approaches were followed: identification of total lipids directly extracted from biomass, and identification of purified fractions of the lipids extracted.

When the different fractions of lipids were studied, they had to be first purified by thin-layer chromatography (TLC). To do so, lipids samples stored in chloroform were evaporated under N_2 gas until complete removal of the solvent, and then the samples were resuspended in 500 µl of hexane and the lipids were separated by TLC on a pre-coated silica gel plate (Merck, silica gel 60). Neutral lipids were separated using hexane/diethyl ether/acetic acid (70:30:1, by vol.) and polar lipids using chloroform/methanol/acetic acid/water (170:30:20:5, by vol.). Lipids were after located by staining with iodine vapour.

After the identification, the fractions of the desired lipids were removed from the TLC plates and transferred to different Pyrex tubes for transesterification and formation of fatty acid methyl esters (FAMEs)(In case of determination od total lipids the process started from this moment on). After that 50 µl of the internal standard heptadecanoic acid (17:0) 2nm and 2 ml of 2.5% (v/v) sulphuric acid in anhydrous methanol were added and the tubes were incubated for 90 minutes at 70°C. After the incubation, 1.5 ml of isohexane and 1.5 ml of H_2O were added, followed by the removal of the hexane phase into 2 ml amber vials. The samples were then evaporated under N_2 until an approximate volume of 0.3 ml and

were kept in the -20°C freezer for its further quantification analysis in the GC/MS, or directly run.

To determine the fatty acid composition of the algae on a DB-23 capillary column (length 30 m, id 0.25 mm, film thickness 0.25 µm). The GC injector was operated in split mode (50:1) with an inlet temperature of 250ºC. The column temperature was hold at 50ºC for 1 minute, and then increased at 25ºC/min to 175ºC and followed by a second ramp with heating of 4ºC/min up to 235ºC maintained for 15 minutes. Assignment of FAMES was made by using a NIST08 MS library.

2.4 Evaluation of the effects of Vitamin-E analogues on lipid oxidation.

2.4.1 Thiobarbituric acid-reactive substances (TBARS) assay.

The amount of malondialdehydes (MDAs) was determined as an index of general lipid peroxidation by TBARS assay since it is a secondary end-product of polyunsaturated fatty acid oxidation. The thiobarbituric acid (TBA) reagent was prepated by dissolving 0.6% (w/v) TBA in 0.05 M sodium hydroxide (NaOH). Moreover, an acid buffer was prepared mixing 90 ml of 0.2 M acetic acid with 0.2 M sodium acetate trihydrate.

After that, 0.2 g of *C. reinhardtii* were homogenised with 0.5 ml of trichloroacetic acid (TCA) (10% w/v). The homogenate was washed twice with 2.5 ml acetone and centrifuged (4000G for 10 minutes). Then, the pellet was incubated at 100ºC for 30 minutes with 1.5 ml of 1% phosphoric acid (H_3PO_3) and 0.5 ml of 0.6% of TBA. After that it was cooled on ince and 1.5 ml of n-butanol were added and the mixture was centrifuged again at 4000G for 10 minutes. The persistence of the butanolic layer was evaluated by measuring the difference between the absorbances at 532 nm and 590. The concentration of MDA was measured using a MDA standard calibration curve.

2.5 Pyrolysis-Gas chromatography-Mass spectrometry (Py-GC-MS) of the algae biomass.

After harvesting, biomass was vacuum dried in an oven at 60ºC overnight until water was completely removed. After that, biomass was grinded and sieved

to obtain homogeneous fraction (<0.25 mm). Analytical flash Py was performed with approximately 3 mg of biomass sample in a quartz tube in a pyroprobe 5000 Series at 500ºC with a total time of 30 s. The devolatilised compounds were transferred via heated transfer line maintained at 310ºC onto the injector, and then to the 1701 Perkin Elmer GC Column (30 m 0.25 mm i.d. 0.25 µm df). The GC oven was held at 45ºC for 2.5 min and then ramped at 2.5ºC to 250ºC hold for 30 s. Mass spectra were obtained for the molecular mass range m/z= 45-300 for a total time of 85 minutes. The GC carrier gas was Helium (25 ml/min) The FID make up mixture was H_2 with flow of 45 ml/min and air with flow of 450 ml/min and were maintained at 250ºC.

2.6 Analytical and statistical analyses.

Calculation of the growth parameters and their representation was performed with Microsoft Excel (Microsoft, Redmond, WA). Only graphs corresponding to biomass growth (10^6 cells/ml) were included. Microsoft Excel was also used for the determination of LC_{50} by the graphical method.

Only were represented and statistically studied concentrations in which growth was observed. Hence, concentrations with total inhibition were not had into account.

Statistical analyses were carried out using SPSS (IBM, Armonk, NY). To compare the statistical significance of the differences and equalities between the treatments, Student's t-test were performed. Maximum concentrations of biomass, maximum growth rates and minimum duplication times for the different compounds concentrations were compared with controls treatments in the case of growth studies, whereas for the analyses of the differences in the lipid profile of the biomass in the different treatments, the percentage of total lipids compared in treatments compared to controls. For TBARS analysis, the total content of MDA for each treatment was statistically compared too. In all analyses a 95% confidence interval was used, so results were considered significant at $p<0.05$. Data were represented as average± standard deviation.

NOAEC was also determined with SPSS using the different tests available and necessary according to the recommendations by EPA.

3. RESULTS.

3.1 Growth parameters.

Growth kinetics for the different compounds is shown in figures 11, 12, 13, 14, 15 and 16. To evaluate the different results obtained with them, maximum values of concentration and growth rate, and minimum duplication times were studied and compared with controls (Tables 2, 3, 4, 5, 6 and 7).

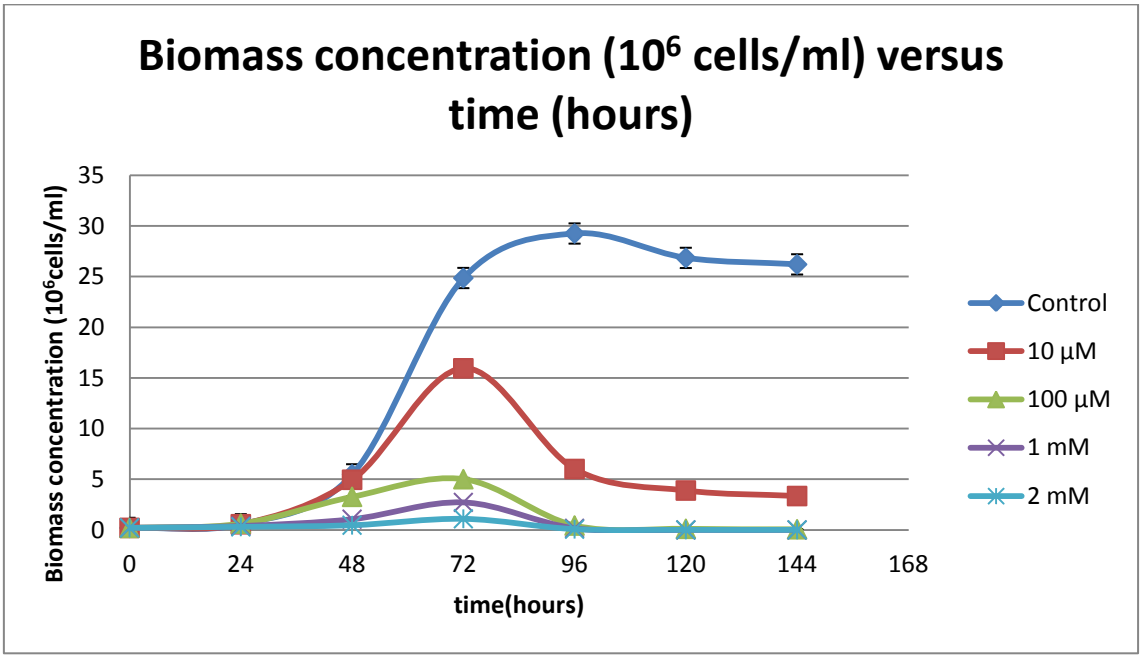


Figure 11. Biomass concentration (10^6 cells/ml) versus time (hours) for different Esculetin concentrations in which growth was observed, and Control (Blue).

In the case of Esculetin, the growth was clearly inhibited from lower to higher concentrations (Figure 11). When the maximum concentrations were analysed, the maximum concentration with all Esculetin concentrations in which growth was observed (15.94±0.346 for 10 µM, 5.01±0.130 for 100 µM, 2.72±0.108 for 1 mM and 1.10±0.122 for 2 mM) was significantly lower than controls (29.25±0.834), however, the maximum

growth rate was the same than control (0.096±0.014) for treatments with Esculetin at 10 and 100 µM (0.091±0.007 and 0.069±0.002 respectively), whereas significant differences were observed for higher concentrations (0.042±0.010 for 1 mM and 0.037±0.006 for 2 mM). For the minimum duplication times no significant differences were observed between control (7.36±1.057) and 10 µM (7.67±0.604), whereas the rest concentrations had significantly higher duplication time, and therefore their growth was slower (10.04±0.356 for 100 µM, 16.95±3.970 for 1 mM and 19.33±3.077 for 2 mM)(Table 2).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	µ	Dt
Control	29.25±0.834	0.096±0.014	7.36±1.057	-	-	-
10 µM	15.94±0.346	0.091±0.007	7.67±0.604	0.002	0.636	0.679
100 µM	5.01±0.130	0.069±0.002	10.04±0.356	0.000441	0.079	0.036
1 mM	2.72±0.108	0.042±0.010	16.95±3.970	0.000420	0.004	0.031
2 mM	1.10±0.122	0.037±0.006	19.33±3.077	0.000257	0.008	0.010

Table 2. Maximum concentrations and growth rate and minimum duplication times observed for different concentrations of Esculetin, and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when p<0.05.

Similar inhibitory effects were observed for carboxylic Trolox (Figure 12), although in this case, the growth was inhibited at lower concentrations (19.91±0.176 and 0.071±0.025 for 10 and 100 µM respectively compared with 24.57±1.848 for the controls). Despite of this, 10 µM and controls showed statistically the same maximum growth rate and minimum duplication time (0.089±0.008, 7.85±0.756 and 0.081±0.005 and 8.55±0.557 respectively for controls and 10 µM), whereas for 100 µM data are not shown since their values are negative because of cell death since the beginning of the experiment (Table 3).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	µ	Dt
Control	24.57±1.848	0.089±0.008	7.85±0.756	-	-	-
10 µM	19.91±0.176	0.081±0.005	8.55±0.557	0.028	0.253	0.249
100 µM	0.071±0.025	N.S	N.S	0.000131	N.S	N.S

Table 3. Maximum concentrations and growth rate and minimum duplication time observed for different concentration of Trolox-COOH and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when p<0.05. N.S: Not shown since negative values were omitted.

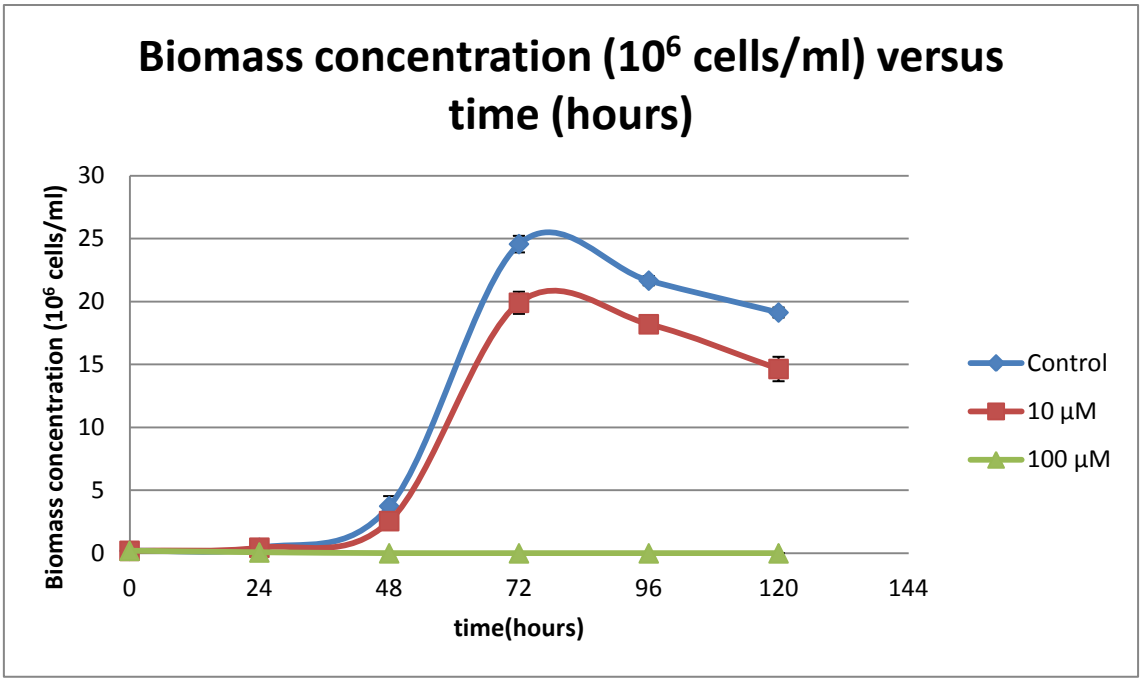


Figure 12. Biomass concentration (10^6 cells/ml) versus time (hours) for different Trolox-COOH in which growth was observed, and Control (Blue).

Surprisingly, for alcoholic Trolox different results were observed (Figure 13). Lower concentrations of the compound resulted in an stimulation of the growth, since significant differences were observed in maximum concentration for 10 (30.32±1.328) and 100 µM (30.75±0.624) with respect to controls (26.95±0.242), whereas higher concentrations turned inhibitory. For 1 mM significant inhibition was observed (1.51±0.182). In the case of maximum growth rate and minimum duplication time, no significant difference was observed for 10 (0.092±0.005 and 7.53±0.422) and 100 µM (0.093±0.004 and 7.43±0.358) with respect to controls (0.090±0.002 and 7.30±0.132), so they can be considered as equal. On the other hand, significant difference was observed for 1 mM, since the growth rate is clearly slower and the duplication time higher (0.042±0.006 and 16.85±2.445 respectively) (Table 4).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	µ	Dt
Control	26.95±0.242	0.090±0.002	7.30±0.132	-	-	-
10 µM	30.32±1.328	0.092±0.005	7.53±0.422	0.048	0.493	0.514
100 µM	30.75±0.624	0.093±0.004	7.43±0.358	0.017	0.278	0.278
1 mM	1.51±0.182	0.042±0.006	16.85±2.445	0.000002	0.007	0.023

Table 4. Maximum concentrations and growth rate and minimum duplication time observed for different concentration of Trolox-OH and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when $p<0.05$.

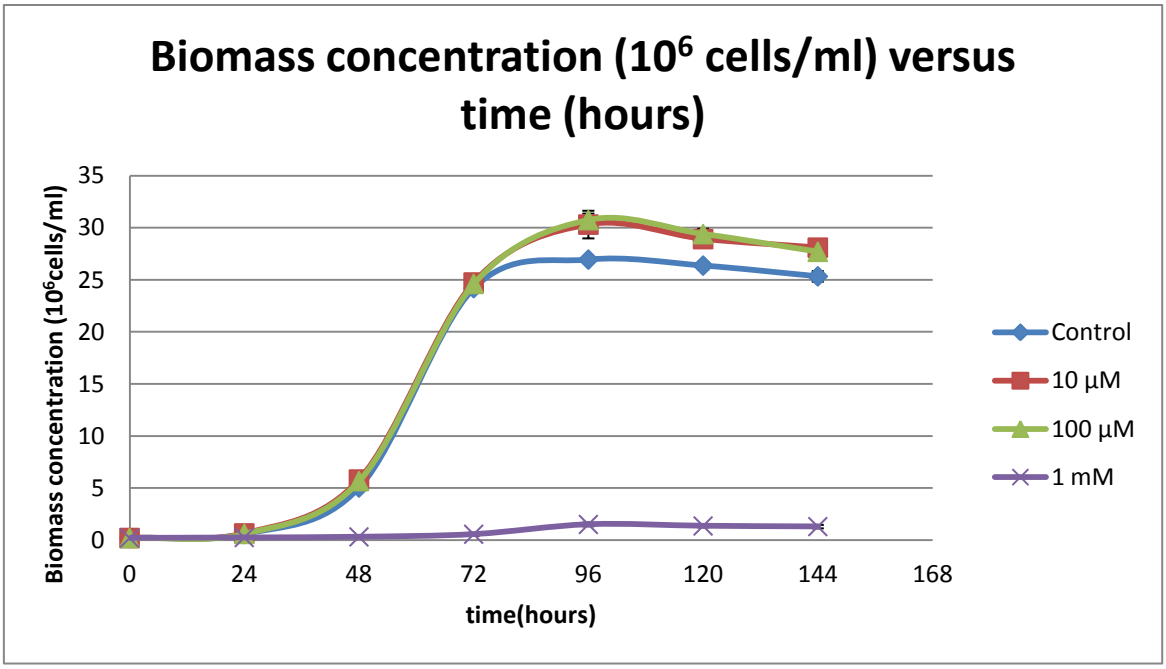


Figure 13. Biomass concentration (10^6 cells/ml) versus time (hours) for different Trolox-OH concentrations in which growth was observed, and Control (blue).

In the case of α-tocopherol (Figure 14), the growth kinetic shown in control and 10 µM is statistically the same, hence the maximum values of concentration (39.71±0.622 for control and 40.34±0.060 for 10 µM) are significantly equals (Table 5). These similarities are also maintained in the study of the growth parameters since the growth rates (0.082±0.005 for controls and 0.079±0.013 for 10 µM) and duplication times (8.51±0.530 and 8.96±1.480 for controls and 10 µM respectively) are again statistically similar. On the other hand, at 100 µM the growth was statistically inhibited (down to 13.83±0.227), and the growth was slower in both growth rate and duplication time (0.060±0.002 and 11.58±0.343 respectively).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	µ	Dt
Control	39.71±0.622	0.082±0.005	8.51±0.530	-	-	-
10 µM	40.34±0.060	0.079±0.013	8.96±1.480	0.223	0.894	0.731
100 µM	13.83±0.227	0.060±0.002	11.58±0.343	0.000351	0.015	0.008

Table 5. Maximum concentrations and growth rate and minimum duplication time observed for different concentration of α-tocopherol and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when p<0.05.

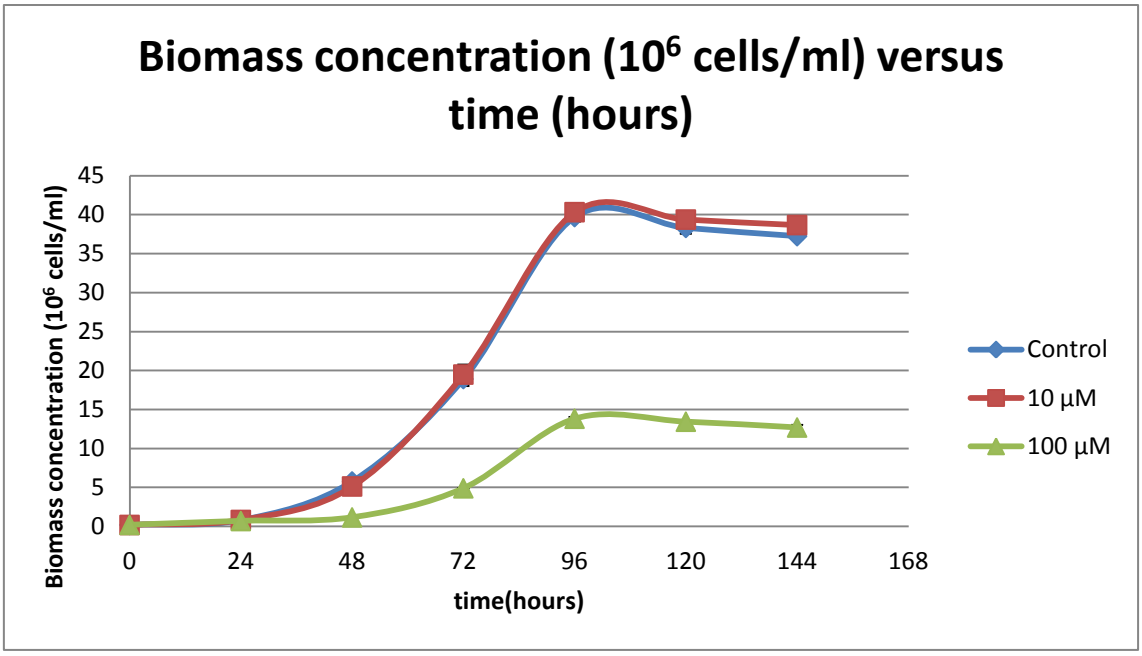


Figure 14. Biomass concentration (10^6 cells/ml) versus time (hours) for different α-tocopherol concentrations in which growth was observed, and Control (blue).

γ-tocopherol showed growth at higher concentrations than α-tocopherol (Figure 15), but again, the maximum growth at low concentrations (10 µM) showed no statistical differences compared to controls (34.71±0.561 for the first and 34.98±0.729 for the second). Moreover the growth rate and duplication times were also significantly equals (0.084±0.008 and 8.24±0.790 for controls compared to 0.075±0.009 and 9.32±1.090 for 10 µM). In the case of 100 µM the maximum concentration of cells was statistically lower compared to controls (24.21±1.308), however, surprisingly, the growth rate and duplication time were significantly the same (0.075±0.012 and 9.44±1.506 respectively). Finally, for 1 mM, maximum concentration (2.67±0.007), growth rate (0.233±0.000) and duplication time (2.98±0.001) were all significantly inhibitory (Table 6).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	µ	Dt
Control	34.98±0.729	0.084±0.008	8.24±0.790	-	-	-
10 µM	34.71±0.561	0.075±0.009	9.32±1.090	0.725	0.155	0.105
100 µM	24.21±1.308	0.075±0.012	9.44±1.506	0.001	0.137	0.140
1 mM	2.67±0.007	0.233±0.000	2.98±0.001	0.005	0.013	0.034

Table 6. Maximum concentrations and growth rate and minimum duplication time observed for different concentration of γ-tocopherol and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when $p<0.05$

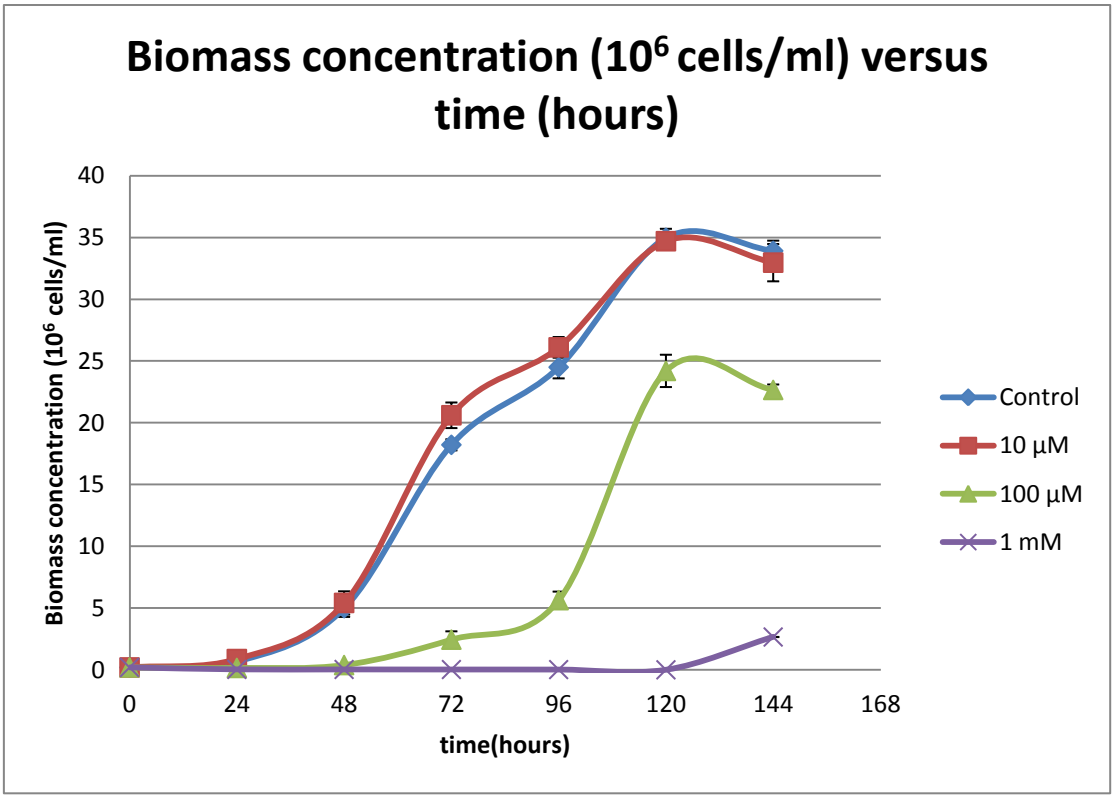


Figure 15. Biomass concentration (10^6 cells/ml) versus time (hours) for different γ-tocopherol concentrations in which growth was observed, and Control (blue).

Finally, δ-tocopherol showed growth up to higher concentrations than α and γ-tocopherol. However, the effects at lower concentrations were similar, no growth stimulation was observed for 10 µM (23.64±0.287 compared to 23.92±0.640 in controls) and the maximum growth rate and minimum duplication time was significantly the same (0.088±0.001 and 7.87±0.058 for control and 0.084±0.007 and 8.25±0.670 for 10 µM respectively). The rest of the concentrations in which growth was observed showed inhibition effects from slight decrease for 100 µM (20.60±2.110) to huge inhibition effects (5.19±0.558 for 1 mM, 2.17±0.260 for 2 mM and 0.88±0.243 for 5mM). However, in this case even when the inhibition was more important, no differences in the growth rates and duplication times were observed for any concentration with respect to controls (0.088±0.001 and 7.87±0.058 respectively for controls, 0.084±0.007 and 8.25±0.670 for 10 µM, 0.086±0.004 and 8.04±0.431 for100 µM, 0.069±0.016 and 10.51±0.984 in the case of 1 mM, 0.067±0.012 and 10.56±2.106 for 2 mM and finally 0.088±0.004 and 7.08±0.316 for 5mM)(Figure 17)(Table 7).

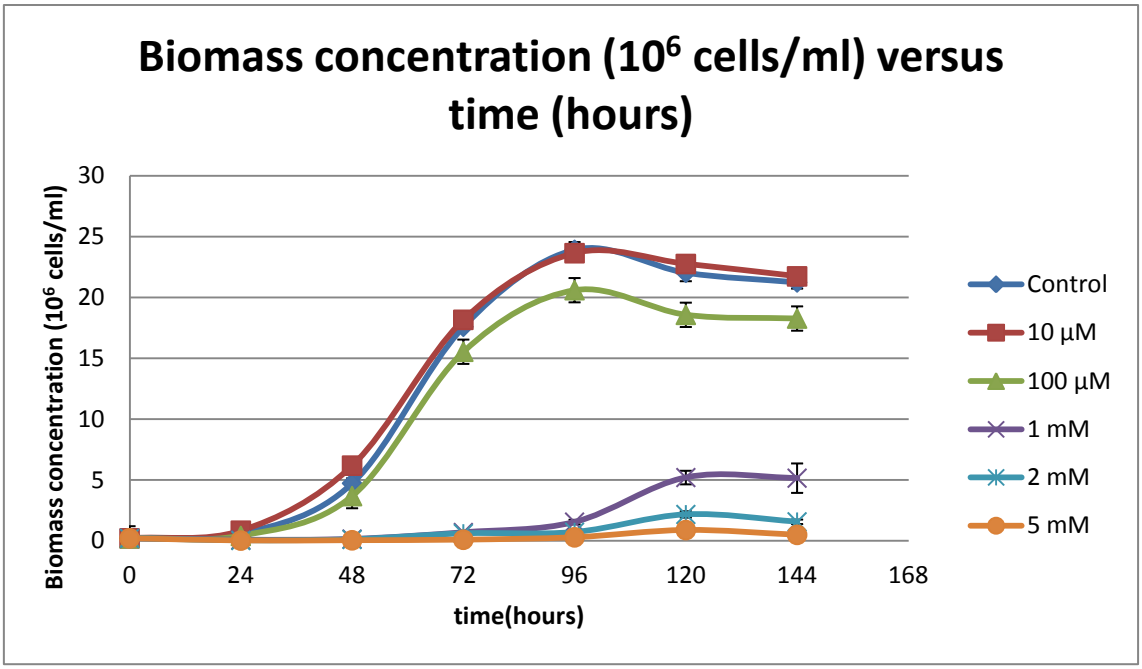


Figure 17. Biomass concentration (10^6 cells/ml) versus time (hours) for different δ-tocopherol concentrations in which growth was observed, and Control (blue).

	C_{max} (Nº of cells ·10^6/ml)	μ_{max} ($hours^{-1}$)	dt_{min} (hours)	Student's t-test (p-value)		
				C	μ	Dt
Control	23.92±0.640	0.088±0.001	7.87±0.058	-	-	-
10 µM	23.64±0.287	0.084±0.007	8.25±0.670	0.204	0.149	0.158
100 µM	20.60±2.110	0.086±0.004	8.04±0.431	0.037	0.617	0.593
1 mM	5.19±0.558	0.069±0.016	10.51±0.984	0.001	0.225	0.299
2 mM	2.17±0.260	0.067±0.012	10.56±2.106	0.000257	0.103	0.163
5 mM	0.88±0.243	0.088±0.004	7.08±0.316	0.000475	0.932	0.944

Table 7. Maximum concentrations and growth rate and minimum duplication time observed for different concentration of δ-tocopherol and Control. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when $p<0.05$

3.2 Toxicity assays.

The trend of the different percentages of inhibition at different concentrations of each compound is shown in figure 18, whereas the results of the LC_{50} and NOAEC are resumed in table 8 and figures19 and 20. The compound which showed inhibition at the lowest concentration was Esculetin (20% of inhibition at 2 µM, the lowest tested), whereas the compound which showed first symptoms of inhibition at the highest concentration was Trolox-OH (4.75% at 200 µM). Among the different tocopherols, α-tocopherol produced higher inhibition than γ-tocopherol at lower concentrations (15% at 20 µM for α-tocopherol, whereas the inhibition at that concentration of γ-tocopherol was 1.84%), while δ-tocopherol showed the best results since low inhibition was showed at higher concentrations (4.17% at 100 µM). Trolox-COOH resulted surprisingly inhibitory

compared to controls, since important inhibition was achieved at very low concentration (19% at 10 µM), showing opposite results to Trolox-OH despite to their similar structure. These results help to explain the reason why no NOAEC was observed for Esculetin and Trolox-COOH, since significant inhibition compared to the controls was observed for all concentrations studied. Trolox-OH showed the highest NOAEC (200 µM), and among tocopherols, α the lowest (10 µM), γ the middle (40 µM) and δ the highest (100 µM) (Table 8, Figure 20). These results were also maintained in the study of LC_{50}, since the compounds which showed inhibition at lower concentrations reached before the 50% of inhibition. The firsts were the inhibitors Esculetin and Trolox-COOH at 8.44 and 20 µM respectively. Curiously, α and γ-tocopherol showed the same 80 µM LC_{50}. The highest LC_{50} were δ-tocopherol and Trolox-OH at 520 and 580 µM respectively (Table 8, Figure 19).

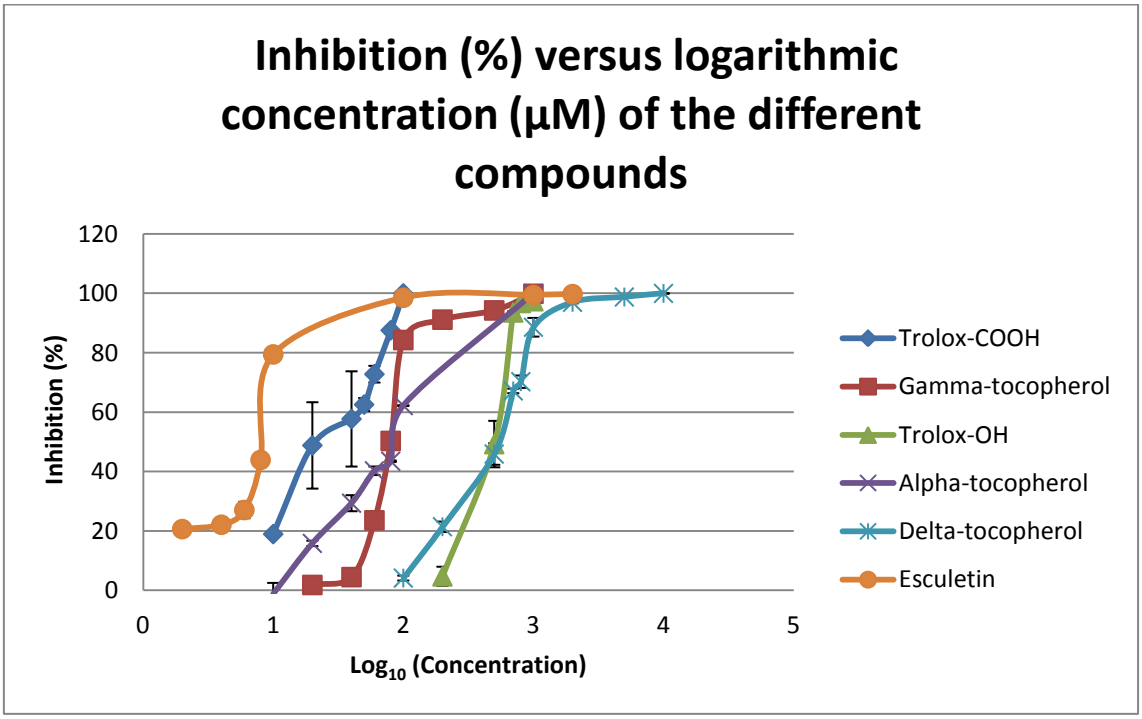


Figure 18. Inhibition % versus logarithmic concentration (µM) for each compound tested.

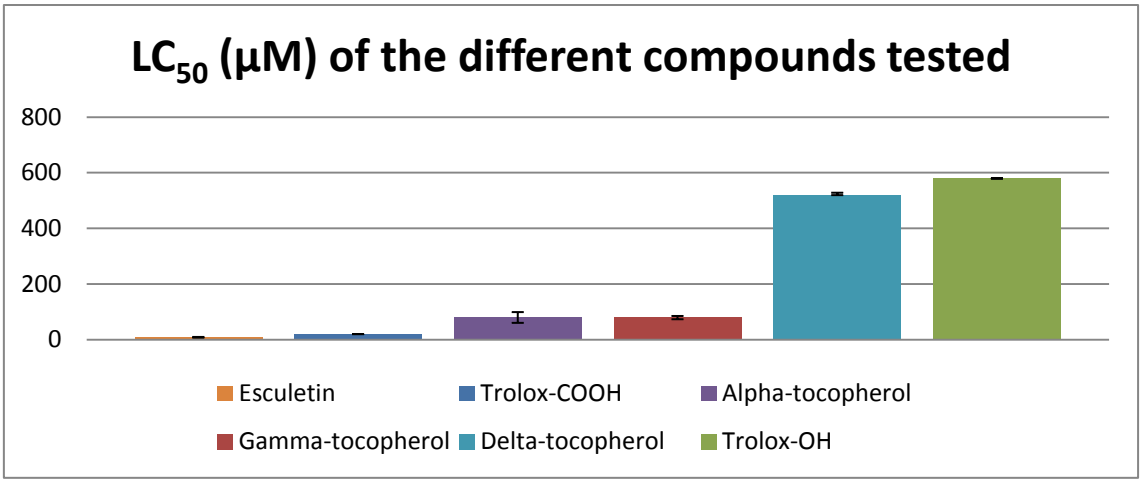


Figure 19. LC50 (µM) of the different compounds tested sorted from the lowest to the highest value.

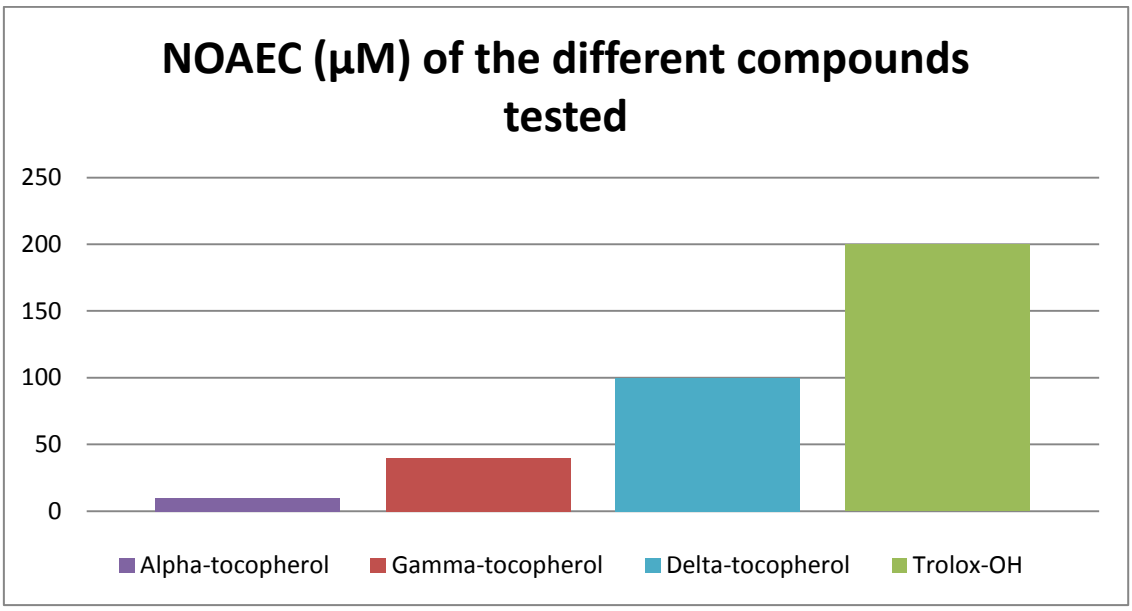


Figure 20. NOAEC (µM) of the different compounds tested, sorted from the lowest to the highest value. Esculetin and Trolox-COOH were omitted since no NOAEC was observed at all the concentrations studied.

Compound	Esculetin	Trolox-COOH	α-tocopherol	γ-tocopherol	δ-tocopherol	Trolox-OH
LC_{50}	8.44 µM	20 µM	80 µM	80 µM	520 µM	580 µM
NOAEC	-	-	10 µM	40 µM	100 µM	200 µM

Table 8. Values of LC_{50} and NOAEC for the different compounds tested.

3.3 TBARS assay.

Results of TBARS assay for the different treatments are summarised in table 9 and figure 21. The highest values of malondialdehyde formation, and therefore, the highest lipid enzymatic oxidation were for controls (40.67±0.4 ng/g). α-tocopherol and Trolox-OH 10 µM showed statistically the same results (31.636±4.620 ng/g and 31.213±13.436 ng/g respectively) whereas γ- and δ-tocopherol treatments statistically inhibited the enzymatic oxidation of lipids compared to the controls (22.090±6.154 ng/g and 21.193±0.444 ng/g respectively). The treatment which showed the less amount of malondialdehyde was

Trolox-OH 100 µM (21.353±3.237 ng/g).

Malondialdehyde (ng/g)	40.67±0.4	31.636±4.620	22.090±6.154	21.193±0.444	31.213±13.436	21.353±3.237
p-value	-	0.221	0.035	0.013	0.196	0.012

Table 9. Malondialdehyde (ng/g) formation for control and the different treatments as an indicator of the oxidation of lipids. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when p<0.05

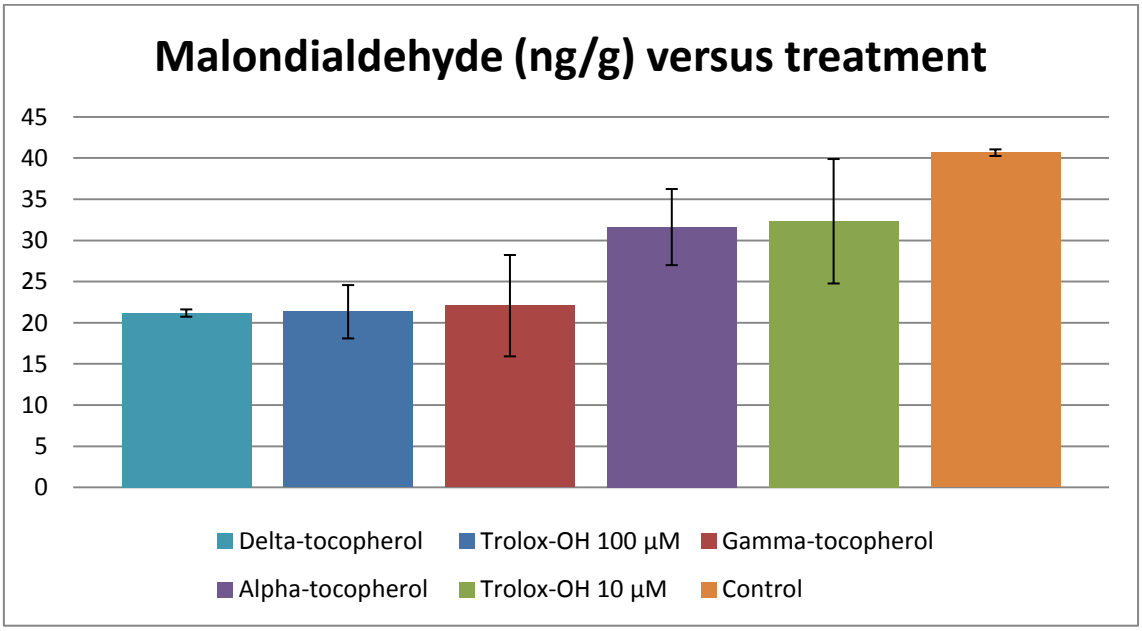


Figure 21. Malondialdehyde levels (ng/g) for each treatment, from the lowest formation, to the highest.

3.4 Total lipid determination.

The results of the total content of lipids is shown in Table 10. The control *C. reinahrdtii* presented a total amount of lipids of 10.87±0.60% and all the Vitamin E analogues showed statistically the same lipid content (7.04±2.29% for α-tocopherol, 12.55±5.62% in the chase of γ-tocopherol, 15.25±5.33% for δ-tocopherol and Trolox-OH 100 µM 10.41±3.45%), with the only exception of Trolox-OH 10 µM, which showed a statistically lower lipid content (4.81±1.21%).

Treatment	Control	α-tocopherol 10 µM	γ-tocopherol 10 µM	δ-tocopherol 10 µM	Trolox-OH 10 µM	Trolox-OH 100 µM
%	10.87±0.60	7.04±2.29	12.55±5.62	15.25±5.33	4.81±1.21	10.41±345
p-value	-	0.258	0.748	0.451	0.005	0.876

Table 10. Content (%) of lipids with respect to the total wet biomass for control and the different treatments. Data are represented as the average ± standard deviation for replicates. The Student's t-test were considered as significant when p<0.05

3.5 Lipid profile.

The TLC plates with the results obtained for the purification of lipids is shown in figure 22.

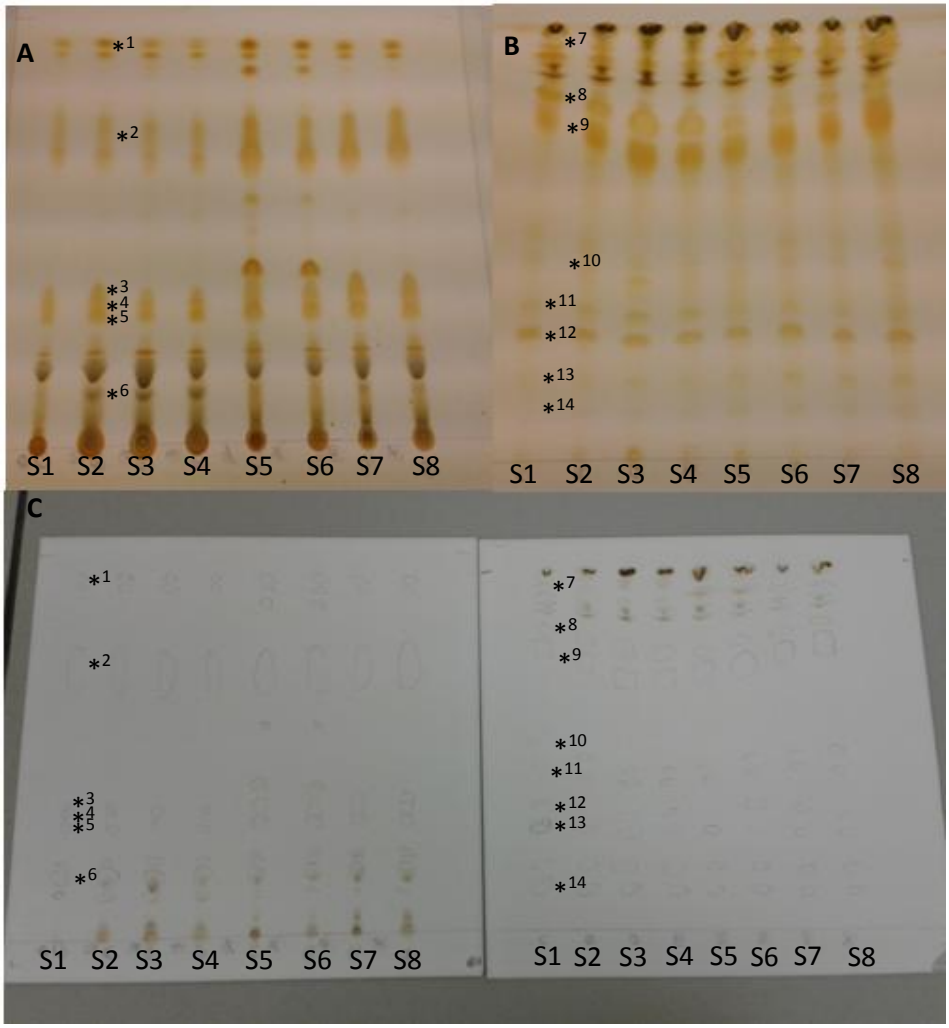


Figure 22. TLC plates showing the results obtained in the purification of the different fractions of lipids contained in the biomass for control and the different treatments. A: Plate run with non-polar solvents after staining with iodine. B: Plate run with polar solvents after staining with iodine. C: Plates when iodine staining disappeared. The position of the different lipids was marked using a pencil. *[1]: sterol esters (SE), *[2]: TAGs, *[3]: Unsaturated fatty acids (UFA), *[4]:1,3-DAGs *[5]: 1,2-DAGs, *[6]: Polar Lipids, *[7]: Polar Lipids, *[8]: Monogalactosyldiacylglycerol (MGDG), *[9]: Diacylglyceryltrimethylhomo-Ser (DGTS), *[10]: Phosphatidylethanolamine (PE), *[11]: Digalactosyldiacylglicerol (DGDG), *[12]: Diradylglycerolipids (DG,) *[13]: Glycerophosphoinositols (PI), *[14]: Liso-phospholipids (or hydrolised lipids). Sample (S) 1: Control; S2: 10 µM α-tocopherol; S3: 10 µM γ-tocopherol; S4: Control; S5: 10 µM δ-tocopherol; S6: 10 µM δ-tocopherol; S7: 10 µM Trolox-OH; S8: 100 µM Trolox-OH; S9: 100 µM Trolox-OH.

In this case the lipids were analysed by GC-MS in order to determine the quantity of the different lipids that could be found in each spot. However, to quantify their levels, the internal standard 17:0 2 nm was added for all the different fractions and all the chromatograms showed always the peak of 17:0 as the only result in the fraction (Results not shown). Despite this, the identification was made by the TLC results conducted with commercial standards. Although this analysis is not quantitative, the strength of the dyeing in each spot gives an idea of the amount of lipids in that spot, so normally, the more intensity, the higher concentration of lipids. For non-polar solvents (Figure 22,A), all the fractions for the different treatments seemed similar, with the only remarkable difference of δ-tocopherol which showed two undefined spots, which might be unsaturated fatty acids owing to the close distance to these spots. The rest of the spots could be easily identified and belong to well established fractions of lipids. Among the different types of lipids, TAGs seemed to be the highest coloured, so probably that fraction of the lipids will be the biggest, but also a high concentration of DAGs can be detected. On the other hand, for polar solvents, the most abundant spot belongs MGDG and DGTS whereas the lowest were in lyso-phospholipids.

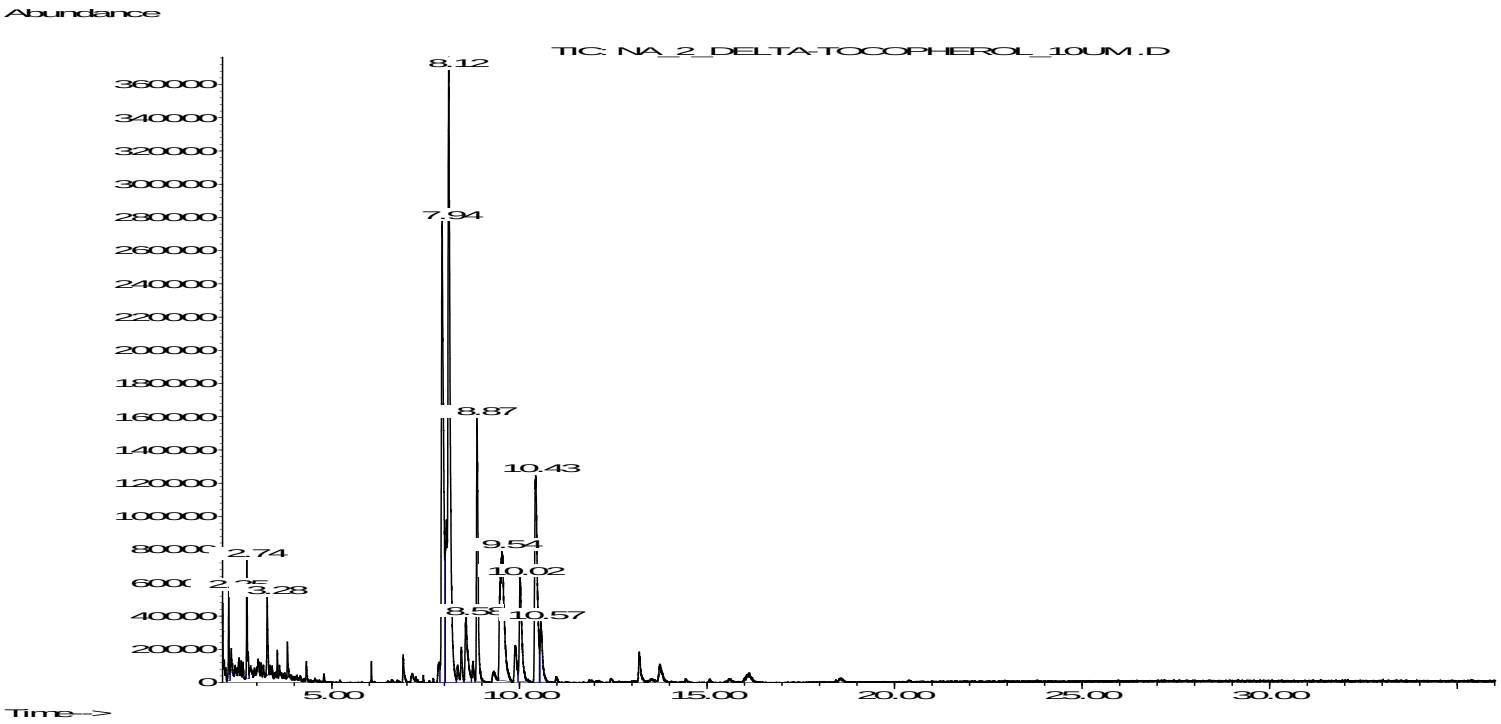


Figure 23. GC-MS chromatogram for 10 µM δ-tocopherol. The different peaks obtained represent the different molecules detected at different retention time.

For the determination of the total lipids GC-MS was used. In this case the lipids were transesterified and then run in the GC-MS without been purified. The chromatogram shown is the one which showed the best resolution, for 10 μM δ-tocopherol(Figure 23). The chromatogram shows different components in the sample and the retention times (RT) in which they were detected. Only compounds determined with peaks with a quality superior to 90 have been commented. The first peak studied belonged to Hexadeconic acid methyl ester (16 carbon atoms, 0 Degree of unsaturation, 16:0) at RT of 7.94 min. After that, at 8.12 appeared 9-hexadecenoic acid methyl ester (16:1), and then at 8.58 the internal standard Heptadeconic acid (17:0). The peak at 8.87 was not had into account because of low quality of the signal (<90%). At 9.54 min appeared 9-octadecenoic acid methyl ester (18:1), and finally at 10.43 min 9,12,15-octadecatrienoic acid methyl ester (18:3).

3.6 Analytical pyrolysis by Py-GC/MS.

Py-GC/MS was used to study the thermochemical behaviour of microalgae *C. reinhardtii* cultured with Vitamin E analogues. The chromatograms derived from the results are shown in figure 24. In this case results for control (A), tocopherols (B) and Trolox (C) is shown.

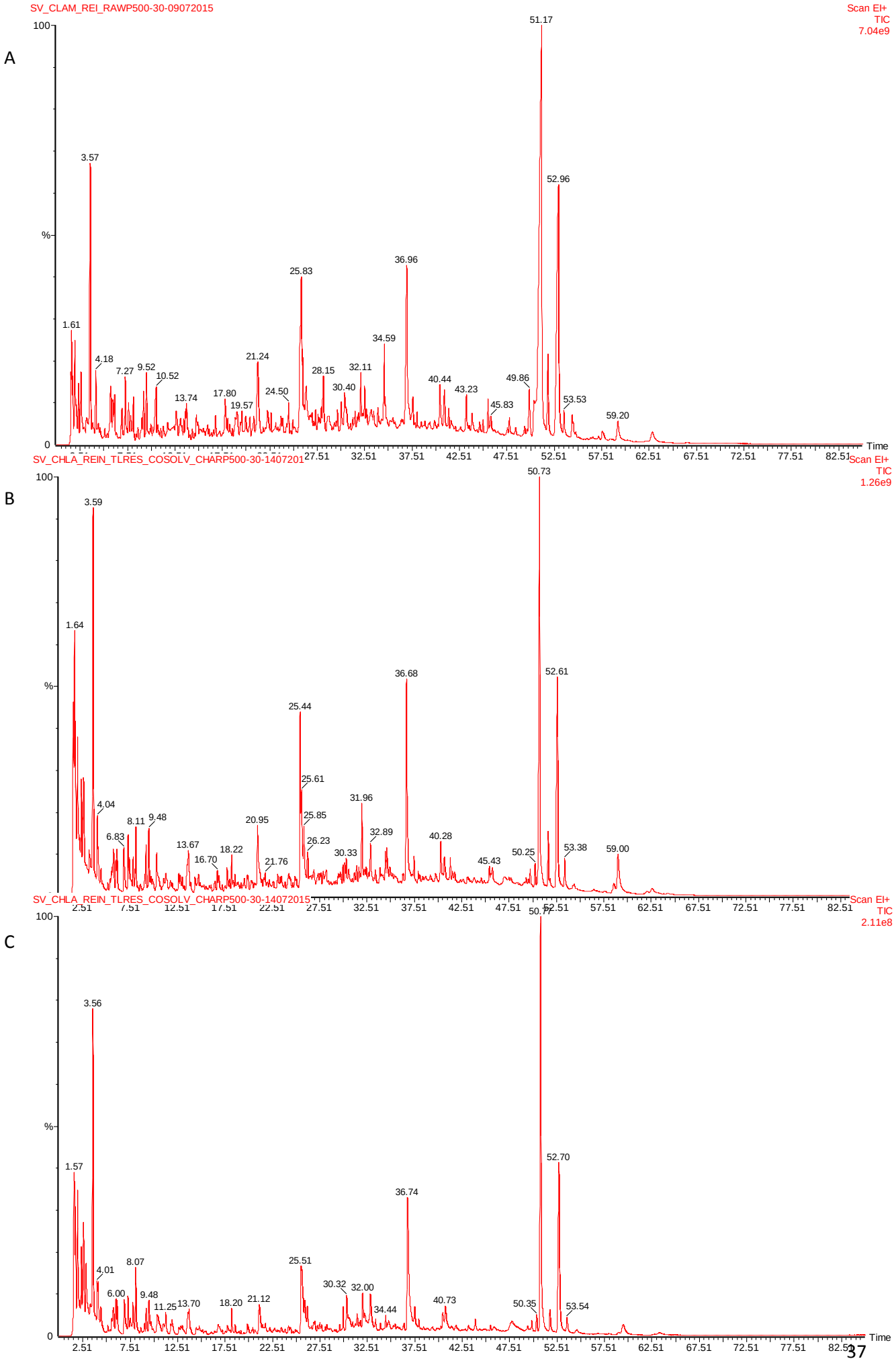


Figure 24. Pyrograms obtained from analytical pyrolysis from Control (A), tocopherols combined (D), 100 µM Trolox-OH (C).

According to the results obtained in the MS, *C. reinhardtii* showed approximately the same compounds in the three studies. In the study of the chromatograms, the peaks indicated by their retention time were analysed, and only in case one component was found indicated as highly abundant in one of the chromatograms, but not in the others, minor peaks were also determined. In total, 22 peaks were indicated for control and tocopherols, and 19 for Trolox-OH. Although the peaks were not always the same compound, all the compounds whose peak was identified in one of the chromatograms was finally found as a minor peak in the others, so the fragmentation pattern of the compounds present in the biomass seems maintained.

Moreover, a semi-quantitative analysis (comparative area (ca) % on pyrograms) was conducted to determine the relative abundance of the different compounds using FID. These results are shown in figure 25.

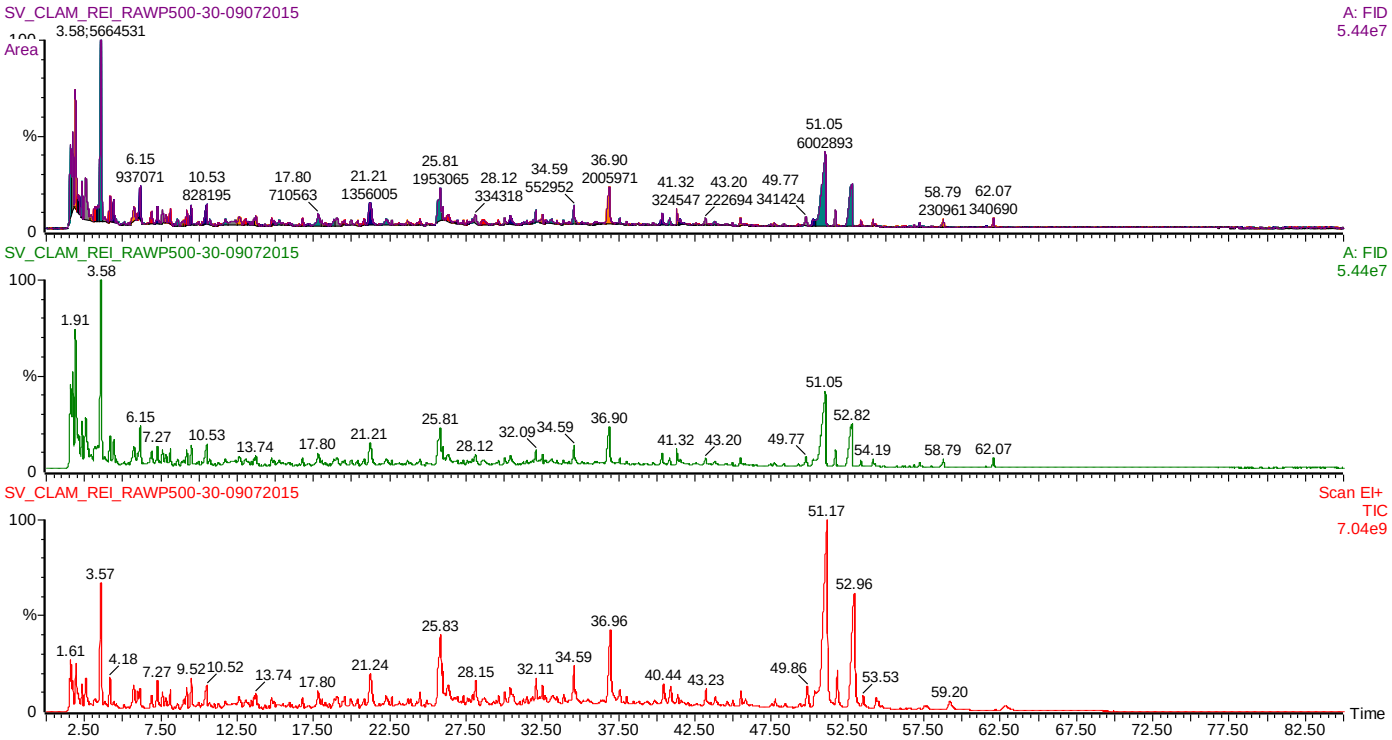


Figure 25. Electron Impact total Ion Chromatogram (EI-TIC) showing the peaks corresponding to different molecules according to the retention time (red), Flame Ionization Detector (FID) chromatogram suitable for the semi-quantitative analysis (green) and the same chromatogram integrated to show the areas corresponding to each peak.

The relative abundance of all the pyrograms was similar, so only controls are shown in this report. The most abundant components in order of elution were toluene (at 3.57 min, ca 16.57%), p-cresol (25.83 min, ca 5.71%), phytols (51.17 min, ca 17.56%), 3,7,11,15-tetramethyl-2-hexadecen-1-ol (52.96 min, ca 9.83%) and finally hexadeconic acid ethyl ester (59.20 min, ca 9.90%). Minor products were also indentified, such as 1,2-dimethyl-cis-cyclopropane (1.64 min, ca 4.82%), 2-furanmethanol (10.52 min, ca 2.42%),

2,3,5-trimethyl-1H-pyrrole (17.80 min, ca 3.96%) or 2-ethyl-4-methyl-pyrrole (18.28 min, ca 2.74%). All the products identified are summarised in table 10. As it can be noticed, some of the compounds have two elution time which is a phenomenon commonly observed in pyrolysis. Moreover, many compounds presented analogues in the final result, but different reaction time.

RT	Compound	%	RT	Compound	%
1,64	1,2-dimethyl-cis-cyclopropane	4,83	34,59	Pentadecane	1,62
1,95	2-methylfuran	0,14	39,96	Indole	5,87
3,57	Toluene	16,57	40,44	3-methyl-1H-Indole	1,37
4,18	Pyridine	1,91	41,32	2,6,10-trimethyl-tetradecane	0,95
6,15	Pyrrole	0,95	43,20	2-methyl-E-7-hexadecene	0,65
7,27	Styrene	1,45	43,23	Heptadecene	0,65
9,52	3-methyl-1H-pyrrole	1,66	47,77	3,7,11-trimethyl-1-dodecanol	1,33
10,52	2-Furanmethanol	2,42	49,77	3,7,11,15-tetramethyl-2-hexadecene	0,68
13,74	2-ethyl-1H-pyrrole	0,76	49,86	3,7,11,15-tetramethyl-2-hexadecene	0,99
17,8	2,3,5-trimethyl-1H-pyrrole	2,08	51,17	Phytol acetate	17,56
18,28	2-ethyl-4-methyl-pyrrole	2,70	52,96	3,7,11,15-tetramethyl-2-hexadecen-1-ol	8,93
21,24	Phenol	3,97	53,53	3,7,11,15-tetramethyl-2-hexadecen-1-ol	0,04
25,83	p-Cresol	5,71	54,38	6,10,14-trimethyl-2-pentadecanone	0,30
28,15	Pentadecane	0,98	58,79	2-methylen-cholestan-3-ol	0,99
30,15	1,2-dihydro-1,1,6-trimethylnaphtalene	0,28	59,20	Hexadeconic acid ethyl ester	9,90
32,11	Benzenepropanenitrile	1,04	62,07	2-methylen-cholestan-3-ol	0,67

Table 10. Main compounds identified found the study of the pyrograms. Colored in orange are the compounds with the higher % in the biomass. On the other hand, in green are the most abundant compounds which showed minor peaks. These compounds were obtained in control sample. . Abbreviations: RT: Retention time.

4. DISCUSSION

The stimulation of the production of lipids inside the cells normally requires stress conditions for them, which has been explained previously in the introduction. However these stress situations can lead to the degradation of the lipids, so the main final goal is lost. Then, the presence of certain compounds in the growth media which could help to avoid these side effects of the stress conditions seems interesting.

When one compound is present in the environment and is absorbed inside the cell, it normally undergoes metabolism, and this process is called biotransformation. There are many possible reactions that these exogenous compounds can undergo, but once of the more important are oxidations (Timbrell, 2002)(Niesink et al., 1996), so when a compound is introduced into the cell and is recognised as dangerous it is probably going to be oxidised, and then other compounds inside the cell which are important for its development can be affected. This is the reason why antioxidants can be important in the growth of biomass for the production of biofuels.

According to the results previously shown, the growth of *C. reinhardtii* was stimulated by Trolox-OH up to 100 μM and it was the only Vitamin E which showed significant enhance in the multiplication of the cells. The rests of the analogues showed no statistical difference at low concentrations for the different tocopherols or even inhibition in the case of Trolox-COOH. The inhibition Trolox-COOH showed might be related with his proven capacity to act as an antioxidant, but also as a pro-oxidant depending on the compound it acts over. Pro-oxidant capacity of Trolox-COOH over proteins has been demonstrated at low concentrations (20 μM) in biological tests, so this seems to be the main reason why the growth is inhibited (Lu et al., 2011). Esculetin showed the highest inhibition among all the compounds, but in this case was related with their role in the inactivation of LOX (Shandhu et al., 2014). This suppression leads to the inhibition of the oxylipins pathway, so the activation of certain signaling cascades in which growth and other vital processes depend on is not possible. Therefore, lower growth has been an expected result.

In order to understand better the effects on growth, LC_{50} and NOAEC were determined for each treatment. Obviously, for the reasons explained earlier, Esculetin and Trolox-COOH showed the lowest LC_{50} and no NOAEC was observed

at any concentration studied. Among tocopherols, α- and γ- showed the lowest values of LC_{50} but NOAEC of γ-tocopherol was a bit higher. The compounds with the higher LC_{50} and NOAEC were δ-tocopherol and Trolox-OH. It is remarkable that in the growth studies the results obtained for 100 μM of δ-tocopherol were considered as inhibitory, the highest NOAEC for the same compound was 100 μM which is contradictory. The possible explanation might be that the values of the standard deviation in the growth assays is high, and also because NOAEC is an statistical tool that only has into account the concentrations studied and in this case the p-value obtained was very close to the limit to consider the values statistically equal (0.048), so the real highest NOAEC could be close to 100 μM, but not been this exact value.

In the case of Esculetin and Trolox-COOH the possible explanation of the toxicological effects has been already described, whereas for the other compounds where there are no toxic effects at low concentrations, the different toxicological profile might be related with the structural particularities of the compounds tested (Table 11).

Compound	α-tocopherol	γ-tocopherol	δ-tocopherol	Trolox-OH
H bond acceptors	2	2	2	3
H bond donors	1	1	1	2
ACD/LogP	11.90	11.44	10.98	2.83
ACD/LogD	10.30	9.74	9.49	2.61
BCF	1000000.00	1000000.00	1000000.00	57.10
KOC	9547463.00	4735059.00	3462730.50	629.51

Table 11. Interesting chemical properties and characteristics of the chemical compounds tested in the experiment. Data from ChemSpider. Abbreviations: ACD/LogD: Distribution coefficient from structure predicted by ACD Labs at pH 7.4; ACD/LogP: Octanol/water partitioning coefficients from structure predicted by ACD Labs; BFC: Bioconcentration factor; H: hydrogen, KOC: Adsorption coefficient.

The different tocopherols have high octanol/water partitioning, which indicates that are more soluble in organic solvents, high bioconcentration factor, which means that the compounds tend to be maintained in the cells, and high adsorption coefficient. This all means that the compounds can go through the membrane and go inside the cells easier than Trolox-OH. α-tocopherol potentially seems to be more adsorbed and concentrated than the others analogues, and this might explain its higher toxicity.

Tocopherols, as other tocochromanols, are anchored in the membranes by their polyprenyl chain. The relative antioxidant activity of the different tocopherols does not only depends on their capacity to donate the phenolic hydrogen to lipid-free radicals but also in their mobility and accessibility within the membrane. It

has been proven that α-tocopherol has a pronounced influence on membrane dynamics and has also showed strong interactions in the membrane stability. Moreover, the best effects have been observed at low concentrations, close to its concentration in the chloroplasts membranes suggesting that this concentrations is very regulated to modulate the stability of the membrane (Falk and Munné-Bosch, 2010). Therefore, the higher absorption of α-tocopherol might led to a higher accumulation of it, and this would imply modifications in the membrane fluidity compromising the survival of the cells. In the case of γ- and δ-tocopherol, they might also cause these side effects in the membranes, but since their absorption according to the properties seems to be lower, more concentration of exogenous antioxidant is necessary to achieve the same effects. Another possible reason could be that the content of γ- and δ-tocopherol are higher in the cells, so to reach the very regulated concentration of them in the membranes is more complicate, and higher amount of exogenous antioxidant is needed. However, this theory seems to be false, since the α-tocopherol seems to be the most abundant tocochromanol in the cells (Li et al., 2008). In the case of Trolox-OH no previously research published has been found, so many reasons could explain the results that have been discovered in this project, from interactions with enzymes related with growth, to regulation of the oxylipins growth signaling through antioxidant properties related with the common structure to known antioxidants such as, vitamin E.

The result of stimulation in growth of Trolox-OH seems promising when production of biofuels is the main goal, since the more biomass is produced, the more lipids can be extracted and transformed. Moreover, in the cases of no statistically differences in growth, the cultivation with antioxidants could help to protect the lipids, and therefore it could be interesting to study if this lipids are being protected in the treatments with vitamin E analogues.

In order to determine this antioxidant protection of vitamin E analogues, TBARS assay was conducted. Furthermore, this assay could be useful to determine whether the possible role of Trolox-OH as lipid antioxidant could be the reason why it showed growth stimulation. Esculetin and Trolox-COOH were not further studied since they produced growth inhibition, which is undesirable for biofuel production.

Lipid enzymatic peroxidation involves a process where PUFAs of membrane phosphoplipids are damaged by oxidative stress, leading to the formation of mixtures of lipid hydroperoxide and aldehydic end-products such as MDA, which is believed to be responsible for many pathological effects related with oxidations. So, the determination of the formation of MDA is a powerful tool to measure the antioxidant effects of different compounds over the cells (Olszewska-Slonina et al., 2011).

In this case, controls and treatments with 10 μM α-tocopherol and 10 μM Trolox-OH showed statistically the same production of MDA, which indicates that these treatments are not inhibiting the peroxidation of lipids. On the other hand, γ- and δ-tocopherol, and 100 μM Trolox-OH showed inhibition in the enzymatic oxidation of lipids. Hence, since 10 μM and 100 μM Trolox-OH showed different inhibition in lipid peroxidation but similar stimulation in growth, the reason why the growth was enhanced is not related with the antioxidant activity of Trolox-OH. Contrary as expected, α-tocopherol showed no statistical variations compared to controls, so instead of showing the highest antioxidant activity (Falk and Munné-Bosch, 2010), the protection was the lowest compared to γ- and δ-tocopherol. The reason for this could be that α-tocopherol is more specialised in avoiding the oxidation on lipids caused by ROS than in the enzymatic peroxidation (Yi et al., 2008) although it also has a role in the second. In the case of γ- and δ-tocopherol, significant peroxidation inhibition was shown, which is explained by their antioxidant property.

Although 10 μM γ- and 100 μM δ-tocopherol did not show enhancement in the growth of cells, these concentrations turned into lipid peroxidation protective. This similar protection over the lipid peroxidation was also observed in 100 μM Trolox-OH, and in this case it also improved the growth of the biomass. These results seems interesting when the protection of the lipids is basic for the production of biofuels, so this is the reason why the lipid profile of the microalgae grown in the different treatments was further evaluated.

First, the content of total lipids in the cells was analysed measuring the percentage of lipids per wet weight. All the treatments showed statistically the same percentage of lipids than controls, except 10 μM Trolox-OH which showed significant decrease in the total content. This might be related with the growth of the biomass, since the cell is focused in growth and all its energy and compounds are been used for cell division so the metabolism is not diverted to the synthesis of lipids (Guschima and Harwood, 2013). These results have been already observed in other experiments where algal growth showed reductions in lipids levels (Cheirsilp and Torpee, 2012). In the case of 100 μM Trolox-OH even although there is stimulation in the growth, its antioxidant property might inhibit its degradation.

The profile of lipids was studied by purification of the different fractions by TLC and then GC-MS. No definitive results were obtained in the chromatogram, since only the peak of the internal standard appeared. Despite of this, the evaluation of the different fraction could be made according to standards previously run in the laboratory. TAGs were the lipids more abundant, probabivly because in the moment of the harvest, cells were in its 5^{th} day of culture close to the stationary phase, when the growth stops, and the cells start to synthesise compounds to store energy (Guschima and Harwood, 2013). Also the levels of DAG are high, maybe for the same reason, since the synthesis of TAGs requires the

previous formation of DAGs. On the other hand, the polar solvents showed high content of MGDG and MGTS, which seems logical since they are very important lipids in the membranes of algae (Ye et al., 2013), whereas the lowest content of lipids is polar solvent plate is for lyso-phospholipids. Low content of lyso-phospholipids was also expected since they are products of degradation of lipids, and they are being protected by the antioxidants.

Since the determination of the purified lipids could not be done the total lipids were studied by GC-MS. In this case different chromatograms were obtained, but not important differences were observed.

Finally, the thermochemical behaviour of the algae at different treatments was evaluated. Analytical pyrolysis studies of *C. reinhardtii* turn out to be quite low if we have into account that it is a model organism. However some studies have been published before, and they allow us to determine that the thermochemical behaviour of *C. reinhardtii* is maintained. Recent publications have demonstrated that the main components that can be found are similar to our results. Phenols, pyrroles and indols and phytols derivates are normally the main peaks visible in the spectra. The main reason why this happens seems to be related with the decomposition of tyrosine and tryptophan, since they are two of the main aminoacids found in microalgae (Hognon et al., 2015). Analytical pyrolysis of *C. reinhardtii* wall mutant has been also studied in previous work at the European Bioenergy Research Institute. In that experiment the determination of the main peaks by semi-quantitative analyses gave as a results pretty similar values to the previously exposed in this report. Both analyses resulted in the determination of phenols, indoles and phytol isomers as the main components. However, the most important difference is the absence of squalene in the results obtained in this research. Squalene is a triterpenic lipid and is an important intermediate in the biosynthesis of cholesterol. It used in the development of vaccines, but since is extracted from shark livers, research focused on trying to find new production alternatives could be interesting. However, although the peak of squalene appears at almost 80 minutes and is one of the most important in the previous research, and in our results any compound similar to squalene was detected (Kebelmann et al., 2013).

5. CONCLUSIONS.

During the investigation of this project, the different main goals proposed at the beginning have been studied. According to the results obtained in the growth experiments, α-, γ-, and δ-tocopherol showed no stimulation or inhibition at low concentrations (10 μM), but at higher concentrations the growth decreased, being α-tocopherol the most toxic analogue (80 μM of LC_{50} and 10 μM of NOAEC) and δ-tocopherol the least (with 520 μM of LC_{50} and 100 μM of NOAEC). The reason for that might be related with the probable higher uptake of α-tocopherol by the cell, which can lead to instability in the membranes. The most toxic compound studied was Esculetin (with LC_{50} at 8.44 μM and no NOAEC observed) since it is an enzyme inhibitor, and the least toxic was Trolox-OH (520 μM LC_{50} and 200 μM NOAEC), which actually showed stimulation in the growth at 10 and 100 μM. Surprisingly, Trolox-COOH resulted inhibitory, probably due to its pro-oxidant activity over different components of the cells.

The stimulation in the growth by Trolox-OH is not related with its antioxidant capacity since 10 μM Trolox-OH did not show statistical inhibition of the enzymatic oxidation of lipids compared to controls. 100 μM of Trolox-OH and 10 μM of α- and δ-tocopherol showed similar inhibition in the oxidation being the most antioxidant compounds.

10 μM Trolox was the only treatment which showed a significant decrease in the percentage of the total lipids, whereas the other had statistically the same levels than control. Despite of this, all the compounds showed similar results in the GC-MS analysis and in the analytical pyrolysis.

In conclusion, tocopherols do not affect to the growth or lipid levels in the microalgae *C. reinhardtii*, whereas Trolox analogues do affect the growth, both inhibiting (Trolox-COOH) or stimulating (Trolox-OH). Since there is not a clear stimulation or protection of the lipids, treatments with tocopherols seem useless for the production of biofuels. The only compound which could be considered as interesting and potentially enhancer of the production is Trolox-OH when the biomass is exposed to 100 μM, since there is more growth and the lipid content is still the same than in the control, which means, more cells, and therefore, more lipid levels available to be transformed to biofuels.

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