TÉCNICO LISBOA

# Extraction of phenolic compounds from *Sargassum muticum* using deep eutectic solvents by conventional solid-liquid extraction, ultrasound and microwave-assisted extraction techniques

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Thesis to obtain the Master of Science Degree in

## Chemical Engineering

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# Declaration

I, Bárbara Cristina Moutinho de Jesus, student number 90437 of Instituto Superior Técnico, declare that this master thesis is the original work of my authorship, and it follows the requirements of the conduct code and good practices of the Universidade de Lisboa and of Instituto Superior Técnico.

# Abstract

Seaweeds are naturally abundant and are spread all over the globe. They have several biologically active secondary metabolites of great interest. Therefore, the interest of their exploitation has increased over the years. In this work *Sargassum muticum* was the algae employed as biomass and the aim was to extract phenolic compounds (PCs) and the pigment (fucoxanthin) using green extraction solvents.

Several betaine-based, proline-based, and choline-based (deep) eutectic solvents ((D)ESs) were tested for the extraction of PCs. All extracts were evaluated according to the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity. Followed by the HPLC characterization of 9 PCs (gallic acid, 3,4-Dihydroxybenzoic acid, caffeic acid, syringic acid, *p*-coumaric acid, ferulic acid, salicylic acid, catechin and quercetin). There were a few (D)ESs that showed higher efficiency in comparison with conventional extraction solvents also evaluated. Proline combined with propylene glycol (Pro:PPG) was the best (D)ES according to HPLC results, followed by proline:1,2-butanediol (Pro:1,2-But) and choline:citric acid (ChCl:CA). Pro:PPG presented high selectivity towards the salicylic acid and ChCl:CA towards the gallic acid. Optimizations studies of water content and temperature were performed for the three best (D)ESs, being the optimum conditions at 30 %(v/v) of water and 60 ºC of extraction temperature.

Ultrasound assisted extraction (UAE) and Microwave assisted extraction (MAE) were two intensification methods evaluated as the objective to enhance the extraction yield. They improved the extraction by significantly reducing the extraction time when compared with the conventional solid-liquid extraction even though the extraction yield could be slight lower. The exception was Pro:PPG with MAE where the extraction was significantly higher in comparison with the conventional extraction.

The results of fucoxanthin extraction indicate that the *S. muticum* studied possesses only a residual value. Given this, no significant amount of fucoxanthin was able to be extracted from *S. muticum*.



**Keywords:** *Sargassum muticum*, green extraction techniques, eutectic solvents, phenolic compounds, fucoxanthin, bio-solvents.

# Resumo

Algas são naturalmente abundantes e encontram-se espalhadas pelo mundo inteiro. Elas possuem uma variedade de metabolitos secundários que são biologicamente ativos. Posto isto, o interesse em explorá-los tem vindo a crescer ao longo dos anos. No presente trabalho, *Sargasso muticum* foi a alga usada como biomassa tendo como objetivo a extração de compostos fenólicos e do pigmento (fucoxantina) recorrendo a solventes verdes.

Vários solventes eutécticos à base de betaína, cloreto de colina e prolina foram testados na extração de compostos fenólicos. Para todos os extratos o conteúdo total de compostos fenólicos, assim como conteúdo total de flavonoides e a atividade antioxidante foram analisados. Seguidamente, foi realizada a caracterização e quantificação de nove compostos fenólicos no HPLC (ácido gálico, ácido 3,4-dihidroxibenzóico, ácido cafeico, ácido siríngico, ácido p-cumárico, ácido ferúlico, ácido salicílico, catequina e quercetina). Alguns dos solventes eutécticos testados retornaram uma eficiência superior na extração comparando com os solventes convencionais também testados. De acordo com os resultados do HPLC, o solvente eutéctico formado pela combinação de prolina e propileno glicol mostrou ser o melhor solvente na extração de compostos fenólicos. Este foi seguido da combinação de prolina com 1,2-butanodiol e posteriormente de cloreto de colina com ácido cítrico. O solvente eutéctico prolina:propileno glicol mostrou também uma elevada seletividade em relação ao ácido salicílico, já a combinação de colina com ácido cítrico mostrou uma seletividade em relação ao ácido gálico. A quantidade de água inserida na preparação dos solventes eutécticos foi otimizada, assim como, a temperatura de extração para uma extração solido líquido convencional. Foi verificado que 30 %(v/v) era a quantidade ótima de água a introduzir no solvente eutéctico para a extração de compostos fenólicos e que 60 ºC correspondia à temperatura ótima.

Métodos de intensificação, tais como a extração assistida por ultrassons ou micro-ondas, foram testados com o objetivo de melhorar e otimizar ainda mais a extração. Estas técnicas reduziram significativamente o tempo de extração quando comparado ao tempo necessário para uma extração convencional, mesmo que o rendimento da extração tenha sido ligeiramente inferior ao convencional. No entanto, ao comparar a extração assistida por micro-ondas com a extração convencional, o solvente eutéctico prolina:propileno glicol retornou rendimentos de extração consideravelmente superiores.

Apesar de várias tentativas para a extração da fucoxantina terem sido realizadas, verificou-se que esta apenas existia em quantidades residuais no *S. muticum.* Posto isto, não foi possível extrair quantidades significativas de fucoxantina do *S. muticum*.



**Palavras-chave:** *Sargassum muticum*, técnicas verdes de extração, solventes eutécticos, compostos fenólicos, fucoxantina, bio-solventes.

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# List of Abbreviations

| | |
|---|---|
| EU | European Union |
| TRL | Technology Readiness Levels |
| SDGs | Sustainable Development Goals |
| CBE | Circular bioeconomy |
| *S. muticum* | *Sargassum muticum* |
| PCs | Phenolic Compounds |
| CAGR | Compound annual growth rate |
| SLE | Solid-liquid extraction |
| MAE | Microwave-assisted extraction |
| GAE | Gallic acid equivalents |
| PGE | Phloroglucinol equivalents |
| TPC | Total phenolic content |
| HPLC | High-Performance Liquid Chromatography |
| UAE | Ultrasound-assisted extraction |
| US | Ultrasound |
| MW | Microwave |
| PLE | Pressure Liquid Extraction |
| SSE | Subcritical Solvent Extraction |
| EAE | Enzyme Assisted extraction |
| SEM | Scanning Electron Microscopy |
| SFE | Supercritical fluid extraction |
| (D)Ess | (Deep) eutectic solvents |
| HBD | Hydrogen bond donor |
| HBA | Hydrogen bond acceptor |
| T | Temperature |
| SLEq | Solid-liquid equilibra |
| NA(D)Ess | Natural (Deep) Eutectic Solvents |
| EFSA | European Food Safety Authority |
| FDA | Food & Drug Administration |
| ChCl | Choline chloride |
| Pro | Proline |
| Bet | Betaine |
| PPG | Propylene glycol |
| CA | Citric acid |
| 1,2-But | 1,2-Butanediol |

| | |
|---|---|
| 1,3-But | 1,3-Butanediol |
| LA | Lactic acid |
| Gly | Glycerol |
| $Na_2CO_3$ | Sodium carbonate |
| $Na_2NO_3$ | Sodium nitrite |
| $AlCl_3$ | Aluminium chloride |
| NaOH | Sodium hydroxide |
| DPPH | 2,2-Diphenyl-1-picrylhydrazyl |
| Trolox | (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid |
| DAD | Photodiode array |
| Reichardt's dye 33 | 2,6-Dichloro-4-(2,4,6-triphenyl-N-pyridino)-phenolate |
| MeOH | Methanol |
| EtOH | Ethanol |
| EtAc | Ethyl acetate |
| DMSO | Dimethyl sulfoxide |
| EtLac | Ethyl(-)-L-lactate |
| IPA | Isopropyl alcohol |
| 2-MeTHF | 2-Methyltetrahydrofuran |
| DMI | Dimethyl isosorbide |
| DMC | Dimethyl carbonate |
| γ-Val | γ-Valerolactone |
| CPME | Cyclopenthyl methyl ether |
| TFC | Total flavonoid content ( |
| QE | Quercetin equivalents |
| TEAC | Trolox equivalent antioxidant activity |
| COSMO-RS | COnductor-like Screening Models – Real Solvent |
| ρ | Density |
| $\eta$ | Viscosity |
| Z | polarity parameter |
| ETN | Normalized polarity |
| α | HBD ability |
| β | HBA ability |
| π* | Polarisability/dipolarity |
| ANOVA | One-way analysis of variance |
| HSD | Honestly significant differences |
| TAMDI | Theoretical maximum daily intake |
| γ | Activity coefficient |

# 1. Introduction

## 1.1. Biorefinery Concept

Refinery consists in transforming fossil fuel-based raw materials into other different chemicals, commodities and other valuable products, and energy always having in mind to valorise the original material. This is a part of the concept of brown economy which is the economy based on finite resources that tend to be environmentally damaging. Biorefinery has the same objective as refinery but using biomass as a source of chemical compounds, and basically is a conjugation of processes to convert and separate biomass into bioproducts and bioenergy. European Commission defines biorefining as "*sustainable processing of biomass into a portfolio of marketable bio-based products, which could include co-production of food and feed, chemicals and materials and bioenergy (power, heat/cold, fuels)*" [1,2].

Biomass is the most significant renewable resource on Earth and, other than fossil fuels, it is considered as the most carbon-rich material available, which has sparked great interest in biorefinery because it is urgent to reduce greenhouse gas emissions and the dependency on fossil raw materials. Reducing this dependency can bring advantages not only in environmental terms but also in economic terms since despite the fact that fossil raw materials are limited, their geographic location might lead to some volatility in their prices [1,3]. In terms of the raw material to use biorefinery is very flexible, meaning that the feed can come from different bio-sources. However, this can also be a limitation when it comes to the rate of utilization and the stock of the feed can contribute to 40 up to 60% of the operating costs [3].

Many companies are already making a slow transition to biorefinery, investing, and supporting the new biorefineries, while others prefer to invest in developing new technologies since the scale-up of new bio-based chemicals and materials is not an easy task. As drivers, governmental agencies are encouraging biorefineries that produce biofuels and bio-products through financial incentives. The global market value of biorefinery was around US$553.7 billion in 2020 and it is expected to reach US$979.5 billion in 2026 [4,5]. In Figure 1, a map of the biorefineries identified in Europe is presented, depicting the reported 803 biorefineries. Two types of biorefineries can be seen: integrated biorefineries, that combine the production of bio-based products and bio-based energy; and non-integrated or classical biorefineries if they just produce either bio-products or biofuels. However, classic biorefineries bring environmental and economic drawbacks and consequently exploitation in cascade should be preferred, to ensure the complete valorisation of the biomass through sequential steps. The EU Waste Framework Directive (2018/851) supports the implementation of integrated biorefineries, with the extraction of added value compounds (fine chemicals) being the priority, and only afterward the residual biomass should be used to produce lower value products (bioenergy and biofuels). Belgium, United Kingdom, Germany, and France are the countries with the most of biorefineries, which represented 51% of the turnover of the European bioeconomy in 2015 [6–8].

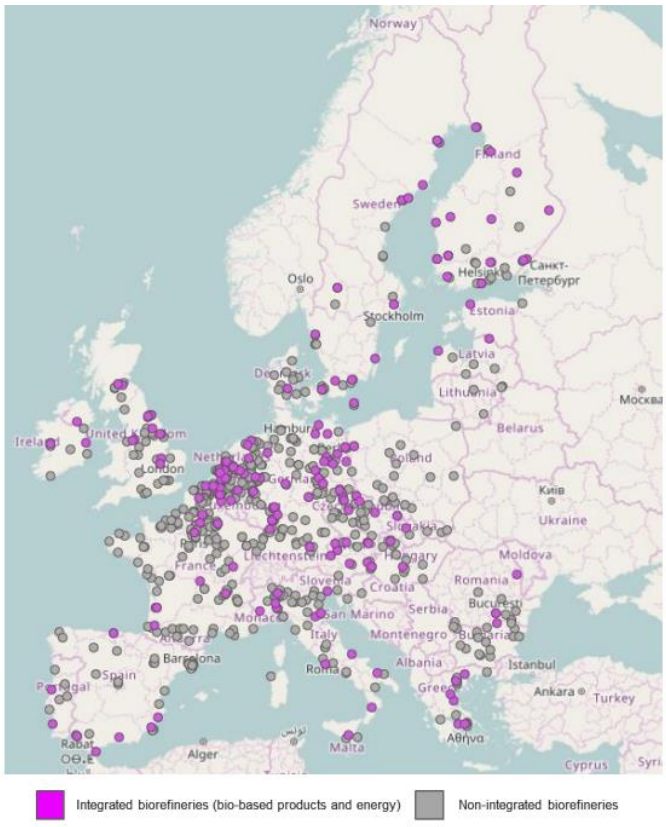



Figure 1. Map of biorefineries identified in Europe that produce bio-based products and bioenergy (integrated biorefineries) and those that only produce one of the two [6].

Just like other technologies, biorefinery technologies can also be evaluated by the Technology Readiness Levels (TRL) system proposed by NASA. This level system measures the maturity of the process itself. The TRL system is depicted in Figure 2. Many biorefinery routes are still rated at low TRL levels, which means that are still in the research phase. Thus, there are routes that still need to be further development to pilot plants, and only then scale-up, which can present some challenges in terms of capacity [2,9,10].

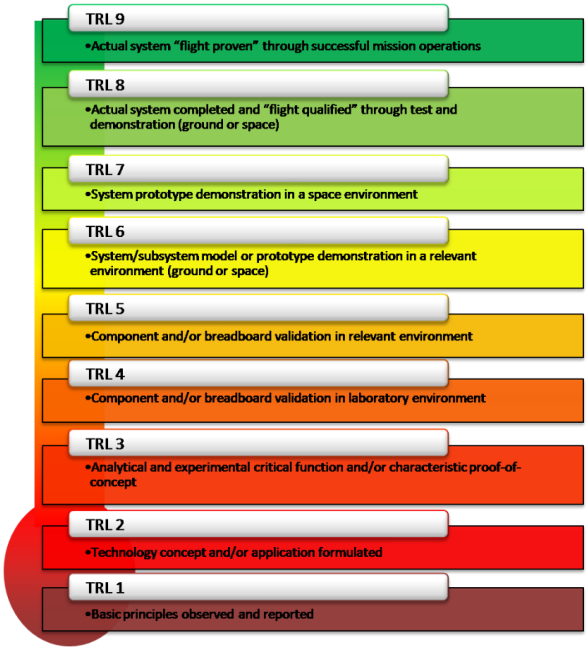



Figure 2. Technology Readiness Level chart (TRL) defined by NASA [9].

The term "bioeconomy" refers to any industry that employs biological raw materials as their sources to obtain bio-based products, such as biorefinery processes. The notion of bioeconomy was introduced in 1966 by a Romanian economist, Nicholas Georgescu-Roegen, and the European Commission defines it as "*using renewable biological resources from land and sea, like crops, forests, fish, animals, and micro-organisms to produce food, materials, and energy."* In 2012, EU stablished a bioeconomy strategy with the following objectives: ensuring food security, managing natural resources sustainably, reducing dependence on non-renewable resources, mitigating, and adapting to climate change, and creating jobs and maintaining EU competitiveness [11]. Having an effective bioeconomy is crucial to achieve the Sustainable Development Goals (SDGs) adopted by United Nations in 2015, where the EU is currently considered a worldwide leader. Given this, in 2018 EU updated the bioeconomy strategy highlighting the key words for sustainability "*economy, society and environment*", in order to have a better match with the SDGs. The European Commission tends to encourage a combination between bioeconomy and circular economy in the so-called circular bioeconomy (CBE), where bio-waste is exploited aiming at the full valorisation of the biomass, including cascade exploitation to obtain several products from the same biomass. As mentioned before, in the cascade exploitation, the extraction of the most valuable compounds should be achieved first, and then the recovery of the other ones can be performed. This hierarchy of products can be organized according to the pyramid presented in Figure 3. The European Commission defends that CBE will help to have a more sustainable future and will help to reduce almost 55% of the greenhouse gas emissions in 8 years. However, there are some concerns, such as the feasibility of some biomass sources and a possible negative impact on water bodies and natural ecosystems [2,7,11–16].

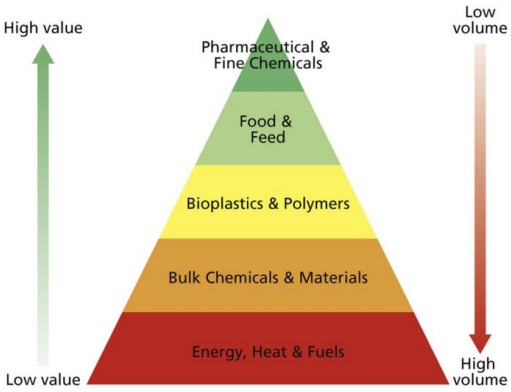



Figure 3. Bio-based products value pyramid [7].

Biorefineries can be classified not only by the different products shown in Figure 3, but also by the conversion processes and complexity of the system and by the feedstock used. Nowadays there are four main conversion processes used in biorefinery that are biochemical, thermochemical, chemical, and mechanical [2,3]. The biomass can be classified as primary, which is the biomass provided by photosynthesis, and due to the photosynthesis process is biomass capable of carbon capture and storage, such as oil crops, aquatic biomass, sugar crops, among others; or secondary biomass that is the biomass resulting from the processing, conversion or decomposition of primary biomass or other organic matter, for example, microbial biomass or several organic residues [2].

### 1.1.1. Biorefinery generations

According to the source of the biomass, biorefineries can be categorized into the first, second, or third generation. The source of first and second generation is mainly from agriculture constituting a part of the green economy, while the third generation does the exploitation of ocean resources incorporating the blue economy [17].

***First Generation***

The first generations of biorefineries use biomass mostly to produce biofuels and are well implemented around the globe. The biomass used normally is also used as food and, consequently, they compete with food production for land which creates a drawback in terms of social sustainability. Despite this fact, first generations of biofuels continue to be a viable option, even in economic aspects, as an alternative to fossil fuels [3].

***Second Generation***

The second-generation biorefineries also consist of the production of biofuels but from a wide range of biomasses; such biomass is usually less expensive compared to that used in the first-generation, and mostly is non-edible biomass such as agricultural, forest, animal, or municipal wastes, being the first predominant. However, this type of biomass can have a higher complexity and thus become more difficult to be transformed, which implies the development of new technologies [6,18]. In the second-generation of biorefineries, the biomass can be classified as homogenous, like white wood, quasi-homogeneous, such as agriculture and forest residues, and non-homogeneous, such as low-value biomass of municipal solid wastes [18].

***Third Generation***

The third generation, which can also be called blue biorefinery, uses algae as biomass and tends to have higher productivity and yield when compared with second-generation of biorefineries. Seaweeds are naturally

abundant and do not present competition to food because also have the ability to grow in complex and extreme environments, while producing several biologically active secondary metabolites of great interest [1,18–21].

As shown in the left axis of Figure 4, the interest in algae-based biorefinery has been slowly increasing throughout the years, not only to produce biofuels but also to extract added value compounds. However, it is also seen that algae still occupied a small fraction (4-7%) of the biomass in biorefinery research leaving plenty of room for more investigation and development. Algae are abundant biomass that do not require crop fields and have a low requirement in terms of land. Comparing with first- or second-generation feedstocks, algae present a more viable option since they have more efficient photosynthetic processes and they absorb a high quantity of carbon dioxide and produce a good quantity of oxygen [1,19].

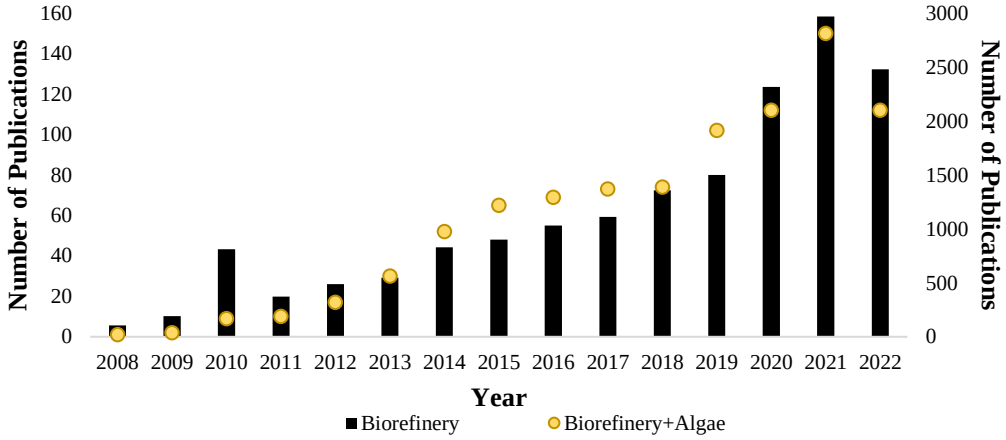



Figure 4. Evolution of publications on “Biorefinery” (black columns, right axis) compared with “Biorefinery+Algae” (yellow dots, left axis) from 2008 until 11/10/2022 [22].

Algae biomass can be divided into two classes: macroalgae and microalgae. Microalgae are microorganisms that use solar energy to perform photosynthesis to store essential biological compounds, and usually they are grown in reactor systems. Macroalgae or seaweeds are microorganisms that are abundant on several coasts and are a sustainable feedstock to biorefinery to be converted into added-value compounds or food. Given this, macroalgae can be considered a high potential biomass, since they only need to be harvested and exist in great abundance [19].

Macroalgae are classified into red (Rhodophyta), brown (Phaeophyceae), and green (Chlorophyta). Each of these different algae grows in a different environment and has its own morphology and composition, which means that their use as feedstock needs to be individually considered. Nowadays, red and brown algae are the most cultivated ones and find use as sources of food sources, hydrocolloids, fertilizers and animal feed. Even though the compositions of the several compounds varies from algae to algae according to the habitat, maturity, and geographical origin, in general, seaweeds are rich in polysaccharides, minerals, phenolic compounds, vitamins, proteins, steroids, and dietary fibers [20,23].

There are two methods to produce macroalgae: aquaculture and harvesting. As shown in Figure 5, for the last two decades, harvesting, mechanically or manually, of algae wild species has been the most used method to produce macroalgae in Europe, representing around 68% of the production of macroalgae. Aquaculture represents almost one-third of the production of macroalgae and most of its production is performed in the sea [24].

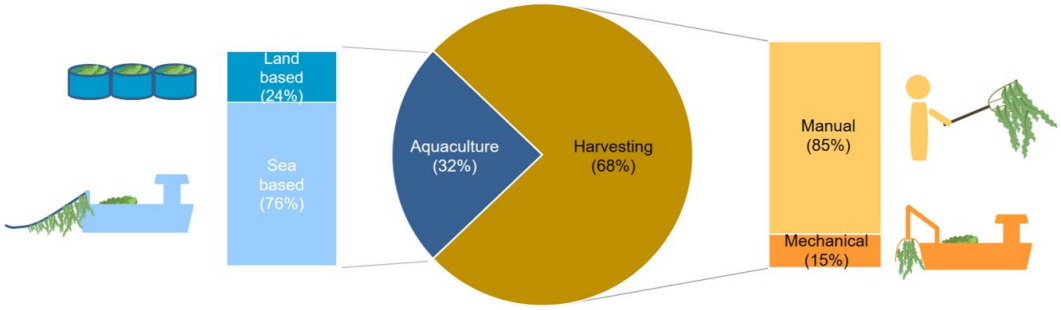



Figure 5. Macroalgae production methods in Europe (shared by the number of companies in 11 countries using these methods excluding pilot plants). Countries covered: Denmark, Estonia, France, Iceland, Ireland, Norway, Portugal, Spain, Sweden, Netherlands, and United Kingdom [24].

## 1.2. *Sargassum muticum* (*S. muticum*)

Brown algae are distributed around the world, with up to 1500 different species, and have a negative ecological impact on the local ecosystems, aquaculture, fishing, and recreational activities. Even though several efforts were made to control the invasion of this algae, none worked successfully, hence their removal is ordered through harvesting. Consequently, these macroalgae are considered biowaste, so their exploitation through the extraction of valuable compound, brings not only considerable economical value but also eases their environmental burden. *Sargassum muticum* (Yendo) Fensholt, is a brown macroalga of Japanese origin that is spread all over the world, being considered invasive in the European coast. Biorefineries using *S. muticum* as biomass have been envisioned with possible uses for nutraceutical, cosmetic and pharmaceutical purposes [21,25–28].

*S. muticum* presents high colonizing potential and its high dispersion all over the world is due to the high growth rates, high fecundity, and self-fertilization. In fact, *S. muticum* has the ability to grow secondary branches and more main branches from one stem annually, as it can be seen in Figure 6. However, this growth of branches tends to happen more in the winter season, decreasing in the latter half of summer when the reproductive phase starts and the algae produces significant quantities of spores [29,30].

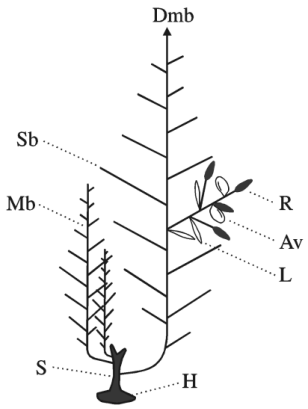



Figure 6. Scheme of the typical morphology of a *S. muticum*. Dmb = dominant (longest) main branch; Mb = main branch; Sb = secondary branch; S = stem; H = holdfast; R = reproductive structure (androgynous receptacle); Av = air vesicle; L = leave-like structures [29].

*S. muticum* is one of the major *Sargassaceae* species in European continental coast, and can also be found in the Western Pacific and western North American coasts [21,26,31]. This macroalgae started being an invasive alga in Europe around 1973 in the United Kingdom coast. *S. muticum* is ecologically and economically important in some regions of Asia, especially in East Asian countries, due to its application as gelling agent and emulsifier in food, aquaculture, and sorption of heavy metals, or as a dye fabric colorant [25,32].

The first time that *S.muticum* was seen in Portugal was in 1989 in the north coast, and eventually spread to the Vicentina Coast (south coast). Chefaoui et al. [33] analysed and developed models for the distribution of *S. muticum* in the Northern Hemisphere, and focusing on Portugal, as illustrated in Figure 7, there is still nowadays a strong presence of the brown macroalgae in the north [33,40]. In the work already published about the Portuguese *S. muticum*, a strong tendency to the macroalgae be collected in north sites is observed, as seen in Figure 8, having a high frequency in Viana do Castelo.

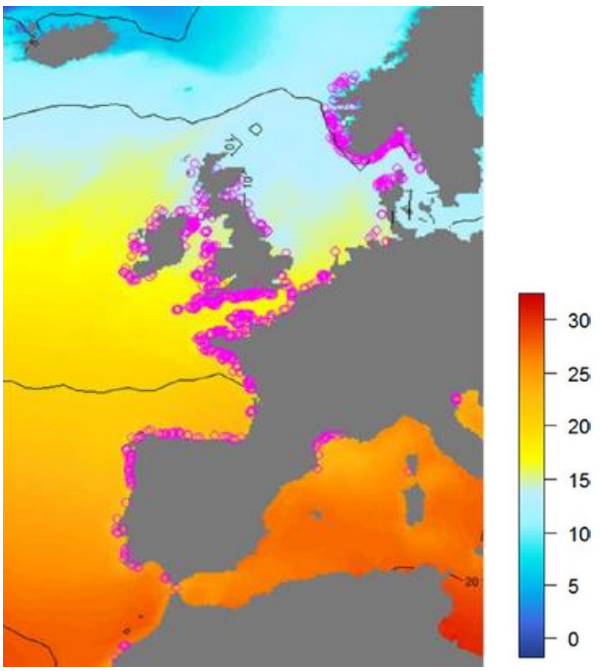



Figure 7. Records of *S. muticum* included in the current analysis (magenta points) showing its distribution in European. The Gradient is the sea surface temperature [33].

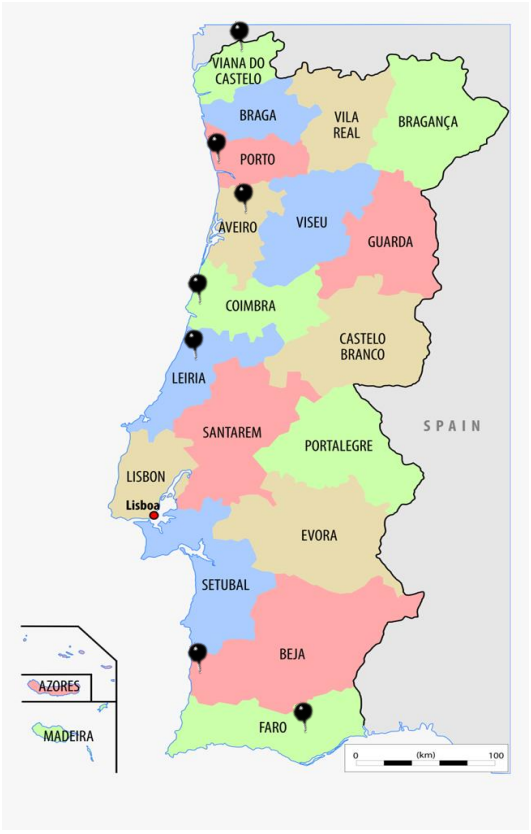



Figure 8. Districts from where there is work already published about *S. muticum*: Viana do Castelo [28,34,35], Porto [28], Aveiro [36,37], Coimbra [20,38], Leiria [39], Beja [34,40] and Faro [41].

*S. muticum* presents several compounds of interest such as phenols (phlorotannins as the major polyphenolic compound found in brown algae), carotenoids, polysaccharides namely alginate and fucoidans, minerals, and lipids, among others, whose composition varies according to geographical location, season, maturity environmental factors, and extraction techniques. Brown seaweeds tend to have a high content of polysaccharides (40-70%) and low content of lipids (usually lower than 3%); *Sargassaceae* species, in particular, are known to have a higher quantity of phenolic compounds (1-15%) and slightly lower protein content (5-15%) when compared to green and red algae. Even the colour intensity of *S. muticum* changes according to the season: in early summer *S. muticum* is dark brown and in mid-summer is pale golden-brown. This can possibly be explained by the

photodegradation of the pigment that occurs with more intensity in summer. Gorham et al. [30] evaluated the seasonal variation of a few organic constituents, namely phenols and polysaccharides, and came to the conclusion that they are more prevalent in the summer [20,25,30,42].

The compounds of interest mentioned before can bring benefits to different applications, such as for example in cosmetic, pharmaceutical, food, and animal feed industries, and especially in the market of functional foods, that has been experiencing fast growth and is expected to keep on growing. Being aware of the added value compounds in high concentrations present in *S. muticum* and their important economic applications, *S. muticum* can be used as biomass in biorefinery processes with the objective to extract these value compounds, and then use them in the industries mentioned before. This way it is possible to simultaneously address two problems: the market demand for natural bioactive compounds (extracted at mild conditions, thus avoiding their degradation, using sustainable methods to achieve high purity), and the remotion the *S. muticum* from the coasts [20,25].

### 1.2.1. Phenolic Compounds (PCs) in *S. muticum*

Phenolic Compounds (PCs) are the largest group of phytochemicals found in several natural bio-sources. They are present in almost all plants as secondary metabolites and have great potential due to the several beneficial properties that they possess such as antioxidant, anti-inflammatory, antibacterial, antimicrobial (antifungal and antiviral), anti-cancer, and antidiabetic, which makes them value-added compounds. In seaweeds, PCs provide protection against herbivores, pathogens, epiphytes and oxidative damage [32,43].

Beyond the interest in PCs' properties, they also shown a market that is expected to expand in a near future due to their various health benefits, which also draws attention to industries such as functional food and beverages, pharmaceutical, cosmetic, animal feed, among others. GRAND VIEW RESEARCH performed market analysis of the growth of PCs market, where a compound annual growth rate (CAGR) of 7.4% between 2022 until 2030 is expected. This yearly PCs market growth can be observed in Figure 9 divided per different bio-sources [44].

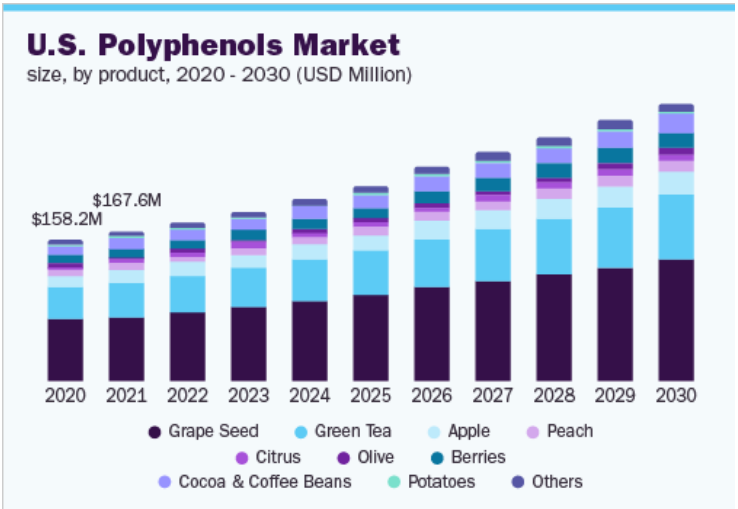



Figure 9. Expected market growth of PCs since 2020 until 2030 divided per different bio-sources [44].

Seaweeds tend to have significant amounts of PCs such as phenolic acids, phlorotannins, bromophenols, flavonoids, phenolic terpenoids, mycosporine-like amino acids and other polyphenols that can exist in smaller quantities. Brown algae tend to have high amounts of polyphenols and *S. muticum* is no exception as can be seen in Table 1. Phlorotannins are the phenolic compounds predominant in brown algae, while bromophenols tend to be predominant in red and green algae [45].

Table 1. TPC found in *S. muticum* from different site and using different extraction techniques and solvents. * no information.

| ***S. muticum* origin** | **Extraction technique** | **Extraction solvent** | **TPC** | **Reference** |
|---|---|---|---|---|
| Venice lagoon, Italy | MAE, 90 ºC during 30 min | Ethanol:Water 70/30 (v/v) | 6.61 mg GAE/g matrix | [46] |
| Pontevedra, Spain | Soxhlet extraction | Ethanol | 79 mg GAE/g ext | [27] |
| | | Water | 72 mg GAE/g ext | |
| | | Ethanol:Water (3:1) | 73 mg GAE/g ext | |
| | | Ethanol:Water (1:1) | 64 mg GAE/g ext | |
| Britanny – Porsmeur, France | SLE under agitation at 40 ºC during 3 h | Methanol:Water 50/50 (v/v) | 0.25 % of PGE equivalents in dry weight | [26] |
| Portugal | Pressurized liquid extraction at 160◦C and 10.3 MPa for 20 min | Ethanol:water (95:5) | 145.02 mg PGE/g ext | [47] |
| France | | | 78.55 mg PGE/g ext | |
| Spain | | | 80.46 mg PGE/g ext | |
| Ireland | | | 94.08 mg PGE/g ext | |
| Norway | | | 148.97 mg PGE/g ext | |
| Peniche, Portugal | * | Dichloromethane | 24.87 mg GAE/g ext | [48] |
| | | Methanol | 71.69 mg GAE/g ext | |
| Viana do Castelo, Portugaç | SLE for 1 hour at 400 rpm | Acetone:water 7:3 (v/v) | 134.59 μg PGE/g dry algae | [28] |
| Denmark | Overnight, at room temperature | Water | 2.308 mg/g dry algae | [49] |
| | | Ethanol | 1.149 mg/g algae | |

As mentioned before, the composition of an algae varies according to geographical location and this variation also affects the PCs content. Tanniou et al. [31] and Montero et al. [47] performed studies focused on the European Atlantic coasts, Norway, Ireland, France, Spain, and Portugal. *S. muticum* from Portugal and Norway revealed to have the higher phenolic content, around 15% of dry weight measure in phloroglucinol equivalents [31,47]. Not only the season and the site where the macroalgae is harvested have impact on the extraction yield

but also the moisture present in the algae can have it as well. Balboa et al. [27] realized extractions with the fresh *S. muticum* and with the dried algae and saw that the extraction yield was higher with the dried algae. However, it is important to be careful when the oven (high temperature) is used for the drying process, as despite being typically employed in industrial factories, this methodology can have an impact in some bioactive compounds [25,27].

***Phlorotannins***

Phlorotannins, whose chemical structures are presented in Figure 10, are hydrophilic compounds that exist mainly in brown algae, although they can also exist in smaller quantities in red algae. They are produced by the polymerization of phloroglucinol, and their molecular weight varies in the range of 126 Da to 650 kDa, due to differences in structure and degree of polymerization. Phlorotannins can present differences in the bonds between the units of phloroglucinol, the number of hydroxyl groups and their bondage to sulfates or halogenates, as shown in Figure 10 (b) and (c), respectively. In fact, according to the types of bonds, phlorotannins can be divided into four subgroups such as phlorethols and fuhaols (ether bond), fucols (phenyl bonds), fucophlorethols (ether and phenyl bonds), and lastly eckols and carmalols, Figure 10 (a) [25,50–52].

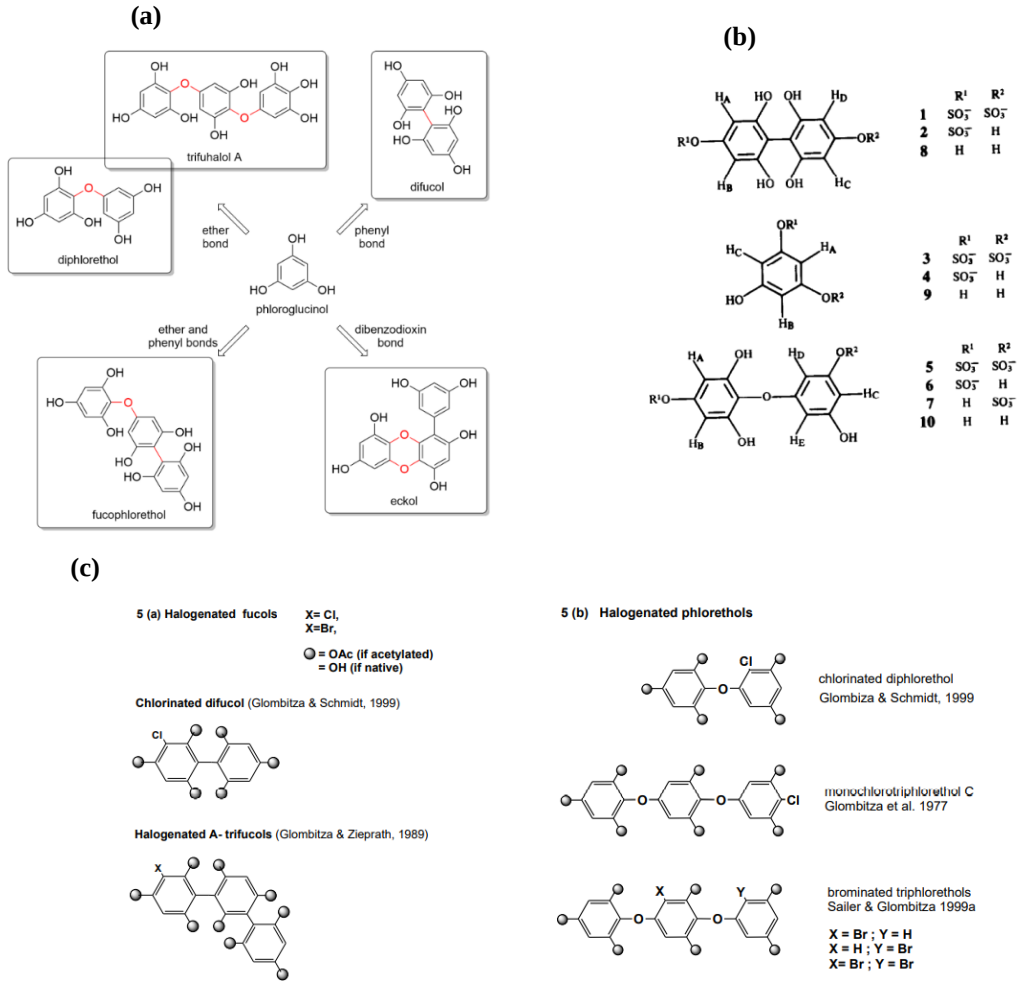



Figure 10. (a) Different phlorotannins structures according to the type of bond created in the polymerization process [52], (b) sulfated phlorotannins [53] (c) halogenated phlorotannins [54].

Phlorotannins correspond to 3-12% of the dry biomass of the algae. Due to the formation of several isomers during the polymerization process, it is impossible to characterize well the different phlorotannins present on the algae, hence they are usually quantified by a general method where the total phenolic content (TPC) is

determined [50,52]. Due to these severe limitations on characterizing the different phlorotannins, research was carried out on other relevant PCs also present in *S. muticum* and possible to characterize, involving the following two groups: phenolic acids and flavonoids [55,56].

***Phenolic acids***

Phenolic acids are small and simple molecules that contain a phenolic and an acid group, and present high antioxidant activity. Phenolic acids contribute to the total amount of phenolic compounds, and they can be easily identified with *High-Performance Liquid Chromatography* (HPLC) [57,58]. Only one published work was found where different phenolic acids were quantified and characterized in *S. muticum* by Sabeena Farvin & Jacobsen [49]. Caijiao et al. [55] and Chakraborty et al. [56] have also performed characterization of a few phenolic acids in other *Sargassum* species. This work already published lead to a few phenolic acids to be characterize for *S. muticum*: gallic acid, 3,4-dihydroxybenzoic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid, and salicylic acid, whose chemical structures can be seen in Figure 11.

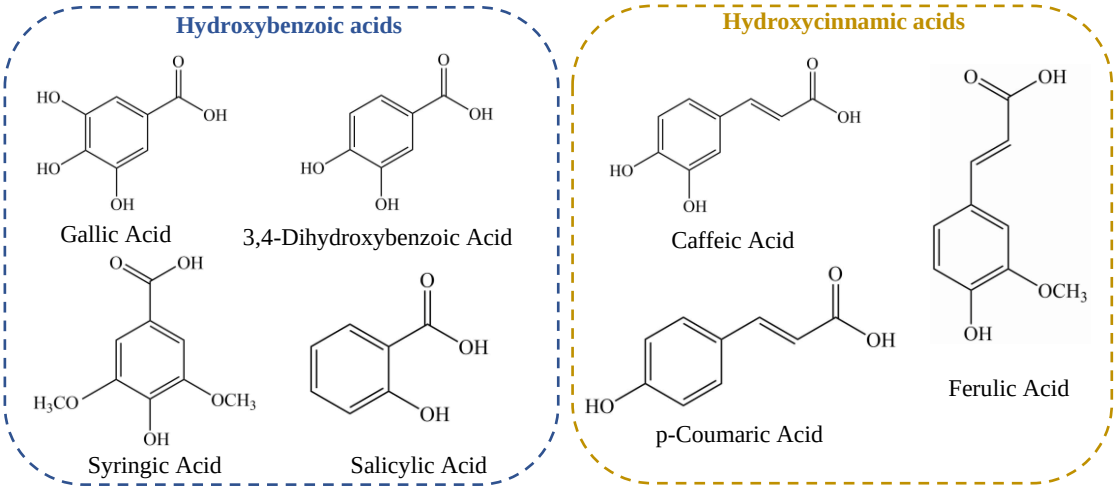



Figure 11. Structures of the phenolic acids present in *S. muticum*.

Besides being easy to characterize phenolic acid are known for the diverse biological applications in therapeutics, cosmetics, and food industries. Despite the applications mentioned above for PCs in general phenolic acids also can act as neuro protective, food preservative and be used in skin care products [59].

***Flavonoids***

Flavonoids are low-molecular-weight PCs that can be found in plants and food such as fruits, vegetables, and wine, among others. Besides the properties mentioned before for PCs in general, flavonoids can also be good inhibitors of several enzymes. They are composed of two benzene rings (Figure 12 – rings A and B of the base structure) bonded through a unit of 3 carbons (chalcones) or through a heterocyclic pyrane (flavones, isoflavones, flavonols, anthocyanins, anthocyanidins and proanthocyanidins). The conjugated double bounds and hydroxyl groups or other functionalities allows the resonance of electrons for stabilization of radicals. Depending on which position the ring B is bonded to the ring C, the degree of unsaturation and oxidation of the C ring flavonoids can be divided into subclasses that are presented in Figure 12. The functional groups bonded to the flavonoid affect the molecule's absorption, which is something to consider when analysing different classes of flavonoids through UV-vis [60–63].

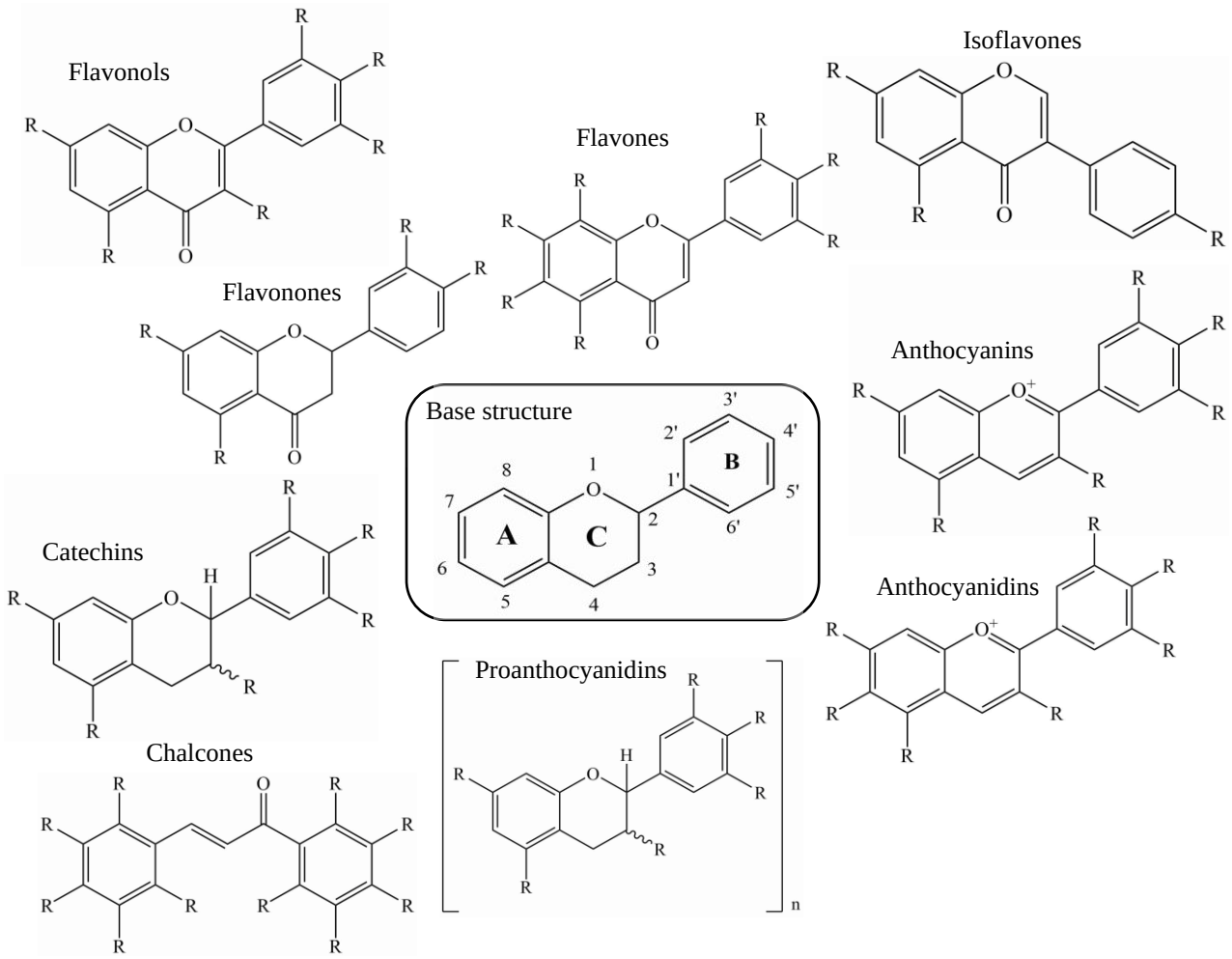



Figure 12. The basic skeleton structure of flavonoids and their classes. Inspired by Routray et al. [61].

Like phenolic acids, several flavonoids also can be individually identified. In this work, catechin and quercetin, two representative flavonoids whose structures can be seen in Figure 13, were chosen to be evaluated.

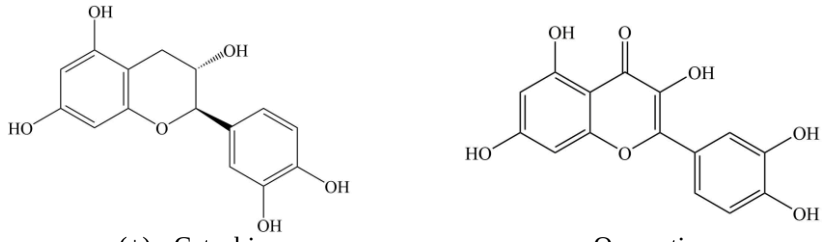



Figure 13. Structures of the flavonols analysed in this work.

### 1.2.2. Carotenoids in *S. muticum*

Carotenoids are tetraterpenoids produced during the photosynthetic process in plants and algae and are among of the value-added compounds found in algae. Carotenoids are composed of carbon chains with saturated and unsaturated bonds with different cyclic or acyclic end groups, and these conjugated unsaturated bonds determine biochemical functions and also their colour. Carotenoids can be divided into two subclasses: carotenes and xanthophylls. Carotenes are composed only of carbon and hydrogen, while xanthophylls possess at least one oxygen atom [64].

Fucoxanthin is the most abundant carotenoid and consequently the major xanthophyll present in brown seaweed, in macro and microalgae, mostly applied in food industry but also in cosmetic and pharmaceutical industries. Its major natural isomer of is all-trans-fucoxanthin whose chemical structure can be seen in Figure 14. This pigment was for the first time isolated in 1914 by Willstatter und Page from brown seaweeds; actually, fucoxanthin was the first allenic carotenoid discovered in brown seaweeds and represents more than 10% of the approximate total carotenoids in nature [65].

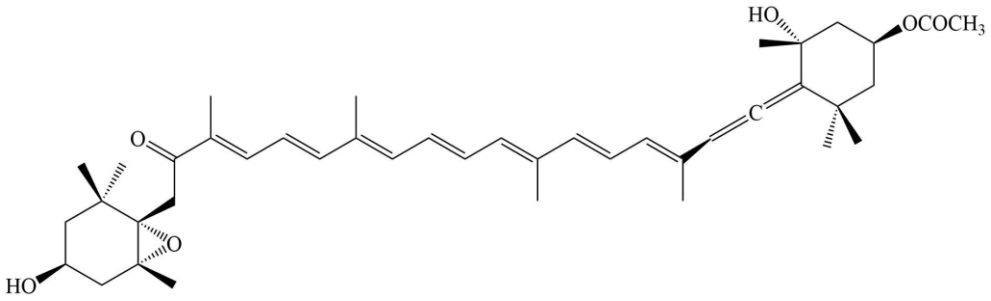



Figure 14. Chemical structure of fucoxanthin.

Fucoxanthin is a compound of great interest due to its properties such as antioxidant, anticancer, antihypertensive, anti-inflammatory, anti-diabetic, anti-obesity, neuroprotective and anti-angiogenic [66]. Also, due to the capacity of light-harvesting and energy transference, fucoxanthin enables an efficient photosynthesis and photoprotection when the algae face environmental fluctuations, which aids the algae survive in shallow coastal waters [67].

The interest in fucoxanthin can be easily seen by the expected growth of its market. A prediction of the fucoxanthin market was made at a CAGR of 3.8% by 2027, also indicating the segmentation between the fucoxanthin analysed with HPLC and UV spectroscopy, and the share per continent, as shown in Figure 15. The HPLC tends to be preferred over the UV, especially because of the ability to isolate and analyse the fucoxanthin present in complex samples such as biomass extracts [68,69]. The main market limitation of fucoxanthin is the price, due to its expensive production process. However, the properties that fucoxanthin possesses makes its use really beneficial, overcoming the price drawback mentioned before [69].

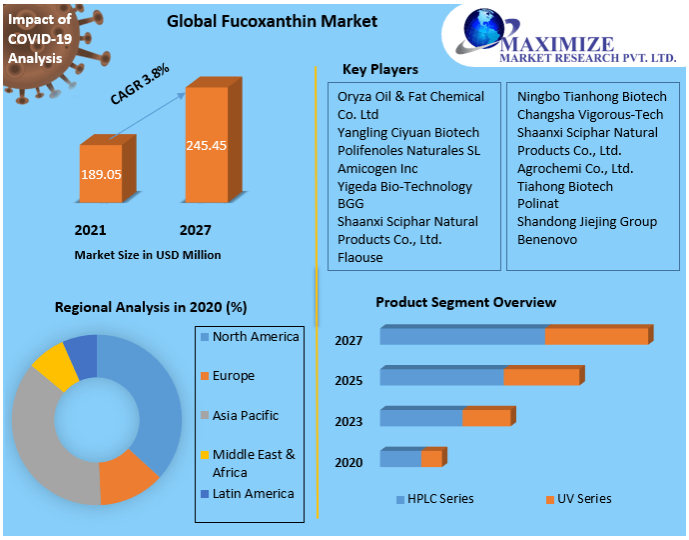



Figure 15. Global market of fucoxanthin: Growth, analysis segmentation and continent share [69].

Finding an easy, cheap and green process to obtain fucoxanthin has been a focus for different investigators in order to solve commercial problems, because the chemical synthesis and purification of fucoxanthin is complex and it is not the most efficient and eco-friendly process [66,70]. As previously said, fucoxanthin was firstly isolated in 1914, but only in 1989 a purification process was developed by Haugan et al. [71] through partition and resorting to a silica chromatographic column [65,67,71]. This molecule has a hydrophobic behaviour, and consequently its extraction is usually performed in the absence of water, i.e., dry biomass is used. Moreover, since fucoxanthin is a thermolabile compound it is important to select the right pre-treatment, and freeze-drying is the recommended technology [72,73].

As mentioned in Section 1.2, the algae composition suffers variations due to different factors, and this leads to an alteration of colour especially at high temperatures. Lewey et al. [42] reports a significant decrease in xanthophylls, such as fucoxanthin and violaxanthin, from brown seaweeds harvested between April and July.

The amount of fucoxanthin present in different *Sargassum* species was a subject of research, noting that it is a valuable compound that exists in small quantities, as can be seen in Table 2. Vieira et al. [73] performed the extraction of carotenoids from *S. muticum* gathered from the Portuguese coast including fucoxanthin, with a limited recovery of 0.1 mg/g of dry weight when comparing with Terasaki et al. [74] who was able to extract 37 times more from *S. horneri* from Japan.

Table 2. Fucoxanthin extraction from different Sargassum species.

| Biomass | Solvent | Extraction method | Extraction yield (mg/g of dry weight) | Reference |
|---|---|---|---|---|
| *Sargassum bonderi* | Acetone/Methanol (7:3 v/v) | Stirred for 10-15min on ice and left under room temperature and N2 atmosphere until the biomass deposits | 0.73±0.39 | [75] |
| *Sargassum duplicatum* | | | 1.01±0.10 | |
| *Sargassum horneri* | Methanol | Room temperature, overnight, under N2 | 3.7 ± 1.6 | [74] |
| *Sargassum thunbergii* | | | 1.8 ± 1.0 | |
| *Sargassum fusiforme* | | | 1.1 ± 0.6 | |
| *Sargassum muticum* | Ethanol | Stirred for 120 min (250 rpm) at 25 ºC | 0.100±0.004 | [73] |
| *Sargassum cymosum* | Ethanol | | 0.089±0.003 | |
| *Sargassum siliquosum* | Methanol | 30 min at 45 ºC | 0.707 | [76] |
| | Ethanol | | 0.491 | |
| *Sargassum polycystum* | Methanol | | 0.521 | |
| | Ethanol | | 0.450 | |

### 1.2.3. Antioxidant activity

Reactive oxygen species are molecules composed by a radical located in the oxygen atom (•OR) with the ability to attack biological macromolecules which can cause damage in humans and animals, including diseases such as cancer, and also skin aging. As previously said, both PCs and fucoxanthin have antioxidant properties, being able to protect the biological macromolecules and help avoiding the problems that the oxygen radicals can cause. Therefore, it is important to evaluate the antioxidant activity of a certain extract [77].

## 1.3. Solid-Liquid Extraction (SLE)

### 1.3.1. Conventional extractions

Conventional extractions are methods that have been used for a long time until the current days, where compounds from the solid matrix are extracted using organic solvents. They are solid-liquid extraction (SLE) such as steam distillation, maceration, and Soxhlet extraction [78].

SLE consists in mixing a certain ratio of solid and solvent and agitating both phases during a certain period of time to promote the mass transfer. When performing a SLE, something that requires attention is the type of solvent used to extract, but also the ratio between the solid and the solvent, the time required, the number of extraction steps, the operating temperature, and the particle size of the solid [79].

***Maceration***

Maceration is a cheap and simple technique easy to do a scale-up [80]. Maceration relies on the leaching of compounds from the solid matrix to the solvent, due to this the solvent used must be choose wisely as its polarity

which has a huge impact on the extraction efficiency; in addition, to enhance the solubility of the compounds of interest, heat or/and agitation can be applied. Maceration followed by filtration or centrifugation is an inexpensive technique simple to operate. This technique has two huge disadvantages that are the time consumed to perform an extraction and the amount of solvent that is required to use [78].

***Percolation***

Percolation is a process that can be operated in continuous or batch mode. The solvent is continuously being replaced, allowing a higher efficiency and a lower time for the extraction when comparing with maceration. In this method, the solution is added from the top and the extract mixture seeps down throughout the time [80,81].

***Decoction***

The decoction cannot be used to extract thermolabile or volatile compounds because it is necessary to boil the crude aqueous extract, and, normally, this is used for tough and fibrous drugs. The drug and the solvent are mixed with a certain ratio, then the system is boiled, and finally the liquid extract settles down and is cooled, strained, or filtered [80–82].

***Soxhlet Extraction***

One of the oldest and most used techniques of solid sample preparation is solvent extraction. Soxhlet is an SLE technique discovered in 1879 by Franz Ritter von Soxhlet. The first application of the Soxhlet extraction was to determine the fat content in milk. This technique has been used for a long time now to extract and recover valuable compounds from different solid matrices. Currently, Soxhlet extraction is used as a standard extraction method to compare the efficiency of extraction techniques [83].

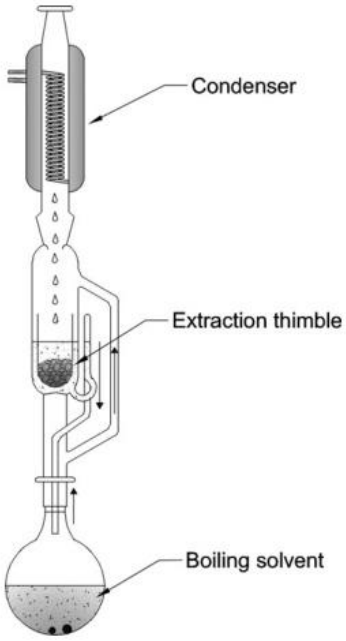



Figure 16. Conventional Soxhlet extractor [83].

This method combines the advantage of reflux extraction and percolation. The thimble, indicated in Figure 16, is where the dry mass is placed, straightway the thimble is filled with a solvent to be used in the extraction for the distillation process. As soon as the solvent achieves the overflow, the extracted liquid is collected

in the distillation flask bellow. The reflux and the condensate go back to the thimble-holder, this enables a feed of fresh solvent to the sample allowing an improvement of the transfer equilibrium, consequently allowing a higher extraction yield and in the end, the extract does not need filtration [78,81,83].

Soxhlet extraction is a simple and efficient technique due to the mass transfer equilibrium promoted by the contact between the solvent and the sample, allowing the extraction of a wide range of components. This technique can reach high temperatures during the extraction, which must be seen carefully because the compounds can be thermally degradable [82,83].

### 1.3.2. Alternative methods – intensification methods

Conventional extraction processes, such as maceration, and percolation, among others, are processes that require high energy consumption – at least 50% of the energy of the industrial process – and large amounts of organic solvents which are not environmentally friendly, tend to be volatile, flammable and toxic. Therefore, there is a need to find alternatives, especially because extraction processes are required in several industries such as cosmetics, medicine, food, biofuels, and other fine chemicals industries. Methods like microwave-assisted extraction (MAE), and ultrasound-assisted extraction (UAE), among others, have been proposed as alternatives, as these methods show a great potential when it comes to reduce the extraction time and improve the extraction yield, hence reducing the energy consumption [78,84,85].

***Ultrasound-Assisted Extraction (UAE)***

For UAE is required to supply ultrasonic energy, this energy passes through the mixture of the solvent and biomass as ultrasound (US) waves. This technique can reduce the operating time by enhancing the extraction, as the US waves break the cell wall due to the cavitation that is produced, making easier the release of the compounds of interest by improving mass transfer and diffusion. UAE is a technique that has been considered lately as a better alternative to conventional techniques, not only by reducing the extraction time but also by being inexpensive, having high efficiency, and the lack of need for high consumption of organic solvents. The US waves can be supply by the usage of a water bath or through a US probe. However, this technique has a huge disadvantage which is the lack of control of the temperature, which tend to be high, especially when the US probe is used. When the target compounds are thermosensitive, the water US bath is more recommendable due to a higher control of the temperature [79,81,86].

***Microwave-Assisted Extraction (MAE)***

Analogous to the other extraction methods, a mixture of solvent and sample is prepared and placed in a closed vessel, and then it is submitted to controlled temperature/pressure conditions, as well to electromagnetic radiations. The vessel can be open, but in this case, the extraction is made at atmospheric pressure. The microwave (MW) energy passes through the vessel and generates heat when interacting with polar compounds, as a consequence of the movement of dissolved ions and the dipolar rotation of polar solvents. Consequently, the cell wall ruptures and the compounds of interest are released [79,81,86].

The time of extraction can be shortened when compared with the normal SLE for the same extraction efficiencies, reducing the cost and the consumption of organic solvents, and decreasing thermal degradation. It is even possible to perform this extraction with no solvent, and the water present in the solid matrix should act as the solvent [56,81,83,87].

***Pressure Liquid Extraction (PLE)***

Pressure Liquid Extraction (PLE) can also be called Subcritical Solvent Extraction (SSE), where the solvent is at high temperature and pressure close to the supercritical region, where the surface tension and viscosity are low, leading to improving the extraction by promoting mass transfer and diffusion. PLE returns a higher extraction efficiency in lower extraction times, as the high pressure allows to reach above the boiling point of the solvents while still being in liquid phase, consequently, allows to reach a higher solubility and diffusion rate. This has been a technique that have been used to extract flavonoids [79,81,87].

***Enzyme Assisted Extraction (EAE)***

Enzyme Assisted extraction (EAE) can be considered a green extraction since it uses an enzyme to extract the desired compounds and mainly uses water as solvent medium. There are several factors that have impact on this extraction such as pH, temperature, time, the selection and concentration of the enzyme and maybe the stirring speed. Using an enzyme will improve the extraction due to its hydrolytic action on the cell wall [81,82,87].

### 1.3.3. Extraction techniques for algae

After harvesting and dewatering, the algae is ready to perform an extraction, although to promote the recovery of the desired compounds from the inner cell, a cellular wall disruption step is typically needed. The cell wall is composed of cellulose microfibrils that are parallel to the cell surface with lack of orientation, creating a fibrillar framework. The cell disruption can be done on a dry or wet base according to the technology being used. Further, new operations can be added to improve the yield of the extraction and/or to purify the final product [1,12]. Vieira et. al. analysed the damages in the cellular wall of *S. muticum* after the solid-liquid extractions through Scanning Electron Microscopy (SEM) [12,73].

Maceration is often the conventional SLE method carried out to extract polyphenols and fucoxanthin from different matrices, although the solvent used has an enormous impact on the extraction yield. Since PCs and fucoxanthin have different polarities, it is likely that the best solvent to extract each one of them will differ. As previously said, the algae is composed of different classes of PCs, despite their similarities, some properties are different, and therefore the extraction solvent has to be carefully chosen according to the compounds of interest [88].

Soxhlet extraction is a conventional technique not very recommendable, since it requires a long extraction time for polyphenols (up to 24-50 h), large quantities of solvent and sample, an evaporation step after the extraction, and the ability to reach high temperatures can end up degrading some PCs and the fucoxanthin [82,83].

With UAE the recovery performance of polyphenols is usually improved, hence this is a method commonly suggested to extract PCs. However, the cavitation, thermal and mechanical effects are not suitable for more sensitive compounds, as it can degrade some polyphenols and fucoxanthin, and can generate reactive hydroxyl radical in the bubbles. Notwithstanding, it is a method that consumes low amounts of solvent and energy, and it is a method suitable for industrial scales [88].

MAE helps in the recovery of polyphenols enhancing the efficiency not only in terms of extraction yield, which can increase by 70%, but also in terms of decreasing extraction times. However, this technique needs to be carefully applied since it can cause thermal degradation of sensitive polyphenols and fucoxanthin, and its scale-up

is not easy to perform. According to Meng et al. [88] MAE can increase the yield when compared to conventional SLE and UAE [88].

Although, EAE has a high cost associated which creates a drawback for industrial applications, it is a technique that can also be used to extract polyphenols. On the other hand, this method can be used to remove polysaccharides and proteins that are side-extracted compounds. PSE and Supercritical fluid extraction (SFE) are also expensive techniques and need specific equipment, which makes them more complex techniques [88].

## 1.4. Extraction solvents

### 1.4.1. Conventional extraction solvents

The extraction solvent is crucial to achieve a good separation performance. The extraction solvent should be chosen carefully according with the target compounds and their polarity. Parameters as selectivity, solubility, cost, and safety should also count for the solvent selection. Playing with solvents polarity may be helpful for the maximum valorisation of a certain biomass. For example, for *S. muticum*, polar organic solvents are typically used to extract PCs, since they have more affinity with polyphenols due to their tendency to also have a polar behaviour. A subsequent extraction using apolar solvents could be performed for fucoxanthin that has an apolar behaviour [52,81].

Normally organic solvents are the ones used to perform SLE extractions, especially alcohols such as methanol or ethanol, and they are usually mixed with a percentage of water when the target compounds have a polar behaviour [81]. Water is considered the most environmentally friendly solvent, it is non-toxic, non-flammable, non-corrosive, abundant in nature, and inexpensive. However, due to its polarity is not recommended to extract non-polar compounds [89].

### 1.4.2. Alternative extraction solvents

Despite the alternative extractions methods helped to improve the conventional extractions, organic solvents still present a problem that needs to be accessed, even if it is in a lower quantity, this still poses a problem for the environment and biocompatible applications. Given this, there is a need to replace these organic solvents for greener solvents and, ideally in some cases, that are biocompatible [87].

***Subcritical water***

Water is a solvent that can have its properties altered by varying the pressure and temperature; in fact, subcritical water is being employed as a solvent to perform extractions but requires high energy consumption. Subcritical water is water at high temperature and pressure close to the supercritical region where the water properties change drastically. In this region, the surface tension and viscosity tend to be low, which ends up improving the extraction by promoting mass transfer and diffusion; therefore, returns a higher extraction efficiency in lower extraction times. The subcritical conditions also have a huge impact on the polarity of water, especially the variation of the temperature, since at these conditions the dielectric constant of water decreases; consequently, the polarity decreases, and this creates an opportunity to extract organic compounds that tend to be more soluble in subcritical water [89,90].

The subcritical conditions can increase the solubility of the target compounds but also the solubility of undesired compounds, which creates a drawback when making the decision to use this technique. Another

disadvantage is that subcritical water cannot be used when the target compounds are thermolabile, as high-water temperatures can cause compounds degradation [87,89].

***Supercritical Fluid Extraction (SFE)***

A fluid that has its pressure and temperature above the critical point is called supercritical fluid. Fluids in these conditions are being used as a replacement to organic extraction solvents, as they present a good efficiency when extracting compounds due to their unique behaviour in terms of having gas and liquid properties [87].

Using supercritical fluids provides a faster extraction, and this is due to the low viscosities and high diffusivities that they possess, which allows for a higher diffusion and mass transfer. The density also has an important role in the extraction with the advantage of being a property that can be manipulated by temperature and pressure. Controlling this property is a vital factor in the extraction efficiency because it is related with the solubility, at a constant temperature, both increase by increasing the pressure [87].

This technique has a large disadvantage which is the cost, is quite an expensive technique and has a low selectivity towards polar compounds [87].

***Biobased solvents***

The synthesis of bio-solvents should lower environmental impacts since they are produced from renewable agricultural biomass. These bio-solvents can be classified into different categories according to the biomass used to produce them: lignocellulosic, sugar and starch, protein and oil-based, and in other forestry and food wastes [90]. Bio-solvents have an advantage when used in extraction processes which is their low viscosity, hence being easy to handle and decreasing limitations to the diffusion phenomena; however, their scale-up it is still challenging for some of these bio-solvents [91].

***(Deep) Eutectic Solvents ((D)ESs)***

(D)ESs were firstly synthesized in 2003 by Abbott et al. [92], and since then they had exponential growth in the amount of publication throughout the years, Figure 17. (D)ESs are generated by mixing two or more compounds that form intermolecular interactions between them, usually these interactions are hydrogen bonds where a hydrogen bond donor (HBD) interacts with a hydrogen bond acceptor (HBA). These interactions cause a shift of the melting temperature to temperatures lower than the melting points of the pure compounds [93–95].

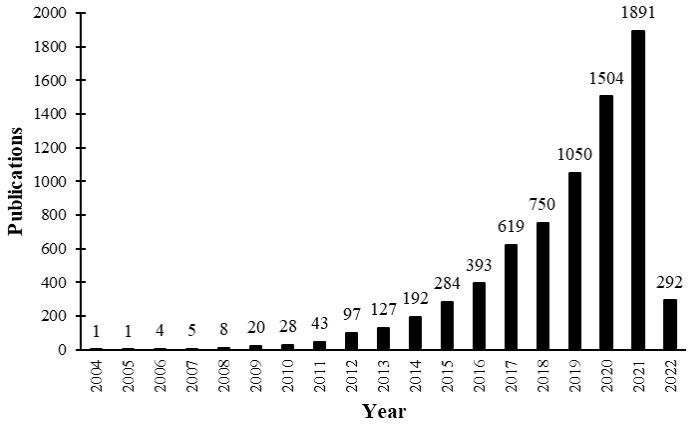



Figure 17. Several publications doing research on “deep eutectic solvent” from 2004 until 30/3/2022 on Web of Science [22].

The solid-liquid equilibria (SLEq) of a mixture of two solids, Figure 18, can be describe by the following expression (1) where $x_i$ is the molar composition of a compound i, $\gamma_i$ is the activity coefficient, $\Delta H_{fus}$ is the fusion enthalpy, $T_{fus}$ is the melting temperature for the pure compound, T is the melting temperature for the solid mixture, R the universal gas constant and $\Delta_{fus}C_p$ is the difference between the molar heat capacity of compound i in the solid and liquid phases. However, an approximation can be made of the expression (1) where the $\Delta_{fus}C_p$ is neglected and a simpler approach is achieved, equation (2) [96,97].

$$ln(\gamma_i x_i) = \frac{\Delta H_{fus}}{R}\left(\frac{1}{T_{fus}} - \frac{1}{T}\right) + \frac{\Delta_{fus}C_p}{R}\left(\frac{T_{fus}}{T} - ln\left(\frac{T_{fus}}{T}\right) - 1\right) \quad (1)$$

$$ln(\gamma_i x_i) = \frac{\Delta H_{fus}}{R}\left(\frac{1}{T_{fus}} - \frac{1}{T}\right) \quad (2)$$

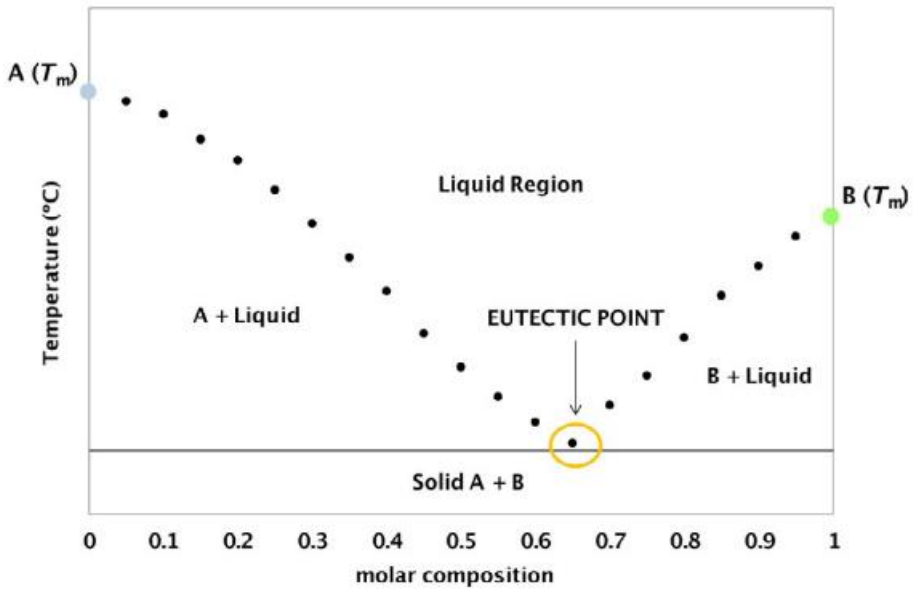



Figure 18. Solid-liquid equilibria diagram for a binary mixture [93].

A subclass of (D)ESs that have shown great interest in green chemistry are Natural (Deep) Eutectic Solvents (NA(D)ESs), are considered type III (D)ESs, which are (D)ESs made with natural compounds and due to this, they are readily biodegradable. Since NA(D)ESs are made with natural compounds, usually, they do not show any biocompatibility problems and can be considered safe to be applied in the food, pharmaceutical, and cosmetics industries [93,97].

A great advantage of (D)ESs is the ease of preparation with 100% of atom efficiency. However, there are more than one way to prepare a (D)ES. The most prevalent one is heating while stirring, until an homogenous liquid is formed. If the melting points of the two compounds are too different, one can tend to degrade while the other it is still in its solid form. This can occur especially when forming NA(D)ESs because of the low thermal stability of most natural compounds. In these situations, an alternative method it is needed, such as a simple dissolution of the compounds in a certain solvent that has affinity with both compounds, followed by its evaporation or a simple griding method [93].

The usage of NA(D)ESs as a solvent in extraction processes has been a subject of several studies. Natural compounds such as PCs, flavonoids, sugar, proteins, and natural pigments, among others from biomass matrices have been successfully extracted using NA(D)ESs, even showing a higher extraction efficiency with lower extraction time. NA(D)ESs also presented high stabilization and solubilization properties. Due to these properties, the good results that NA(D)ESs present in extractions, and their economic and environmental advantages, they are emerging as the most encouraging alternative solvent to replace conventional solvents. Some companies such as BASF already started using NA(D)ESs in their products [90].

### 1.4.3. Extraction solvents selection

This work aims to develop green extraction processes for PCs and fucoxanthin from *S. muticum* using (D)ESs and bio-based solvents, respectively. Therefore, research about potential (D)ES and bio-based solvent that could be employed need to be made.

***(D)ESs as extraction solvent of PCs***

To find a (D)ESs that is able to replace conventional toxic solvents in the extraction of PCs, intensive research was performed to understand the (D)ESs that would show higher efficiency for PCs extraction. To do so, a summary of (D)ESs from different publications was compiled as presented in Table 3. It can be seen that the (D)ESs are mostly choline chloride (ChCl), proline (Pro), and betaine (Bet)-based. The water content was not an object of interest because an optimization of it was already intended to be performed.

Table 3. Several (D)ESs already used for extractions of phenolic compounds.

| Compound 1 | Compound 2 | Ratio | Reference |
|---|---|---|---|
| **ChCl** | Lactic Acid | 1:1<br>1:2<br>1:3 | [98] |
| **ChCl** | Propylene Glycol (1,2-Propanediol) | 1:1<br>1:2 | [99] |
| **ChCl** | 1,4-Butanediol | 1:2<br>1:5 | [100,101] |
| **ChCl** | Glycerol | 2:1<br>1:1<br>1:2<br>1:3<br>1:4<br>1:5<br>1:7 | [100,102–105] |
| **ChCl** | Citric Acid | 2:1<br>1:1<br>1:2 | [102,105–107] |
| **Pro** | Lactic Acid | 1:1 | [102] |
| **Pro** | Glycerol | 2:5 | [102] |
| **Bet** | Lactic Acid | 1:1<br>1:3<br>1:5<br>1:7 | [98] |
| **Bet** | Glycerol | 1:2 | [104] |
| **Bet** | Propylene Glycol (1,2-Propanediol) | 1:3<br>1:5<br>1:7 | [108] |
| **Bet** | 1,3-Propanediol | 1:3<br>1:5<br>1:7 | [108] |
| **Bet** | 1,2-Butanediol | 1:3<br>1:5<br>1:7 | [108] |
| **Bet** | 1,3-Butanediol | 1:3<br>1:5<br>1:7 | [108] |
| **Bet** | 1,4-Butanediol | 1:3<br>1:5<br>1:7 | [108] |

It cannot be forgotten that the proposed (D)ESs need to be biocompatible, so it is important that they are approved by the European Food Safety Authority (EFSA) and Food & Drug Administration (FDA), Table 4. According to EFSA, choline chloride has restrictions when ingested that varies with the sex and age [109]. In terms of cosmetic application, only choline chloride is prohibited to be used [110]. There is no information about 1,2-butanediol in FDA, and the European commission affirms that is inconclusive if 1,2-butanediol is toxic or not when ingested. However, it can be used to cosmetic applications [111,112]. Proline is already used orally through nutritional supplements ranging between 0.5 and 1g/day. Also, in smaller amounts, proline has been employed in a variety of vaccines already, may be used as food additive and it has no restrictions for cosmetic applications [113–115].

Table 4. Compounds allowed to be used as food additives by EFSA and FDA.
* has restrictions, ** no information

| | **EFSA** | **FDA** |
|---|---|---|
| Choline Chloride* | [109] | [116] |
| Lactic Acid | [117] | [116] |
| Glycerol/Glycerin | [117] | [116] |
| Citric acid | [117] | [118] |
| Propylene glycol (1,2-Propanediol) | [117] | [118] |
| Proline | [115] | [118] |
| Betaine | [119] | |
| 1,3 – Butanediol | [120] | |
| 1,2 – Butanediol** | | |

***Bio-based solvents as extraction solvent of fucoxanthin***

The use of bio-based solvents to extract carotenoids or specifically fucoxanthin has not yet received much attention. The scarce information available has been reported by Nie et al. [121] and Pilar Sánchez-Camargo et al. [122], both studying the extraction of fucoxanthin from *Sargassum fusiforme* and *Phaeodactylum tricornutum*, respectively, using limonene, ethyl acetate and ethyl lactate. Mussagy et al. [123] used different bio-based solvents, such as isopropanol, cyclohexane and 2-methyl tetrahydrofuran. However, their study focused on the extraction of all carotenoids from *Rhodotorula glutinis* [121–123].

# 2. Motivation

Pursuing green paths that are environmentally friendly to obtain chemical compounds of interest is crucial in the current days to reduce the greenhouse gas emissions. Extraction processes create an opportunity to discover a green path through the extraction of value-added compounds from different biomass. Among the different biomass that can be exploited, bio-waste is the preferential one, as it does not compete with food and helps to reduce the waste in the planet.

Macroalgae have been shown to be a biomass with much potential as a natural source of functional compounds that have biological activity. They tend to be invasive, have a negative ecological impact, and their removal is normally necessary. Consequently, they are considered bio-waste. *Sargassum muticum* was the brown macroalgae employed as biomass in this work, and it is a seaweed whose pigment, fucoxanthin, is an expensive compound of great interest and whose content in PCs is high. Both fucoxanthin and PCs possess relevant properties that bring increasing attention to them. Also, both these markets are expected to grow, there is room for an increase in the production of these compounds and for the development of new technologies of production.

Conventional production routes tend to have negative environmental impacts which creates major drawbacks nowadays, and therefore it is important to search for alternative green technologies to obtain these compounds. This motivated the opportunity for this work, where the objective is to develop a greener pathway to obtain such valuable compounds, not only by replacing toxic conventional solvents by (D)ESs and bio-based solvents for the extraction of PCs and fucoxanthin respectively, but also by reducing the energy consumption in the extraction process through novel intensification techniques. This project also aims to obtain final extracts that could be applied in formulations of pharmaceutical, food and cosmetics, hence the solvents should be non-toxic and biocompatible.

# 3. Materials and Methods

## 3.1. Materials and reagents

The brown seaweed *Sargassum muticum* was collected at Praia Norte beach, Viana do Castelo, Portugal (41° 41' 44.2'' N 8° 51' 8.1'' W) in Spring/Summer of 2015 and immediately transported to the laboratory. After cleaned and washed, firstly with seawater to remove invertebrate organisms, epiphytes, and detritus, then with distilled water. *S. muticum* was frozen at -20°C and freeze-dried (Scanvac Cool Safe, LaboGene, Lynge, Denmark). The dried algal material was ground into a powder in a grinder and stored protected from light, at room temperature.

### 3.1.1. Phenolic compounds extraction

The solvent used to perform the conventional extraction of phenolic compounds was ethanol absolute (Scharlau). The standards used were gallic acid (purity 97.5%-102.5% titration), 3,4-dihydroxybenzoic acid (also called Protocatechuic acid) (purity ≥97% T), (+)-catechin (purity ≥98%), caffeic acid (purity ≥98%), syringic acid (purity ≥95%), p-coumaric acid (purity ≥98%), ferulic acid (purity 99%), salicylic acid (purity ≥99%) and quercetin (purity ≥95%), all purchased from Sigma-Aldrich.

The compounds used to prepare the (D)ES were L-proline (Pro) (purity 99%), choline chloride (ChCl) (purity ≥98%), betaine (Bet) (purity ≥98%), propylene glycol (PPG) (purity 99%), citric acid (CA) (purity 99%), 1,2-butanediol (1,2-But) (purity ≥98%), (±)-1,3-butanediol (1,3-But) (purity 99.5%), lactic acid (LA) (purity ≥85%), all purchased from Sigma-Aldrich; and glycerol (Gly) (purity 99.5%), purchased from Panreac. For weight measurements, an analytical balance Sartorius M − POWER AZ124 with a repeatability of ± 0.0002 g was used. The pH of the selected (D)ESs for the optimization was measured at room temperature using a Consort SP28X pH electrode connected to a Consort multiparameter C3010 analyser. The densities were measured with an Anton Paar DMA 500 densimeter and the viscosities with Anton Paar (model SVM 3000) automated rotational Stabinger viscometer-densimeter with a temperature uncertainty of ±0.01 K.

To perform the conventional extraction at a controlled temperature, the Orbital Labnet International, Inc. Vortemp 1550 was used, followed by the centrifuge unicen 21, orto alresa. For the intensification extraction methods, a Hielscher ultrasound probe and a Microwave Anton Paar Monowave 400 were used.

The Folin & Ciocalteu phenol reagent used to perform the Folin-Ciocalteu assay was acquired from Sigma-Aldrich and the sodium carbonate ($Na_2CO_3$) (purity 99.5 to 100.5%) from Labkem. The sodium nitrite ($Na_2NO_3$) used in the flavonoids assay was purchased from Emsure, aluminium chloride ($AlCl_3$) from Sigma-Aldrich (purity ≥98%) and sodium hydroxide (NaOH) from J.T.Baker. The 2,2-Diphenyl-1-picrylhydrazyl (DPPH) for the antioxidant activity assay was purchased from Sigma-Aldrich. The UV–Visible Spectrophotometer used in the colorimetric methods was JASCO V-730. The (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) was purchase from Sigma-Aldrich (purity≥ 97%).

The characterization of the phenolic acids and flavonols was performed through a reverse high-performance liquid chromatographic (HPLC) analysis using the JASCO 4000 Series HPLC system with a Fortis C18 column (250 mm x 4.6 mm, 5 µm) and a photodiode array (DAD) detector at 25 ºC. The water and the acetic

acid (purity 99.5%) used for the polar mobile phase were acquired from Honeywell and Labkem, respectively, and for the apolar mobile phase acetonitrile (purity ≥99.9%) was purchased from Honeywell.

For the polarity evaluation through solvatochromic probes, 2,6-Dichloro-4-(2,4,6-triphenyl-N-pyridino)-phenolate (Reichardt's betaine dye 33), 4-nitroaniline, and N,N-diethyl-4-nitroaniline were purchased from Fluka (≥97% mass fraction purity), Sigma Aldrich (≥99% mass fraction purity) and Frinton Laboratories, respectively. To acquire the UV-vis absorption data for the polarity assays a Shimadzu UV-1800 spectrophotometer (UV/vis) was used and all spectroscopic measurements were performed in triplicate.

### 3.1.2. Fucoxanthin extraction

Fucoxanthin purchased from Sigma-Aldrich was used as standard and analysed with the same HPLC used to characterize the PCs. For fucoxanthin extraction, several bio-solvents and conventional solvents were used as follows: methanol (MeOH) (Honeywell, purity≥99.9%), ethyl acetate (EtAc) (Honeywell, purity≥99.5%), dimethyl sulfoxide (DMSO) (PanReac AppliChem, purity ≥99.5%), ethyl(-)-L-lactate (EtLac) (Emplura), isopropyl alcohol (IPA) (purity≥99.5%), R-(+)-Limonene (purity≥97%), 2-methyltetrahydrofuran (purity≥2-MeTHF) (≥99%), dimethyl isosorbide (DMI) (purity≥99%), dimethyl carbonate (DMC) (purity≥99%), γ-valerolactone (γ-Val) (purity≥99%), cyrene (purity≥98,5%) and cyclopenthyl methyl ether (CPME) (purity≥99,5%), these last eight compounds purchased from at Sigma-Aldrich.

## 3.2. Methods

### 3.2.1. (D)ES formation

The eutectic mixtures were prepared by adding HBA (ChCl, Bet and Pro) with HBD (PPG, Gly, LA, CA, 1,2-But, 1,3-But) in different ratios, as presented in Table 5. The mixtures were heated up to 80 ºC, except the (D)ESs composed of ChCl and CA that were heated until 100 ºC and stirred at 1000 rpm. After achieving a homogeneous liquid solution, the heat was turned off until the room temperature was reached, always stirring. The (D)ESs composed of ChCl and CA were the only ones that had to be prepared with a small quantity of water (6.4 %(w/w)) due to high viscosity.

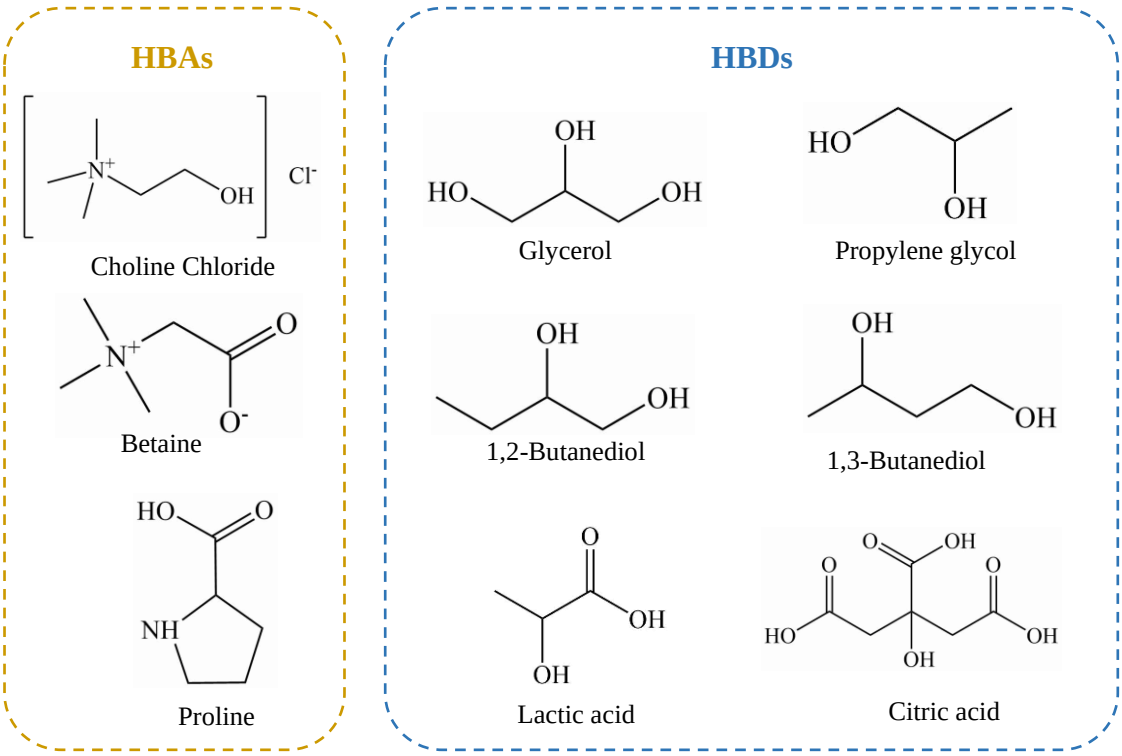



Figure 19. Molecular structures of the compounds used to make the eutectic mixture divided in HBA and HBD.

Table 5. (D)ES combinations tested on the screening.

| HBA | HBD | HBA:HBD ratio | Abbreviations |
|---|---|---|---|
| Betaine | Glycerol | 1:4 | Bet:Gly (1:4) |
| | Propylene glycol | 1:4 | Bet:PPG (1:4) |
| | 1,2-Butanediol | 1:3 | Bet:1,2-But (1:3) |
| | | 1:4 | Bet:1,2-But (1:4) |
| | 1,3-Butanediol | 1:4 | Bet:1,3-But (1:4) |
| | Lactic acid | 1:1 | Bet:LA (1:1) |
| | | 1:2 | Bet:LA (1:2) |
| | | 1:3 | Bet:LA (1:3) |
| Proline | Glycerol | 1:4 | Pro:Gly (1:4) |
| | Propylene glycol | 1:4 | Pro:PPG (1:4) |
| | 1,2-Butanediol | 1:3 | Pro:1,2-But (1:3) |
| | | 1:4 | Pro:1,2-But (1:4) |
| | 1,3-Butanediol | 1:4 | Pro:1,3-But (1:4) |
| | Lactic Acid | 1:1 | Pro:LA (1:1) |
| | | 1:2 | Pro:LA (1:2) |
| | | 1:3 | Pro:LA (1:3) |
| Choline chloride | Glycerol | 1:4 | ChCl:Gly (1:4) |
| | Propylene glycol | 1:4 | ChCl:PPG (1:4) |
| | 1,2-Butanediol | 1:3 | ChCl:1,2-But (1:3) |
| | 1,3-Butanediol | 1:4 | ChCl:1,3-But (1:4) |
| | Lactic acid | 1:1 | ChCl:LA (1:1) |
| | | 1:2 | ChCl:LA (1:2) |
| | | 1:3 | ChCl:LA (1:3) |
| | Citric Acid | 2:1 | ChCl:CA (2:1) |
| | | 1:1 | ChCl:CA (1:1) |
| | | 1:2 | ChCl:CA (1:2) |

### 3.2.2. Extraction

***Conventional SLE***

All the extractions were performed in a solid-liquid ratio of 1:10 (w/v) in the orbital shaker for 100 min, at temperatures of 50 ºC, 60 ºC and 70 ºC. After agitation, the sample was subjected to 15 min of centrifugation at 4200 rpm.

***Ultrasound-assisted extraction (UAE)***

Again, the ratio of 1:10 (w/v) was used and the time was modified in order to perform an optimization using times of 1.5, 3, 6 and 9 min. Two values of power were tested, 10 and 20 W.

***Microwave-assisted extraction (MAE)***

Analogous to the other extraction techniques the ratio used was 1:10 (w/v) and the time was modified at 1.5, 3, 6 and 9 min, as when using the US; however, for the (D)ES composed of ChCl and CA, the time of 12 min was also tested to evaluate the impact of viscosity in the extraction. The temperature was changed between 60 ºC and 100 ºC.

### 3.2.3. Folin– Ciocalteu method

The TPC of a certain extract can be evaluated by the Folin-Ciocalteu assay, where the transfer of electrons phenolic compounds to phosphomolybdic/phosphotungstic acid complexes in alkaline conditions occurs and these reactions cause a colour change then it is detected on UV-Vis spectrometer at 765 nm [51].

The experimental steps for the Folin method is described in detail by Canãdas et al. [124]. Initially, 100 µL of the sample was added to a vial followed by 100 µL of the Folin reagent, and after 3 min in the dark at room temperature, 2 mL of a 2% (v/v) of $Na_2CO_3$ aqueous solution was added followed by 30 min in the dark at room temperature. After the 30 min passed, the absorbance was measured at 765 nm using distillate water as blank. The Folin-Ciocalteu used gallic acid aqueous solution as standard, therefore the TPC was expressed in mg of gallic acid equivalent per g of dry weight (mg GAE/g). For some samples where the absorbance was too high, a dilution had to be made before performing this assay using distilled water. This assay was also performed for the pure (D)ES used in the extraction, to subtract the result obtained for the sample in order to determine the TPC extracted from *S. muticum.* Note that for the ChCl-based (D)ES, the ChCl tends to precipitate.

This method has some disadvantages, namely, the impossibility to characterize the phenolic compounds such phenolic acids, flavonoids, lignans, coumarins, and phlorotannins, as all of them make part of the count of the total phenolic content measured with Folin assay. Another major disadvantage is that other molecules such as sugars, thiols, and proteins can also react, returning a signal that can also count as phenolic content [51].

### 3.2.4. Christ-Müllers method

The Christ-Müllers assay allows to quantify the total flavonoid content (TFC) which was determined by $AlCl_3$ complexation, where the formation of a complex between the flavonoid and $AlCl_3$ occurs, which is detected by a colorimetric method at 510 nm in the presence of $NaNO_2$. The scheme of the formation of the Al(III) complex is presented in Figure 20 [125,126].

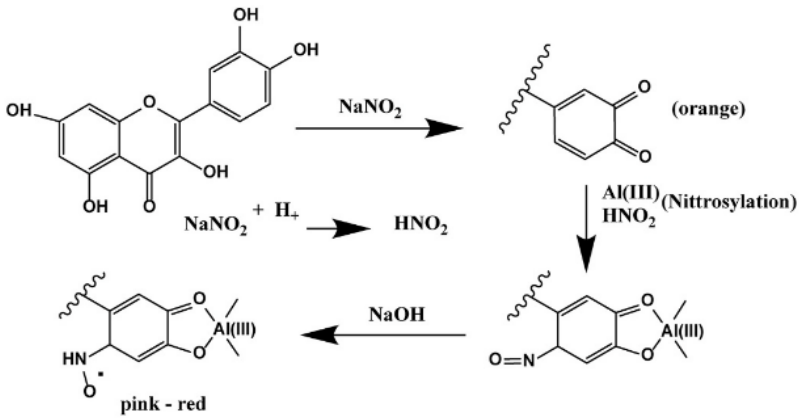



Figure 20. Scheme for the sequential chemical reactions that occurs to form the complex AL(III)-flavonoids in the presence of NaNO2, the quercetin is used as an example since is typically the flavonoid used to perform a calibration curve [125].

The method is described by Canãdas et al. [124], with slight modifications using quercetin aqueous solution as standards. Initially, 2 mL of distilled water was added to a vial, followed by the addition of 150 µL of 5% (w/v) $Na_2NO_3$ aqueous solution, and then 450 µL of the sample; the vials were then agitated at a vortex and kept at room temperature with no need to hide from the light. After 5 min, 150 µL of the 10 %(w/v) $AlCl_3$ aqueous solution was added, and after agitated the vials, they were kept still at room temperature for another 5 min; lastly, the addition of 1 mL of 1 M NaOH aqueous solution was made, and again agitated, but this time the vial was kept still for 15 min. Afterwards, the absorbance was measured at 510 nm using distillate water as blank. The TFC was expressed in mg of quercetin equivalent per g of dry weight (mg QE/g). Again, dilutions were performed before this assay for the samples showing high absorbance, and this method was also performed for the pure (D)ES used in the extraction.

### 3.2.5. DPPH free radical scavenging

In 1958 Blois et al. [127] proposed a method to evaluate the antioxidant activity using a stable free radical, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), that allowed an assessment of the antioxidant activity. However, the method needed some improvements made by Brand-Williams et al. in 1994 [128]. Usually, a solution of DPPH in methanol is used, and when placed with an antioxidant, the reduction of DPPH occurs; the reduction scheme of DPPH is presented in Figure 21 [128].

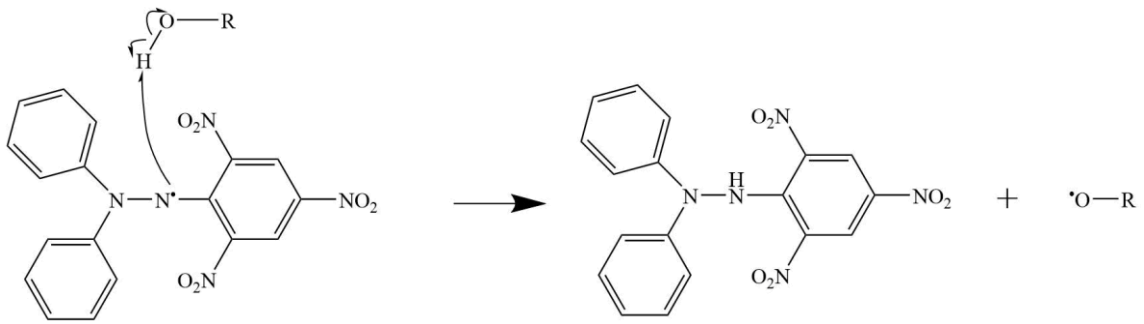



Figure 21. Reduction mechanism of the DPPH radical when an antioxidant is present.

The DPPH radical absorbs at 515 nm and when it suffers the reduction reaction, the colour purple tends to turn yellow, hence causing a loss of absorbance at 515 nm. Measuring the absorbance of the extract after the

DPPH suffer reduction ($A_{sample}$) and having the absorbance of the solution of DPPH in MeOH ($A_{blank}$), it is possible to calculate the inhibition percentage making use of expression (3) [76,124].

$$\%\text{Inhibition} = \frac{(A_{blank} - A_{sample})}{A_{blank}} \cdot 100\% \quad (3)$$

The steps followed to execute this assay are described by Canãdas et al. [124]. Initially, a volume of 100 µL of the sample was added to a vial followed by the addition of 2.9 mL of a solution of 24 mg/L of DPPH in MeOH, and then the vials were kept in the dark at room temperature for 30 min. Afterwards, the absorbance was measured at 515 nm. Every time this method was performed for the samples, it was also performed for pure MeOH in order to calculate the percentage of inhibition. This method was also performed for the pure (D)ES in order to evaluate the antioxidant activity of the (D)ES itself. Note that all the proline-based (D)ES tend to form flakes.

Trolox was used as a standard antioxidant and the measurement of the percentage of inhibition was performed for different Trolox concentration in order to build a calibration curve. Given this, the Trolox equivalent antioxidant activity (TEAC) will be present in mg of Trolox equivalents per g of dry weight.

### 3.2.6. HPLC analysis

***Phenolic compounds HPLC method:***

To be able to separate the phenolic compounds, a gradient elution had to be imposed using different ratios of a polar phase (aqueous solution (A) with 1.25 % (v/v) of acetic acid) and an apolar phase (acetonitrile (B)). The initial composition of the mobile phase was 90% of phase A and 10% of phase B until the method reached 8 min, at these minute a decrease of 10% in phase A was performed staying with 80% of phase A which was maintained until 25 min, at the minute 25 the percentage of phase A was decreased to 75% of phase A, from 30 min to 37 min a more accentuated decreased was imposed to 55% of phase A, followed by another decrease from 37 min to 43 min to 20% of phase A, lastly at 43 min an accentuate increase was performed up to 90% of phase A until the end of the method with a total duration of 48 min. The injected volume was 100 µL and a 0.5 mL/min of flow. To be able to detect all the different phenolic compounds studied herein, three channels of the DAD detector with different wavelengths had to be used, one with 271 nm where the gallic acid, 3,4-dihydroxybenzoic acid, (+)-catechin, syringic acid, and salicylic acid were detected; the second channel had a wavelength of 284 nm, where the caffeic acid, p-coumaric acid, and ferulic acid were identified; and the third wavelength was 323 nm to detect quercetin. To obtain the calibration curve a multipattern was prepared adding 100 ppm of each phenolic compound, and dilutions were made in other to have sub-standards with concentrations of 0.5, 1, 2.5, 5, 10, 20, and 50 ppm; for each compound at each concentration, the peak was area was measure.

***Fucoxanthin HPLC method:***

For fucoxanthin there was no need to impose a gradient since it was the only compound, and was taken into account that the fucoxanthin has a more apolar behaviour than polar; due to this, the elution composition was fixed at 15% of A and 85% of B. The injected volume was imposed at 100 µL with a 1 mL/min of flow. The detection of fucoxanthin was made at a wavelength of 445 nm in the DAD detector and the method lasted for a total of 27 min.

### 3.2.7. COSMO-RS

COSMO-RS (COnductor-like Screening Model for Real Solvents) is a practical tool developed by Klamt to predict thermodynamic properties of fluids without the need of *a priori* experimental data. COSMO-RS method is based on a combination between statistical thermodynamics and computation quantum mechanics, which helps characterizing the molecular interactions and evaluating the solution behaviour of a certain solute in a solvent through the activity coefficient (**γ**). This is valuable information to provide some guidance for the experimental stage, saving time and resources [129–131].

In addition, COSMO-RS method allows to obtain the σ-profile, as a probability distribution of a molecule's or mixture's surface-charge density obtained from quantum chemical calculations. The σ-profile is divided in three zones, the more shifted to the right where the molecule tends to be HBA, in the range more to the left the molecule presents HBD capacity and in the middle zone, typically the region $\sigma \pm 0.01$ e/Å$^2$, is considered weakly polar or even non-polar [129–131].

In this work the molecules that were not included in the COSMO-RS database, they were optimized to their minimum energy structure using Turbomole, and after the structural optimization the *.cosmo file was generated at BP86/TZVP computational level.

### 3.2.8. Thermophysical properties: Density and Viscosity

Density and viscosity are crucial thermophysical properties due to their impact in the mass transport phenomena that will affect the extraction capacity [132]. For the densities it is perform a quadratic adjustment, equation (4) where a, b and c are fitting parameters, ρ is the density in g/cm$^3$ and T the temperature in K.

$$\rho = a \cdot T^2 + b \cdot T + c \quad (4)$$

The viscosity data can be adjusted using a Vogel-Fulcher-Tammann (VFT) model given by expression (5), where $A_\eta$, $B_\eta$ and $C_\eta$ are fitting parameters, η the viscosity in m.Pa.s and T the temperature in K. For the VFT adjustment there was a need to resort to *SOLVER* from excel [132].

$$\ln(\eta) = A_\eta + \frac{B_\eta}{T - C_\eta} \quad (5)$$

### 3.2.9. Betaine dye scale and Kamlet-Taft parameters

There are two scales that can be employed to evaluated the polarity of a certain solvent: Betaine dye scale through the calculation of $E_T(30)$ and $E_TN$ parameters that measures the overall polarity and Kamlet-taft parameters, α, β and π* that measures the ability to be HBD, HBA and polarizability, respectively.

***Betaine dye scale***

The solvent polarity, $E_T(30)$ is an empirical parameter introduced due to the solvent-induced shift of the visible $\pi \rightarrow \pi^*$ absorption band of intramolecular charge-transfer character. This shift is what makes the betaine dye to change colour depending on the solvent employed. The charge-transfer character occurs between the phenolate to the pyridinium moiety. Besides the effect of the solvent in the long wavelength, also affects the band shape. Accordingly, to Reichardt et al. [133] with the increase of solvent polarity, the long wavelength tends to suffer a bathochromic shift (red shift) [133].

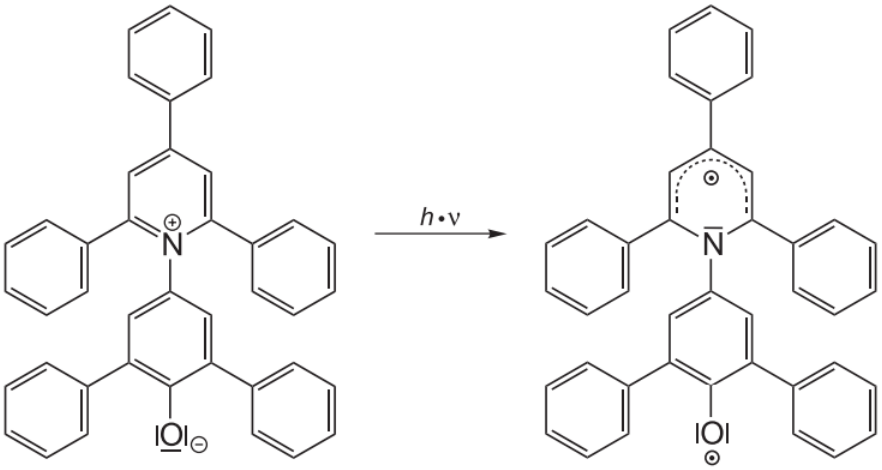



Figure 22. Transition π → π* of the ground-state zwitterion of betaine dye 33 to the less dipolar radical pair in the excited state [133].

When the solvent presents a high stabilization of the ion-pair in the ground state, it means that the energy of the transition band is high. This energy transition is proportional to the polarity parameter, Z, and for that reason higher transition energy transitions means high solvent polarity. The $E_T(30)$ is nothing more than the energy of transition of the dissolved betaine dye 30 [133].

The betaine dye 30 typically used to polarity evaluation presents limitations to be employed for acidic solutions. In acidic environment the protonation of the oxygen in the phenolate occurs and UV-Vis absorption band tends to disappear. Therefore, it is recommended to use other betaine dyes with other substituents (p.ex. Cl) that reduces the basicity of the phenolate moiety. The betaine dye having chlorine atoms as substituents, betaine dye 33, as a way to reduce the basicity is something was already studied [133]. The transition energies of betaine dye 33, $E_T(33)$, have already been correlated with $E_T(30)$ according with expression (6) since it is usually recommend to use $E_T(30)$ in the further calculations [134].

$$E_T(30) = 0.9953(\pm 0.0287) \cdot E_T(33) + 8.1132(\pm 1.6546) \quad (6)$$

The $E_T(33)$ parameter can be determined with expression (7), after the measurement of the wavelength corresponding to the maximum absorbance, $\lambda_{max}$ [134].

$$E_T(33) = \frac{28591}{\lambda_{max}} \quad (7)$$

The normalized polarity, $E_TN$, measures the overall polarity of a solvent and can be determined using the $E_T(30)$ parameter through equation (8).

$$E_TN = \frac{E_T(30) - 30.7}{32.4} \quad (8)$$

***Kamlet-taft scale***

Kamlet taft scale has three parameters: α that is the HBD ability, β which is the HBA ability, and π* that is the polarisability/dipolarity of a certain solvent.

The parameter π* gives information about the polarisability and dipolarity of a solvent and it can be calculated with equation (9), where $\bar{\nu}$ is maximum the wavenumber in $cm^{-1}$. From the UV-Vis data only the wavelength is provided, however, the wavenumber can be determined through expression (10).

$$\pi^* = 0.314 \cdot (27.52 - \bar{\nu}_{N,N-diethyl-4-nitroaniline}) \quad (9)$$

$$\bar{\nu}(cm^{-1}) = \frac{10^7}{\lambda_{max}(nm)} \quad (10)$$

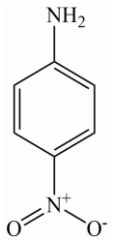
HBD ability, α, measures the capacity of a solvents to donate hydrogen bonds and this can be calculated using expression (11) using the $E_T(33)$ and π* obtained from equation (7) and (9), respectively.

$$\alpha = 0.0649 \cdot E_T(33) - 2.03 - 0.72 \cdot \pi^* \quad (11)$$

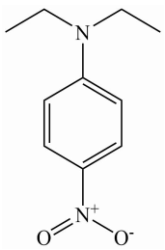
HBA ability, β, compares the solvent-induced shifts of 4-nitroaniline and NN-Diethyl-4-nitroaniline, and this comparison can be then postulated into an equation given by expression (12).

$$\beta = \frac{1.035 \cdot \bar{\nu}_{N,N-diethyl-4-nitroaniline} + 2640 - \bar{\nu}_{4-nitroaniline}}{2800} \quad (12)$$

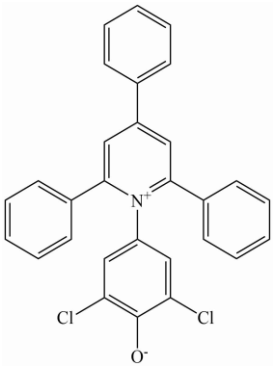
The experimental procedure was based in Florindo et al. [134] where dichloromethane solutions for all three probes presented bellow in Figure 23 were prepared, with concentrations of 0.1 ppm for 4-nitroaniline and N,N-diethyl-4-nitroaniline and 0.5 ppm for reichardt's betaine dy 33. In three different glass vials where added 80 μL of the probe solution for 4-nitroaniline and N,N-diethyl-4-nitroaniline and 400 μL for reichardt's betaine dy 33 in each vials. This step was followed by the evaporation of dichloromethane under vacuum. After being sure that the dichloromethane is all evaporated, 800 μL of the (D)ES was added to each vial. After the (D)ES be thoroughly mixed, it was transferred to a 1 mm light path quartz cuvette [132,134].




4-nitroaniline




N,N-diethyl-4-nitroaniline




2,6-Dichloro-4-(2,4,6-triphenyl-N-pyridinio)-phenolate (Reichardt's

Figure 23. Solvatochromic Probes used to evaluate the polarity.

# 4. Results and Discussion

## 4.1. Phenolic Compounds

### 4.1.1. (D)ESs Screening

The objective of this work is to try to replace conventional organic solvents by new green solvents that could at least return an equivalent extraction efficiency followed by the use of intensification techniques and optimization. In order of trying to find a (D)ES that could play the role of a green extraction solvent a screening was carried out. The (D)ESs selection to be tested in the screening was inspired in Table 3 according to available literature about the extraction of phenolic compounds from different biomasses, and all (D)ESs can be organized in three groups: betaine-based, proline-based and choline-based. Notwithstanding, as a comparison for the extractions employing (D)ESs, extractions with conventional solvents, water and EtOH:water (70:30 v/v), were also carried out. Although, a few of the (D)ESs tested in the screening were not verified to be previously published, they were added to the screening's list with the belief that they could return prosperous results.

The general methods, Folin-Ciolcalteu assay, Christ-Müllers assay and antioxidant activity assay, usually employed to characterize the extracts, were used to analyse all the extracts obtained using the solvents in the screening, Figure 24. It was detected immediately that proline tends to generate flakes in the antioxidant activity assay and that ChCl precipitates in the Folin-Ciolcalteu assay, making them more difficult to assess in the UV-Vis. Given this, it is important to acknowledge that the TPC and TEAC are not very reliable for choline-based and proline-based (D)ESs, respectively.

The TPC was higher for the proline-based (D)ESs, being the higher value 15.30 mg of GAE/g of dry weight for Pro:1,2-But (1:3), followed by Pro:PPG (1:4) with 15.26 mg of GAE/g of dry weight, Pro:1,2-But (1:4) with 14.84 mg of GAE/g of dry weight and Pro:Gly (1:4) with 14.14 mg of GAE/g of dry weight. In comparison with the two conventional solvents, water and EtOH:water (70:30 v/v), 5.64±0.03 and 3.70±0.13 mg of GAE/g of dry weight, respectively, it is seen a notable increase in the extraction yield.

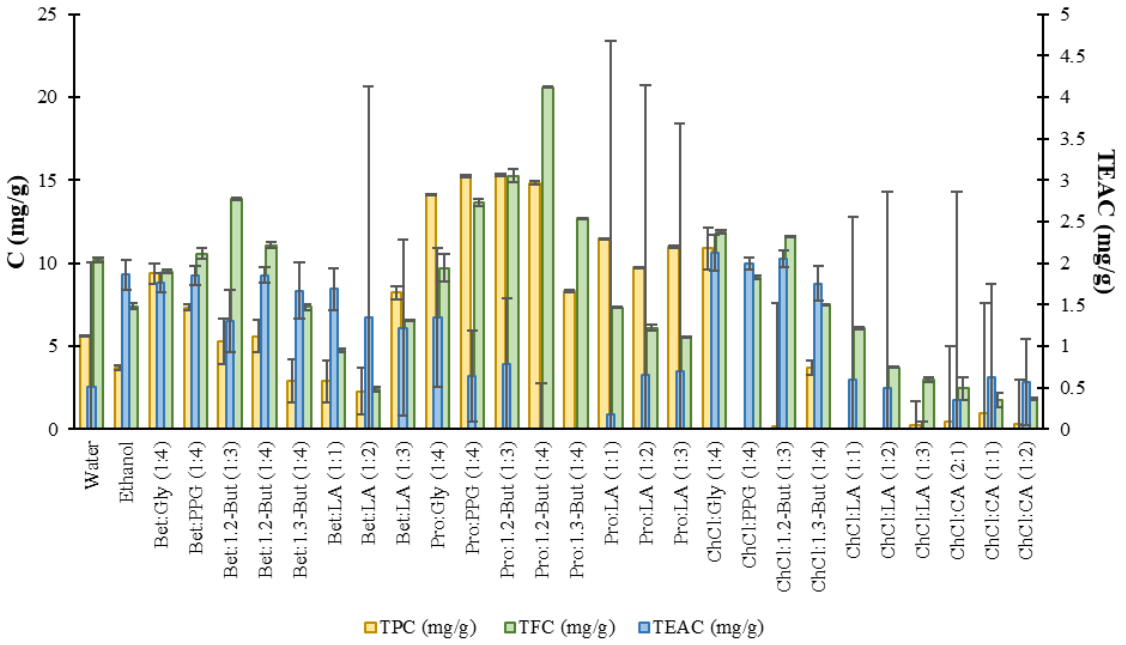



Figure 24. Evaluation of the total phenolic content (TPC, in yellow), total flavonoids content (TFC, in green) and the antioxidant activity (TEAC, in blue) for all the solvents tested as extraction solvents: water, ethanol and other 26 (D)ESs.

Due to the differences in the extraction processes (conditions and solvents) and differences in the quantification of TPC, namely the standard used, it is quite challenging to compare the results obtained with other results already published. Silva et al. [135] tested a variety of conventional organic solvents with different polarities, among them EtOH, using maceration as the extraction technique. Silva et al. [135] used pure EtOH and a total extraction time of 24 hours at 50 ºC obtaining a TPC of 8.31±0.33 mg of GAE/g of dry extract. In the present study extraction times of 100 min were imposed at 60 ºC, which makes a time at least 14 times lower for an extraction yield 2 times higher, relatively to EtOH:water in 7:3 (v/v) ratio as extraction solvent, when comparing with Silva et al. [135] results. Note that Silva et al. [135] results are presented per g of dry extract and in this study the results are present per g of dry biomass.

From the results in Figure 24 it is notable a discrepancy between the TPC and TEAC, as theoretically, a higher TPC should return a higher TEAC. However, in the Folin-Ciolcalteu method other non-phenolic compounds also present in the extract can have impact in the absorbance and consequently will influence the final result. This causes an overestimation of the TPC which could be the cause of the inconsistency between TPC and TEAC. Actually, the (D)ESs that returned higher TPC (proline-based (D)ESs) tend to return the lower antioxidant activity. Silva et al. [135] observed this same effect with extraction of polyphenols from *S. muticum*, where the TPC tendency was not totally concordant with the TEAC.

The TFC is not something that is usually evaluated in algae extracts and the Christ-Müllers assay was not found to be reported to be applied in *S. muticum* extracts. However, Sobuj et al. [136] performed this assay for another *Sargassum* specie, *Sargassum coriifolium*, obtaining the TFC of 31.75±1.01 mg quercetin/g using water as extraction solvent. In this work, the extraction with water gave only a TFC of 10.17±0.14 mg quercetin/g which comparing with Sobuj et al. [136] is 3 times lower. On the other hand, Sobuj et al. [136] extraction time is 24 h which is much more than the 1 h 40 min used as extraction time for the extractions presented in Figure 24. The

*Sargassum* species are also different, therefore, *a priori* must be known that the algae's compositions should not be equal. Although, the same patron seen for TPC was not observed for TFC where the best (D)ESs were proline-based, the (D)ES that returned higher TFC, 20.61±0.02 mg of quercetin/g was Pro:1.2-But (1:4), returning twice as much of the extraction using water and 2.8 times higher than EtOH.

It is important to have in mind that these methods were originated for conventional solvents. As previously said, it was observed a few difficulties when performing these general methods for the (D)ESs selected, namely difficulties with ChCl-based (D)ESs in the Folin-Ciolcalteu assay and with proline-based (D)ESs in the antioxidant activity assay. These difficulties will have impacts on the results and can cause an unrealistic increase or decrease of the results. Another occurrence that was observed is the (D)ESs returning a higher absorbance than the extraction itself, and this represented negative results when the blank's value was subtracted to the extract's result. This can also be a consequence of the precipitation and formation of flakes when in happens.

In order to identify certain compounds that might be present in the extract a more precise, robust and reliable analysis was carried out. This led to the characterization of the extracts in the HPLC, where was possible the identification and quantification of a variety of phenolic compounds present in the extract, as presented in Figure 25. The two proline-based (D)ESs, Pro:PPG (1:4) and Pro:1,2-But (1:4), returned the best extraction yields according with the HPLC results, Figure 25. These two (D)ESs were also found to be among the best (D)ESs for the TPC obtained. This implies that, although non-phenolic compounds can count for the TPC, the Folin-Ciolcalteu method might still indicate what are the extracts with higher TPC, except for ChCl-based (D)ESs.

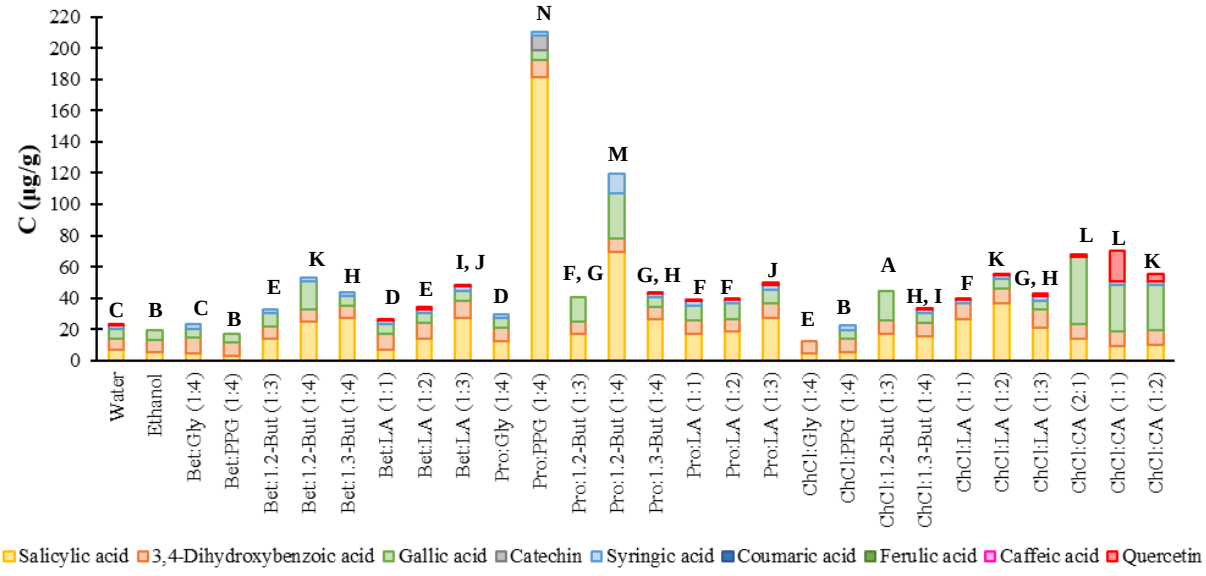



Figure 25. HPLC results for all the solvents tested as extraction solvents: water, ethanol and other 26 (D)ESs. Compounds characterized by HPLC: gallic acid (light green), 3,4-dihydroxybenzoic Acid (orange), catechin (grey), caffeic acid (pink), syringic acid (light blue), coumaric acid (dark blue), ferulic acid (dark green), salicylic acid (yellow), quercetin (red). The results not sharing the same letter are considering significantly different.

From the HPLC results in Figure 25, it can be seen that a significant amount of salicylic acid could be extracted from *S. muticum* just like such as *Sargassum horneri* studied by Caijiao et al. [55]. As mentioned previously in section 1.2, the composition of the algae varies from season to season, site to site, and specie to specie, which means that the PCs characterized by Caijiao et al. [55] and Chakraborty et al. [56], and the amount that they were able to extract, should be taken just as an indicative reference. Actually, in all the extractions

performed for the (D)ESs screening, the presence of 2 of the 9 studied compounds (ferulic acid and *p*-coumaric acid) were not observed, which were the two most extracted phenolic acids following the salicylic acid by Caijiao et al. [55] from *S. horneri*, and were also extracted by Chakraborty et al. [56] from *Sargassum plagiophyllum* and from *Sargassum myriocystum* in small quantities. Also, it is observed from Figure 25 that the studied flavonoids, catechin and quercetin are present in very small quantities as well as syringic acid. Salicylic acid was not only the most extracted compound but also it was possible to discover a (D)ES that presented a high selectivity towards him.

All the phenolic acids studied in this work were evaluated in Sabeena Farvin & Jacobsen [49] work, and as previously said, it was the only work found to be already published about a *S. muticum* characterized through HPLC. Their *S. muticum* was collected in Denmark which is a country with an environment slightly different from Portugal. Therefore, the composition of the algae caught in Denmark was not expected to match the composition of Portuguese *S. muticum*. This difference in the compositions is clearly seen, as Sabeena Farvin & Jacobsen [49] performed extractions using EtOH and water and could not extract salicylic acid in both extractions. In this work, salicylic acid, 3,4-dihydroxybenzoic acid, syringic acid and gallic acid were the four phenolic acids capable be to extract in most of the assays. Among the seven phenolic acids studied, Sabeena Farvin & Jacobsen [49] observed that when using EtOH, 3,4-dihydroxybenzoic acid was the most extracted phenolic acid at 27.9±0.1 mg/g extract, followed by gallic acid at 2.2±0.0 mg/g extract. This behaviour was also seen when using EtOH:Water (70:30 v/v) in this work, obtaining 8.10±0.33 and 6.26±0.22 μg/g dry weight for 3,4-dihydroxybenzoic acid and gallic acid, respectively. However, when using water, Sabeena Farvin & Jacobsen [49] saw a significant decrease in 3,4-dihydroxybenzoic acid extract to 1.1±0.0 mg/g extract. This significant decrease was not observed in the present work with the solvent change from EtOH to water. Sabeena Farvin & Jacobsen [49] not only did HPLC analysis but also the TPC by Folin-Ciocalteu assay and saw that both results were not concordant. Their TPC was higher when using water, even though the extraction of phenolic acids measured with HPLC was higher when using EtOH. Water tends to be less selective, hence extracting other compounds that can react during the TPC assays, giving higher TPC values due to other co-extracted impurities; therefore, providing an overestimation of the TPC value. This indicates that not only for (D)ESs but also for conventional solvents the TPC may not be confirmed with HPLC results. Extraction of other PCs besides the ones studied and other non-phenolic compounds could be related with this inconsistency.

Another observation was made contrasting the outcomes obtained with general methods in Figure 24 with the HPLC results in Figure 25, as for a few (D)ESs they are not concordant with each other and; again, this could be related with the problems that the general methods demonstrated when working with (D)ESs. For example, ChCl and citric acid combinations are not as good as Pro:PPG (1:4) and Pro:1,2-But (1:4) but they have still a considerable extraction yield in the HPLC. This information goes against the general methods, Figure 24, where the three ratios tested for this combination present the lower results, not only for the TPC were ChCl precipitates but also for TFC and antioxidant activity.

A Tukey's test was performed to the sum of all PCs extracted and analysed with HPLC, Figure 25. Tukey's test was the post hoc selected to evaluate if the data in Figure 25 presented significant different among them or not. This is a type of one-way analysis of variance (ANOVA) test that is used to evaluate if the means are indeed significantly different or not. Tukey's test, who also can be called honestly significant differences (HSD)

method, it is a method that performs pairwise comparison of the means using studentized range distribution which is the difference between the largest and smallest data point in a sample [137,138].

Although Tukey's test could be handmade, due to high number of data would be complex to perform any post hoc test without a proper software. In this regard, Brown et al. [139] recommends several software's, among them is *IBM SPSS Statistics v26* which was the one selected, using a degree of significance of 95%.

Performing a post hoc test is not trivial, and firstly the data must be analysed before a type of post hoc test is selected. For the Tukey's test to be correctly applied, the data must be normal distributed and homogeneous, which means equal variances among all groups. According to Shapiro Wilk test presented in Supporting Information C, the data can be considered normal distributed for each solvent. On the other hand, according with Levene's test presented in Supporting Information C, the data it is not homogenous which creates a drawback to apply this test.

Herberich et al. [140] proposed a model created in *Rstudio* to multiple means under heteroscedasticity, this test was also performed, and it is present in Table S.30 in Supporting information C. However it has a great disadvantage comparing to the Tukey's test, only does pairwise comparison one by one and does not return an organization in subsets which makes it harder and time consuming to interpret the results of this post hoc test. Given this, the conclusions were practically based on Tukey's test even though it violates the homogeneity requirement it showed reasonable results and so, to further statistical analysis only the Tukey's test was performed.

Based on the above mentioned information, that sometimes the general methods are not concordant with the HPLC results, especially for ChCl-based (D)ESs, the selection of the best (D)ESs to be used in further studies was mainly based on the HPLC results. With no doubt, Pro:PPG (1:4) and Pro:1,2-But (1:4) were the (D)ESs that returned a higher extraction capacity. The Tukey's test corroborates this observation since it indicates that are two results significant differently from the rest. Not only the extraction capacity of Pro:PPG (1:4) was something that brought attention to but also the selectivity towards the salicylic acid. The combination of ChCl with citric acid showed to be the third best option when it comes to extraction yield; statistically, according to Tukey's test the ratio 2:1 and 1:1 of ChCl:CA are not significantly different, hence the ratio 2:1 was selected due to the higher selectivity towards the gallic acid.

The salicylic acid is a phenolic compound with current applications in cosmetic as an acne reductor, as a food preservative, and as a start compound to produce dyes and aspirin. Scientific Committee on Consumer Safety of the European Commission considers salicylic acid safe when used at 0.5 % concentration in cosmetic formulations and 3 % in hair products. Salicylic acid can be used in concentrations from 15-40% when used for a few local dermatological treatments. As previously said, proline has no restrictions in cosmetic use, and this also applies to propylene glycol [114]. Being the extract obtained by Pro:PPG (1:4) enriched in salicylic acid, cosmetics could be a prosperous application for it. The compound 1,2-butanediol has no information about its ingestion, as previously mentioned in 1.4.3. However, no restrictions about cosmetic applications are imposed. Since proline is also allowed in cosmetic applications, the extract obtained with Pro:1,2-But (1:4) could follow this purpose.

Choline chloride is forbidden to be used in cosmetics; therefore such application should be discarded for ChCl:CA (2:1). However, it can be used for food applications, as ChCl and citric acid are allowed by both EFSA and FDA to be used as food additive, Table 4. ChCl:CA (2:1) was selected due to the selectivity toward the gallic

acid. According to EFSA reports, gallic acid is naturally contained in some fruits, such as cherries and grapes, as well in some drinks such as whiskey, wine and beer, having their maximum concentration in beer (35 mg/kg). EFSA also reports that the theoretical maximum daily intake (TAMDI) for gallic acid is 1600 μg/person/day [141].

The selectivities might be related with the pH of the (D)ESs aqueous solutions, since the ChCl:CA (2:1) with 30 %(v/v) of water presents a lower pH (pH 0.60), where all the PCs characterized with the HPLC should be in their protonated form. In Table 6, the pKas of the PCs are compiled. In addition, a scheme of the deprotonation steps is presented in Figure 26. The two proline-based (D)ESs selected have a neutral pH, 6.68 and 6.46 for Pro:PPG (1:4) and Pro:1,2-But (1:4), respectively. At neutral pH only catechin and quercetin are fully protonated. This also indicates that both gallic acid and salicylic acid are deprotonated. However, this does not explain why there is a significant extraction of gallic acid in Pro:1,2-But (1:4) and thus the extraction mechanism is dependent on other factors besides pH.

Table 6. Dissociation constants for the nine phenolic compounds characterized in the HPLC.

| Phenolic Compounds | pKa | Reference |
|---|---|---|
| **Gallic Acid** | $pKa_1$ = 4.4±0.1<br>$pKa_2$ = 8.8 ±0.1<br>$pKa_3$ = 10.0 ±0.1<br>$pKa_4$ = 11.4 ±0.1 | [142] |
| **3,4-Dihydroxybenzoic acid** | $pKa_1$ = 4.35 ± 0.05<br>$pKa_2$ = 8.79 ± 0.05<br>$pKa_3$ = 13.0± 0.1 | [143] |
| **Catechin** | $pKa_1$ = 8.68±0.23<br>$pKa_2$ = 9.70±0.24<br>$pKa_3$ = 11.55±0.20 | [144] |
| **Caffeic Acid** | $pKa_1$ = 4.43 ± 0.02<br>$pKa_2$ = 8.69 ± 0.03 | [145] |
| **p-Coumaric Acid** | $pKa_1$ = 4.360 ± 0.003<br>$pKa_2$ = 8.982 ± 0.001 | [145] |
| **Ferulic Acid** | $pKa_1$ = 4.52<br>$pKa_2$ = 9.39 | [145] |
| **Salicylic Acid** | $pKa_1$ = 2.80±0.04<br>$pKa_2$ = 13.4±0.2 | [143] |
| **Syringic Acid** | $pKa_1$ = 4.30<br>$pKa_2$ = 9.10 | [146] |
| **Quercetin** | $pKa_1$ = 7.10±0.12<br>$pKa_2$ = 9.09±0.11<br>$pKa_3$ = 11.12±0.36 | [147] |

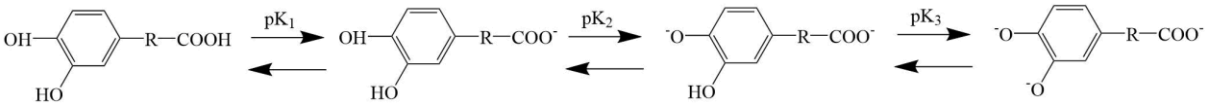



Figure 26. Deprotonation step for a general phenolic acid. Inspired by Beltran et al. [145].

***COSMO-RS***

The interpretation of the σ-profiles will be focused on the gallic acid and salicylic acid present in Figure 28, since were the phenolic acids that shown variation in the extraction yield for the different (D)ESs, especially the salicylic acid. From the σ-profiles, Figure 27, there is no doubt that ChCl is HBA, and the CA tend to be HBD, which creates a very polar (D)ES. The σ-profiles of proline, PPG and 1,2-butanediol show similarities, as they are all more deviated to the HBA zone even though closer to the center, this indicates that both Pro:PPG (1:4) and Pro:1,2-But have a more non-polar behaviour when compared to ChCl:CA (2:1). This difference of polarity could explain the high yields towards the salicylic acid in both proline-based (D)ESs, and the higher yield towards the gallic acid in the ChCl-based (D)ES, since gallic acid shows higher polarities, deviated the HBD zone, when compared with salicylic acid. This information is corroborated by looking at the structure of both phenolic acids, Figure 11, where the gallic acid possesses two more hydroxyl group than salicylic acid. However, this does not explain why there is a small amount of gallic acid being extracted with Pro:1,2-But (1:4). Silva et al. [135] evaluated different organic extraction solvents with different polarities and a relation between the polarity of the solvent and the TPC was not possible to conceive.

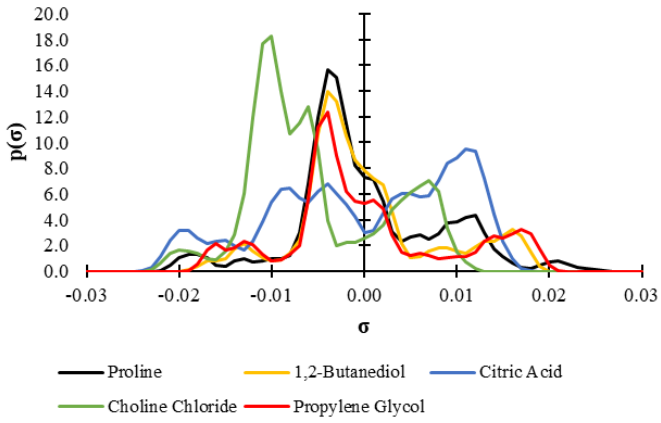



Figure 27. σ-profiles of the compounds that compose the three best (D)ESs selected.

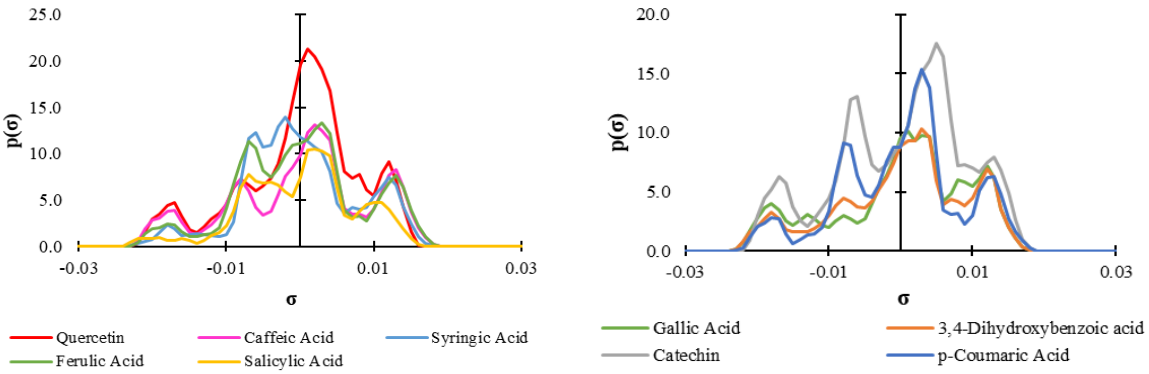



Figure 28. σ-profiles of the PCs characterized through HPLC.

In the best attempt to try to explain the amount of gallic acid extracted with Pro:1,2-But (1:4), the solubility was estimated with COSMO-RS, where lower values of γ means higher solubilities, as explained in the Supporting Information B.1. In terms of logarithmic values of **γ,** the more negative values for ln(γ), the better the solubility is going to be according to COSMO-RS. However, according to the activity coefficient computed from COSMO-RS, Figure 29, all nine PCs present similar solubilities in Pro:PPG (1:4) and Pro:1,2-But (1:4), which still does not explain the extraction of gallic acid when using Pro:1,2-But (1:4).

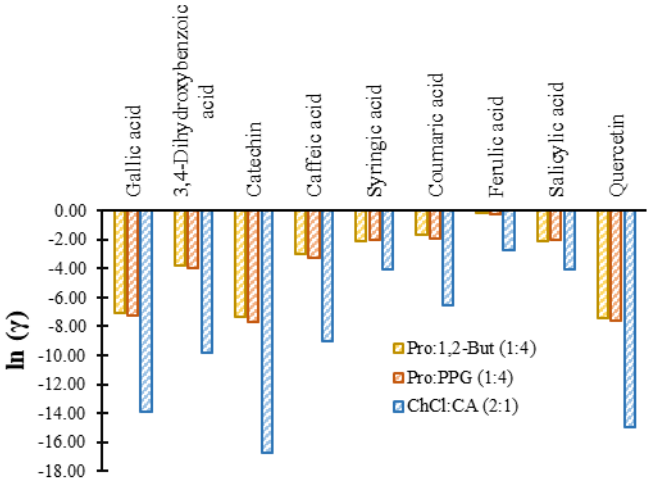



Figure 29. Neperian logarithm of the activity coefficients of the 9 phenolic compounds characterized in the HPLC in the (D)ESs that showed the best results.

### 4.1.2. Optimization

***Water content***

The content of water introduced when preparing the (D)ES has a great influence in the extraction capacity. From a certain amount of water, the hydrogen bonds of the (D)ES might start to break and a transition to an aqueous solution can succeed [148]. Besides the 30 % (v/v) already reported in the screening, 10, 20, 40 and 50 %(v/v) were also evaluated herein.

Accordingly, with the TPC for the two proline-based (D)ES, Figure 30 (a) an (b), the water content of 30% (v/v) returned the better extraction yield. The TPC of ChCl:CA (2:1) is not reliable since the ChCl precipitates as previously mentioned. The TFC of Pro:PPG (1:4) tends to increase with water content, being practically equal at 40% (v/v) and 50% (v/v) of water. For Pro:1,2-But (1:4), it was also observed that for lower water content the

TFC is also lower. The TFC of Pro:1,2-But (1:4) for 30% (v/v) and 50% (v/v) is very similar. However, for 40% (v/v) is lower than the ones mentioned before, which does not allow for a clear behaviour interpretation from 30% (v/v) on. The TFC of ChCl:CA (2:1) increases with the water content, being its maximum at 50% (v/v) of water.

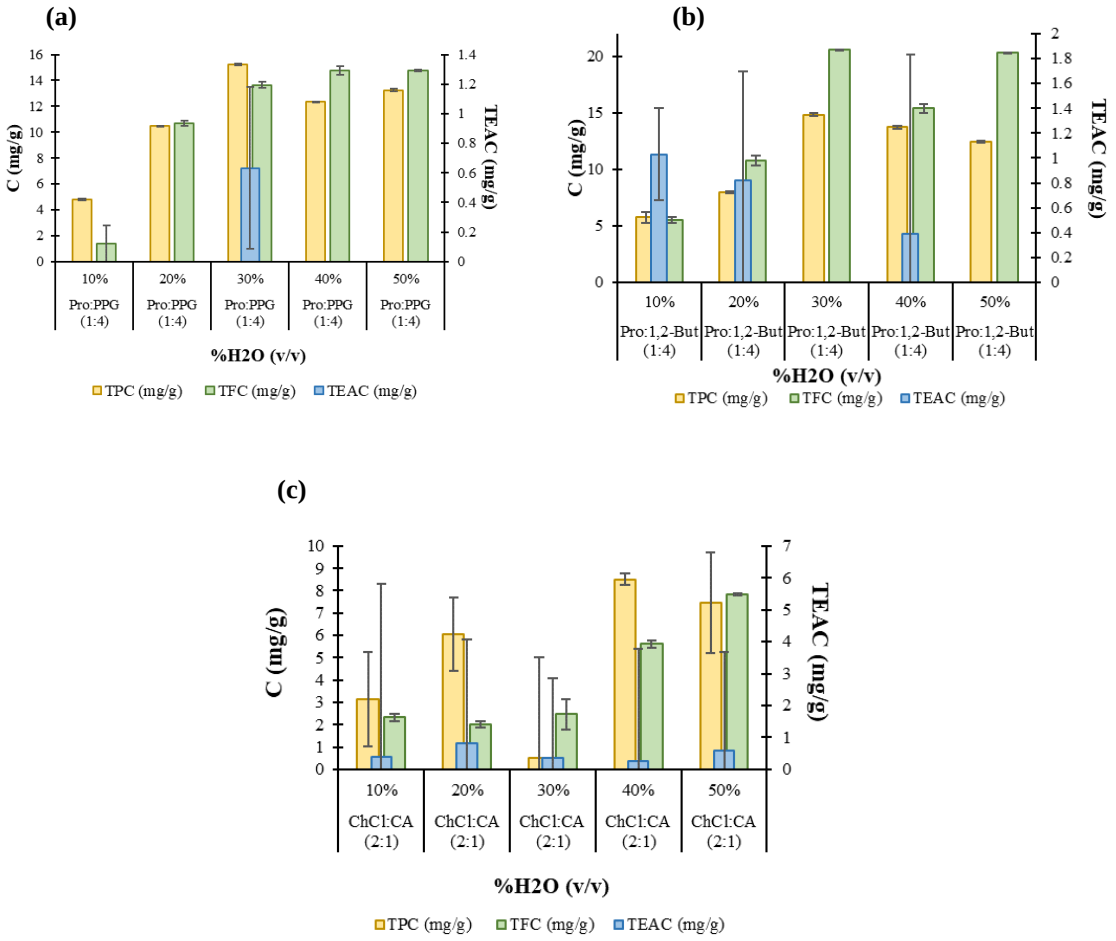



Figure 30. Water content optimization for (a) Pro:PPG (1:4), (b) Pro:1,2-But (1:4), (c) ChCl:CA (2:1). Evaluation of the total phenolic content (TPC, in yellow), total flavonoids content (TFC, in green) and the antioxidant activity (TEAC, in blue).

The HPLC results for the water content optimization are presented in Figure 31. The 30% (v/v) of water content returned the best extraction yield for the 3 (D)ESs, Figure 31. These results are concordant with the TPC for the two proline-based (D)ESs. Although the HPLC results of ChCl (D)ES are not concordant with the TPC, it was something already expected due to the precipitation of ChCl. For further studies, the water content was fixed at 30% (v/v).

An interesting result seen in the optimization of water is that the water content present in the (D)ES makes it possible to improve or decrease the extraction capacity of a certain compound. For example, looking carefully at Figure 31 (a), it can be seen that the yield towards catechin increases for higher amounts of water for Pro:PPG (1:4). This increase of catechin yield when using this (D)ES agrees with the increase of TFC seen in Figure 30 (a), that also tends to increase with the water content.

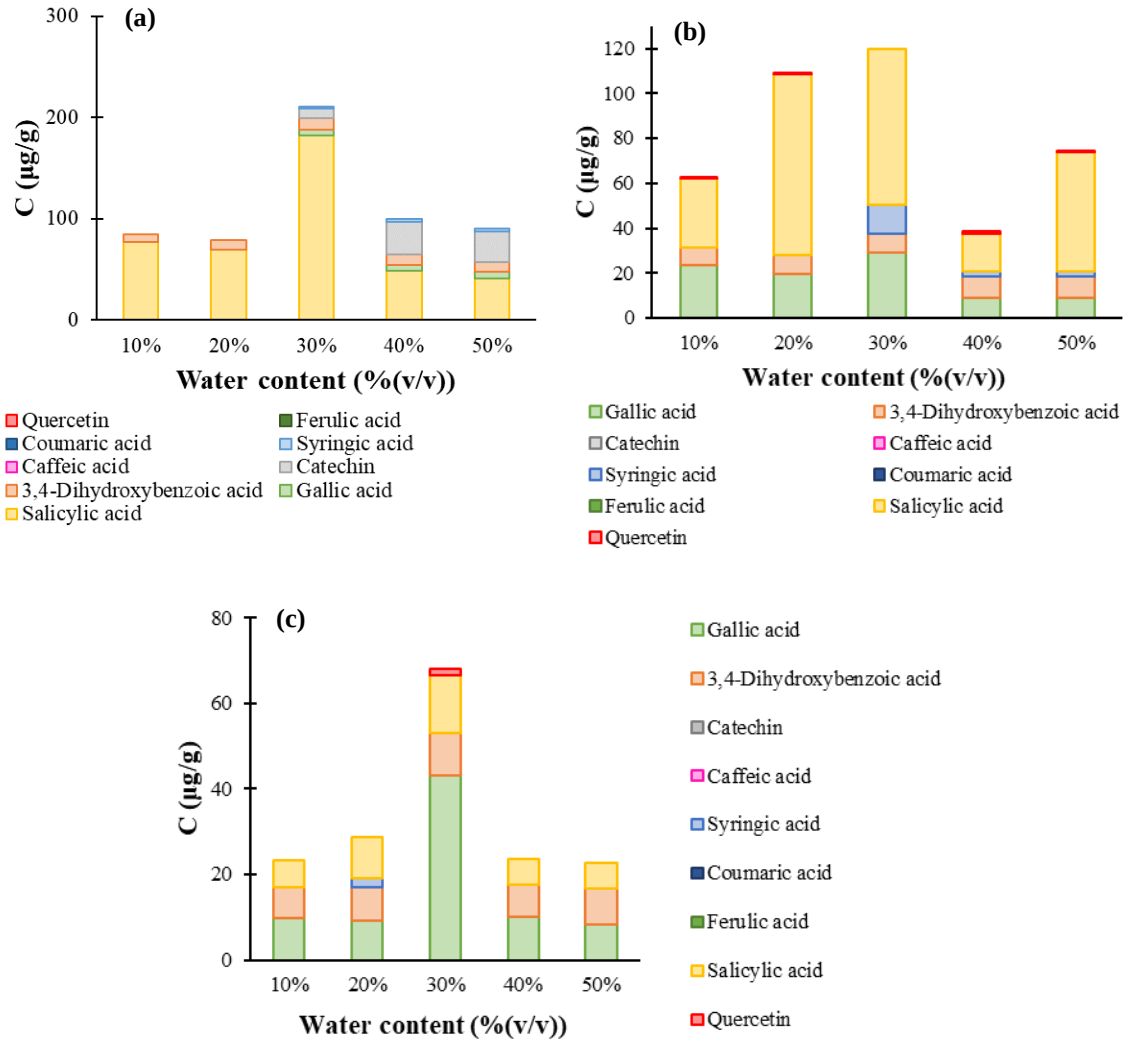



Figure 31. HPLC results for the water content optimization for the three best (D)ESs: (a) Pro:PPG (1:4), (b) Pro:1,4-But (1:4), (c) ChCl:CA (2:1).

The amount of water from where it can be considered that the hydrogen bonds began to rupture can be evaluated by the excess molar volumes at different contents of water. The determination of the excess molar volumes can be made through the measurement of the densities from each water content, as explained in Supporting Information A.3. [149]. The densities and viscosities of each (D)ES were measured and are presented in Supporting Information A.2. It should be mentioned that (D)ES tend to be viscous, especially ChCl:CA (2:1). This study could not be performed for ChCl:CA (2:1) because its density and viscosity are so high that was not possible to handle it without the addition of water. An attempt to add the smallest amount of water to ChCl:CA (2:1) was made to allow the measurement of densities in order to perform the water optimization study. At 6.4 % (w/w) of water was possible to measure densities and the calculations for this (D)ES were made with the assumption that it has no excess volume.

Figure 32 below shows the variation of the excess molar volumes for each water content. For all water contents a contraction of volume was observed, and the minimum is placed at 30 % (v/v) for both proline-based (D)ESs studied. This corroborates the results in Figure 31, since it could mean that at this water content is where the hydrogen bonds can start to rupture. Also, it may indicate that the hydrogen bonds are stronger at 30% (v/v)

since it shows the higher volume contraction, which can result in a more stable (D)ES. This stability could be the reason why the (D)ESs at 30% (v/v) is able to have a higher extraction capacity.

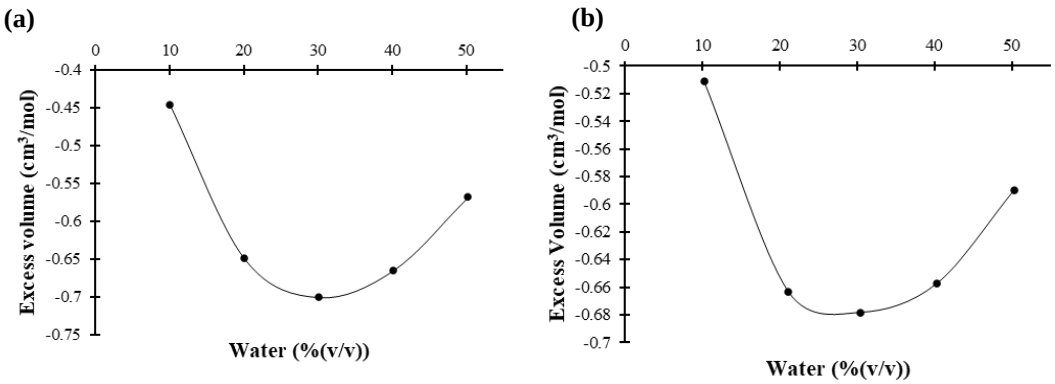
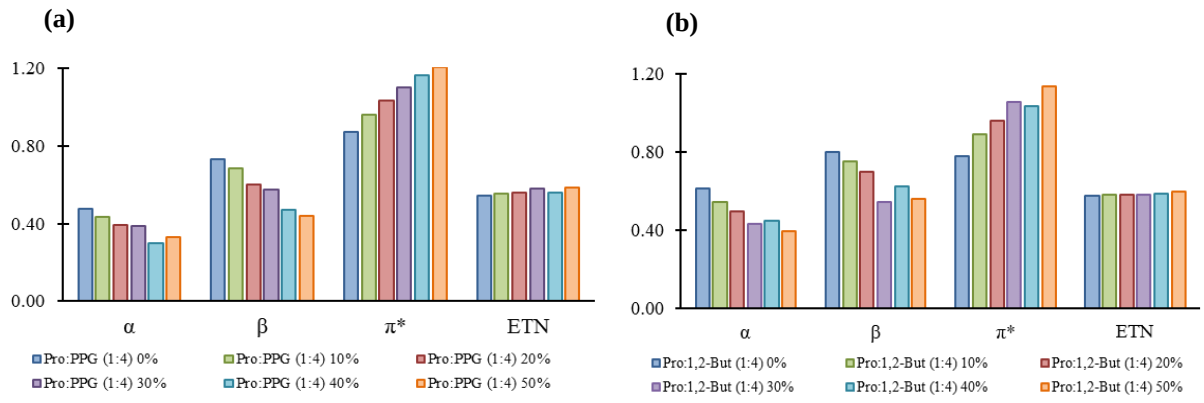



Figure 32. Variation of the excess volumes with water content for two of the three best (D)ESs: (a) Pro:PPG (1:4), (b) Pro:1,4-But (1:4)

Although the σ-profiles in Figure 27 gave insights about the polarity of the compounds used to formulate the best performing three (D)ES, they do not quantify the polarity of the (D)ES. This quantification was conducted through the usage of solvatochromic probes for the different water contents. The overall polarity can be quantified through betaine scale with the $E_TN$ parameter in Figure 33. Clearly, ChCl:CA (2:1) possesses a higher overall polarity (1.668 for 30% v/v of water) than the two proline-based (D)ESs. This result was something expected from the σ-profile of ChCl and citric acid in comparison with the σ-profile of proline, propylene glycol and 1,2-butanediol. Pro:PPG (1:4) and Pro:1,2-But (1:4) have similar $E_TN$ values, 0.579 and 0.581, respectively for 30 %(v/v) of water content. Florindo et al. [134] observed that the $E_TN$ did not vary much by changing the HBD and maintaining the same HBA. This is seen with the proline-based (D)ESs evaluated in this work as mentioned before.

Although it is a very small increase, the overall polarities of all three (D)ESs increase with the water content, which was expected since water is an extremely polar solvent. The exact values of $E_TN$ are presented in Supporting Information Table S.13. The polarity tests were not performed for ChCl:CA (2:1) with a water content lower than 20% (v/v) due to the high viscosity. The high viscosity created limitations for the probes dissolution and were extremely difficult to transfer from the glass vial to the quartz cuvette.



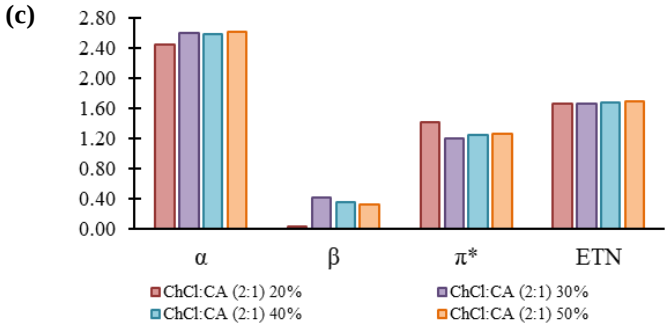



Figure 33. Solvatochromic results for the proline-based (D)ESs (a) Pro:PPG (1:4), (b) Pro:1,2-But (1:4) and (c) ChCl:CA (2:1) for the different water contents.

Pro:1,2-But (1:4) presents a higher HBD ability, α, in comparison with Pro:PPG (1:4) for all water contents, which can indicate that 1,2-Butanediol has a slight higher HBD capacity than propylene glycol. These two molecules have the same quantity of hydroxyl groups placed in the same positions, the difference is one additional methyl group attached to the end of the chain for 1,2-Butanediol, which can be related with a slightly higher HBD capacity. However, the α values for these two (D)ESs are no comparison to the α values of ChCl:CA (2:1), that shows a very strong ability to donate hydrogen bonds. This high values for α can be related to the carboxylic groups and the hydroxyl group present in the citric acid. For the proline-based (D)ESs it is observed a behaviour where the HBD ability decreases with the increase of water content. On the contrary, for ChCl:CA (2:1) the α tends to slightly increase along with the water content. According to Husanun et al. [150] the α parameter is connected with the extraction efficiency of phenolic compounds. A higher α indicates a stronger interaction between the biomass and the (D)ES. Despite this explanation does not explain why the Pro:PPG (1:4) presents the higher extraction yield and one of the highest TPC, this might justify the selectivity towards the gallic acid.

The same compound is used as HBA in the (D)ESs combinations of both Pro:PPG (1:4) and Pro:1,2-But (1:4), so it would be expected that these two (D)ESs would have similar HBA ability. This expectation was fulfilled with the determination of the HBA ability Kamlet-taft parameter, β, that is very similar for both Pro:PPG (1:4) and Pro:1,2-But (1:4). Both of them show the same tendency for β as the one seen before for α, which is to decrease the HBA ability with water content. Basically, the water reduces the HBA and HBD for both proline-based (D)ESs with the solvatochromic probes. The β values for ChCl:CA (2:1) are very low compared with the α values, 6 to 8 times lower. This information could indicate that the polarity of this (D)ES could be mainly due to the HBD ability. For 20 %(v/v) water content in ChCl:CA, the β is practically zero; this could be related with the high viscosity that this (D)ES already presented to this water content. Neglecting this value, a decrease behaviour of the β for ChCl:CA (2:1) can be noted.

The π* Kamlet-taft parameters give a quantitative information about the polarizability and dipolarity. The π* values for Pro:PPG (1:4) were slightly higher than the Pro:1,2-But (1:4) values. ChCl:CA (2:1) demonstrated to be more polarizable and dipolar than the proline-based (D)ESs, returning higher values of π*. Once again, ChCl:CA (2:1) with 20 %(v/v) does not follow the trend, which can be again related with the viscosity and difficult

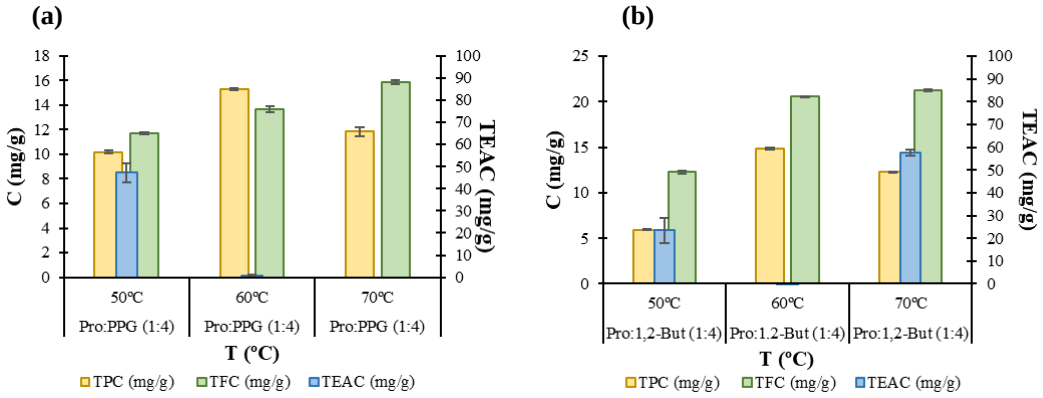
solubilization of the probes, especially of the N,N.diethyl-4-nitroaninline according to equation (9) presented in section 3.2.9.

In Figure 33 (a), the α value for Pro:PPG (1:4) with 40 % (v/v) is lower than the α value for Pro:PPG (1:4) with 50 %(v/v). This creates an incoherency in the trend observed, which is that α values decrease with the increase of water content. However, this incoherency only happens in α, which can mean that this may be due to experimental errors. On the other hand, this behaviour it is seen for Pro:1,2-But with 30 % (v/v) for all Kamlet-taft parameters (α, β and π*), where its values are lower than Pro:1,2-But (1:4) with 40 % (v/v). The way how the probes interact with the solvent has a great impact on these parameters. From the excess molar volumes in Figure 32, it was observed that the maximum volume contraction happens at 30 % (v/v), and this may be due to the formation of strong hydrogen bonds for that water content. This strong hydrogen bonds can generate a robust hydrogen bond matrix. Due to this strong matrix, the probe could face more difficulties to interact with the (D)ES and to form the hydrogen bonds itself, returning lower values than expected. Although the Pro:PPG (1:4) also have its maximum volume contraction at 30 %(v/v), the trend seen of all Kamlet-Taft parameters for Pro:1,2-But it is not observed for Pro:PPG (1:4). The 1,2-Butanediol possesses one additional methyl group than propylene glycol, providing two more hydrogens to its structure which can have influence on the results seen in Figure 33.

***Temperature***

Temperature is another parameter that has a huge impact on the extraction and should be optimized, because if the temperature is too low the extraction yield tends to decrease. Increasing the temperature helps to improve the extraction but can cause degradation of the phenolic compounds. This optimization was performed with 30 % (v/v) of water content and with a total of 100 minutes of extraction time in the orbital shaker. Two additional experiments at 50 and 70 ºC were performed, besides the 60 ºC already performed in the screening.

The TPC values shown in Figure 34 indicate that for Pro:PPG (1:4) and Pro:1,2-But (1:4), 60ºC is the optimum temperature. This could indicate that after 60 ºC compounds' thermodegradation probably occurs. In TFC is observed an increase for higher temperatures; therefore, at 70 ºC, the extraction capacity of flavonoids overcomes the thermodegradation if occurs. From ChCl:CA (2:1), it can be seen that better results are obtained at 70 ºC for all three parameters: TPC, TFC and TEAC. The better extraction at 70 ºC for ChCl:CA could be related with the decrease in viscosity at higher temperatures, as can be observed in Figure S.10, reducing the mass transfer limitations in the extraction process. The TEAC for the temperature optimization presented drawbacks not only for the two proline-based (D)ESs but also for ChCl:CA (2:1), where negative values were also obtained.



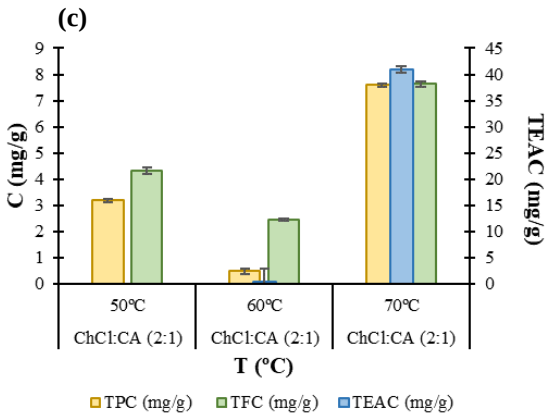



Figure 34. Evaluation of the total phenolic content (TPC, in yellow), total flavonoids content (TFC, in green) and the antioxidant activity (TEAC, in blue) for the temperature optimitzation in the orbital shaker: (a) Pro:PPG (1:4), (b) Pro:1,2-but (1:4) and (c) ChCl:CA (2:1).

With the analysis of the HPLC results, Figure 35, it can be observed that 60 ºC is the optimum temperature to perform the extraction for all three (D)ESs. Once again, the TPC of both Pro:PPG (1:4) and Pro:1,2-But (1:4), Figure 34 (a) and (b), showed to be concordant with the HPLC results, Figure 35 (a) and (b). This indicates that the (D)ESs' screening, Figure 24, was performed in optimal conditions, not only in terms of temperature but also in terms of water content as seen above. Once more the ChCl:CA (2:1) do not match the HPLC results. Actually, when compared both results in Figure 34 (c) and Figure 35 (c), opposing behaviours are seen.

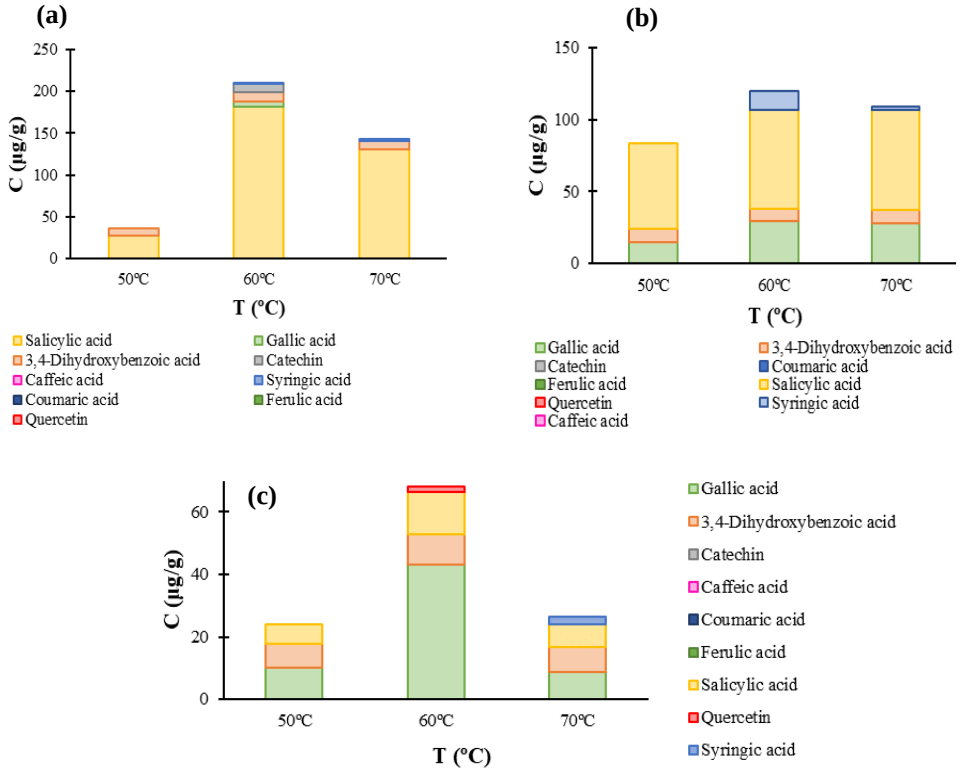



Figure 35. HPLC results of the temperature optimization for the three (D)ESs chosen: (a) Pro:PPG (1:4), (b) Pro:1,4-But (1:4), (c) ChCl:CA (2:1).

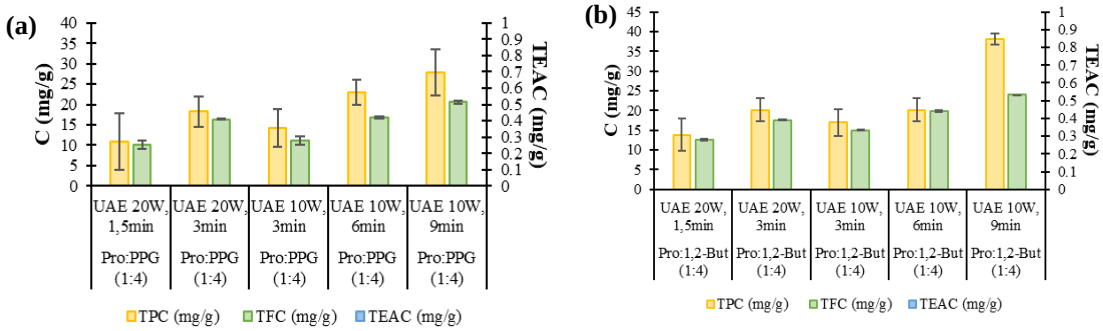
### 4.1.3. Intensification techniques

In order to improve and optimize even more the extraction process, two intensification techniques were tested, UAE with a probe and MAE. An optimization about the parameters of each one was also executed; in UAE the time and power were optimized, while MAE were the time and temperature.

***UAE***

Firstly, it is important to have in mind that with US, especially with the US probe, it is extremely difficult to control the temperature because the US probe tends to heat so much that the temperatures reach easily 90 ºC and sometimes go beyond 100 ºC. However, it is possible to control the power imposed in the probe or the amplitude. In this case, the power was the parameter chosen to be controlled besides the time. Essays were performed at 10 W and 20 W with times of 1.5, 3 and 3, 6, 9 minutes, respectively. As mentioned, the probe tends to reach high temperatures and for safety reasons and to avoid degradation of PCs, at 20 W it was only used small times.

The first thing noticed in Figure 36 is that practically no coherent data for the TEAC was collected, as practically all values were negative. In fact, Grillo et al. [46] were not able to perform either the Folin-Ciocalteu essay or the antioxidant activity essay using DPPH for the extractions performed with UAE. However, difficulties performing the Folin-Ciocalteu method were not observed in this work for UAE, besides the ones already mentioned before. The TFC for all three (D)ESs present an increase with time at 10 W of power. For Pro:PPG (1:4) and Pro:1,2-But (1:4), the TFC also increases with the time for higher power values (20 W). The TPC of the two proline-based (D)ESs presents the same behaviour than TFC, where the tendency to increase with time is observed.



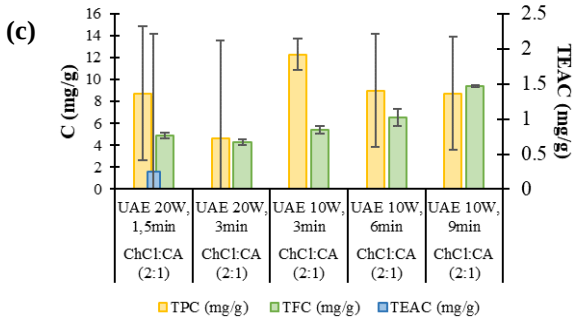



Figure 36. UAE results of the total phenolic content (TPC, in yellow), total flavonoids content (TFC, in green) and the antioxidant activity (TEAC, in blue): (a) Pro:PPG (1:4) with 30%(v/v) of water, (b) Pro:1,4-But (1:4) with 30%(v/v) of water, (c) ChCl:CA (2:1) with 30%(v/v) of water.

HPLC results using UAE as the extraction technique for Pro:PPG (1:4) and Pro:1,2-But (1:4), Figure 37 (a) and (b) have a concordant behaviour with both TPC and TFC seen in Figure 36 (a) and (b). The ChCl:CA (2:1) HPLC results also present this behaviour, where the extraction yield improves with time and having the higher yield for longer times with less power. When shorter times are to be employed, working at higher power helps to improve the extraction.

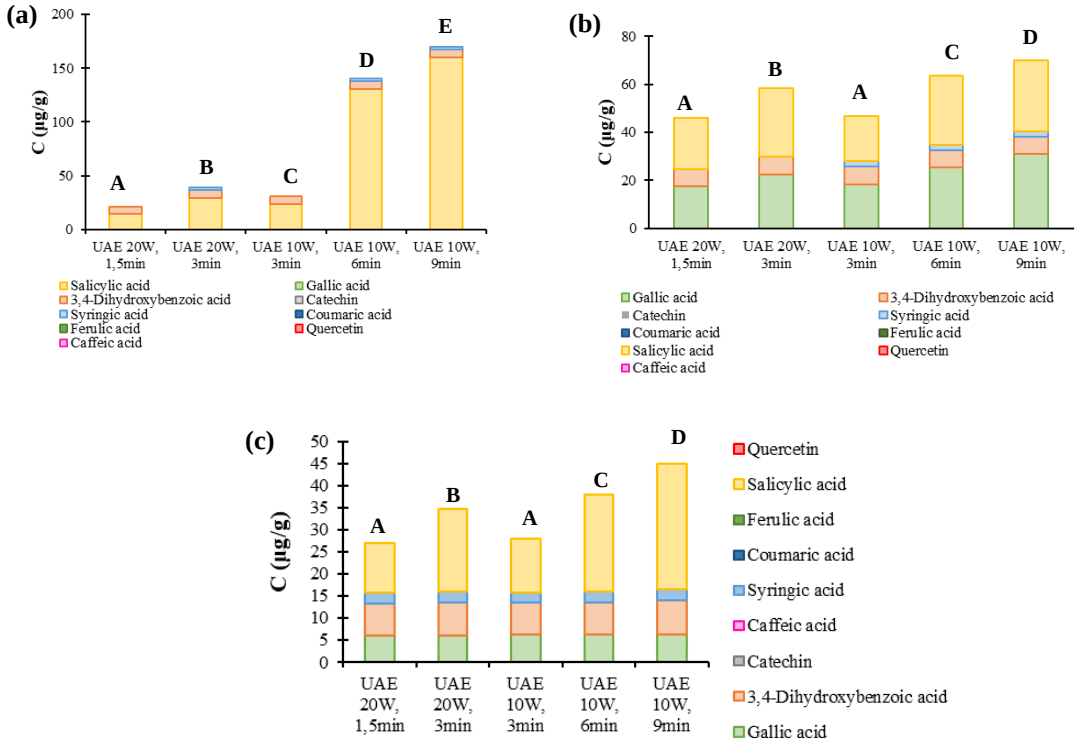



Figure 37. HPLC results for the UAE for the following (D)ES that have 30 %(v/v) of water: (a) Pro:PPG (1:4), (b) Pro:1,4-But (1:4), (c) ChCl: CA (2:1). The results not sharing the same letter are considering significantly different.

According to the HPLC results, lower power and higher extraction times increase the extraction yields for all three (D)ESs. Beyond that, an interesting phenomena occurred with ChCl:CA (2:1), where the selectivity switched completely when using this intensification method, as can be easily seen by comparing Figure 25 with Figure 37 (c). This observation gives a valuable information, that depending on the extraction technique the selectivity can be changed. However, it is important to keep in mind that this change will always depend on the

(D)ES employed. For example, in the two proline-based this change is not seen, Figure 37 (a) and (b). The ultrasound waves must have some influence in the hydrogen bonds established between ChCl and citric acid that allows this selectivity exchange.

The response surface models were plotted resorting to *Design Expert*, and for the three (D)ESs a tendence where the time has more impact on the extraction yield than the power was observed. The response surface methodology uses the central composite design with a quadratic model. For Pro:1,2-But (1:4) and ChCl:CA (2:1), the surface response models, Figure 38 (b) and (c), indicate that more time and power should return higher extraction yields; however, these models do not take into account the heating of the US probe which can easily reach up to 100 ºC and can cause degradation of phenolic compounds. The response surface model of Pro:PPG (1:4), Figure 38 (a), indicates that higher times but a lower power it is prefereble to use as extraction conditions.

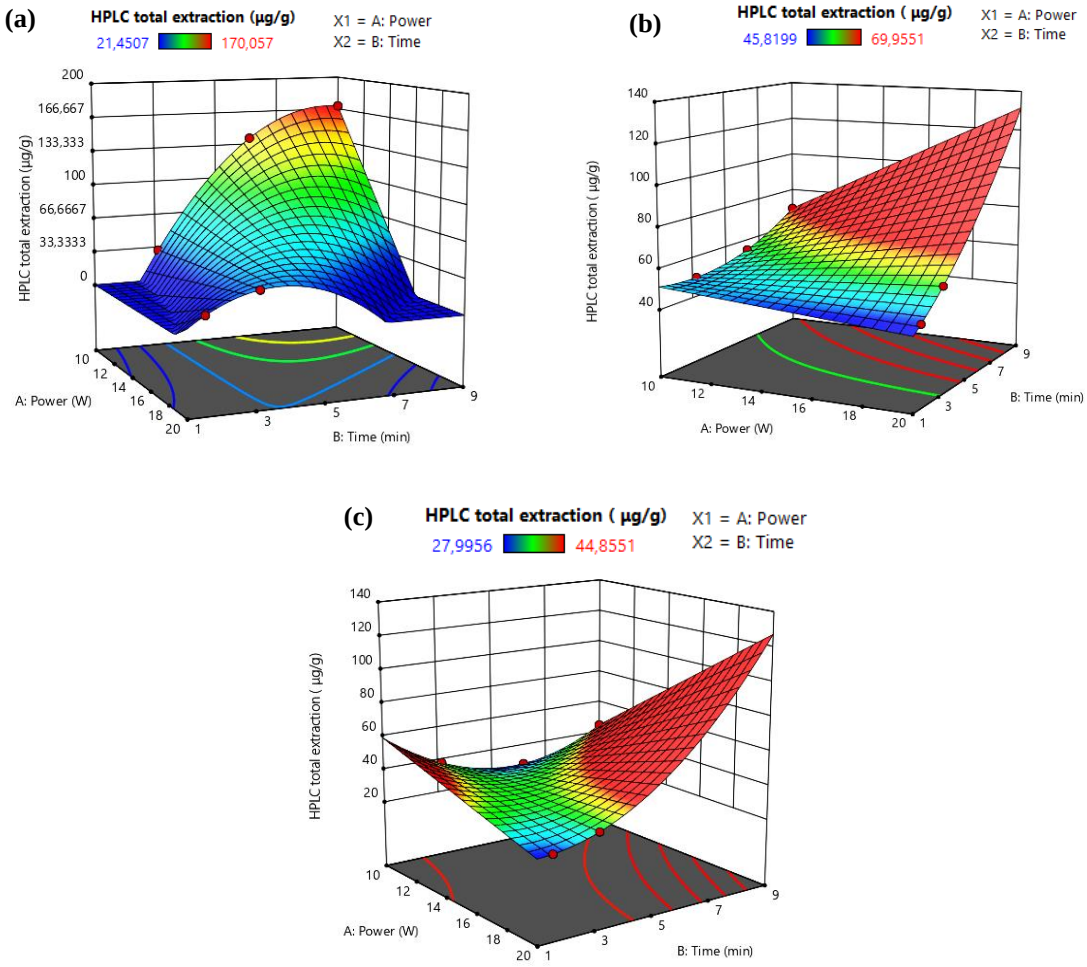



Figure 38. Response surface model for the total HPLC results for UAE performed for different times and powers. (a) Pro:PPG (1:4), (b) Pro:1,2-But (1:4) and (c) ChCl:CA (2:1). The red dots represent the experimental data.

***MAE***

In MAE, unlike in UAE, the temperature here can be controlled, allowing to test higher temperatures such as 100 ºC. The same tendency was demonstrated by TPC and TFC for the MAE extractions when varying with time and temperature, Figure 39, it is notable that both TPC and TFC increase significantly with 100 ºC in

comparison with the 60 ºC. This indicates that in MAE a higher temperature is preferred. The TFC for ChCl:CA (2:1) also returns higher values when the temperature is set to 100 ºC. Once again, the TEAC presented unreasonable results.

The higher TPC obtained for MAE was 24.43±2.36 mg/g of dry weight for Pro:PPG (1:4) at 100 ºC with 6 minutes of extraction time, which is 1.6 times higher than the TPC obtained with the orbital shaker in the (D)ESs screening (15.26±0.08 mg/g of dry weight). This value can be considered high when comparing with the TPC obtain by Grillo et al. [46] also from *S. muticum* and using MAE, which was 6.61±0.17 mg/g dry matrix using EtOH as extraction solvent. Once again for TEAC the results achieved were unrealistic.

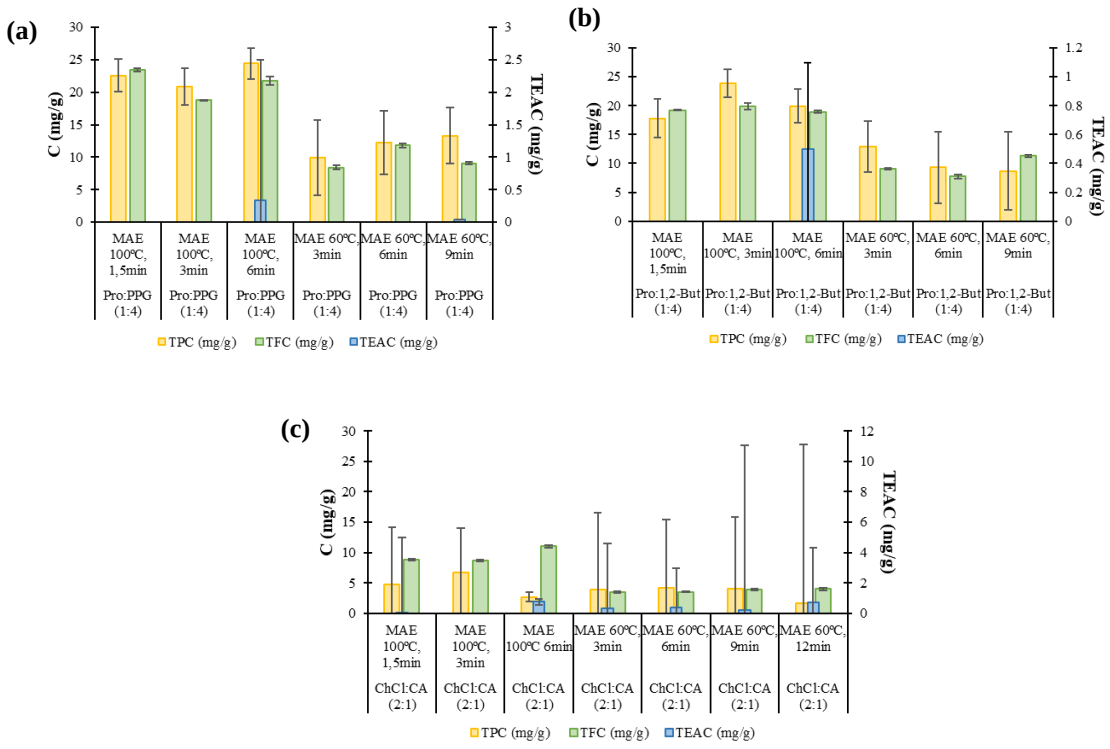



Figure 39. Evaluation of the total phenolic content (TPC, in yellow), total flavonoids content (TFC, in green) and the antioxidant activity (TEAC, in blue) for the microwave assisted extractions: (a) Pro:PPG (1:4) with 30%(v/v) of water, (b) Pro:1,4-But (1:4) with 30%(v/v) of water, (c) ChCl:CA (2:1) with 30%(v/v) of water.

The behaviour of the two proline-based (D)ESs for TPC and TFC, Figure 39 (a) and (b) is coherent with the behaviour seen in the HPLC results, Figure 40 (a) and (b). Clearly, a higher temperature (100 ºC) revealed to improve the extraction, probably because the contact times are low compared with 100 minutes employed for the conventional extractions; otherwise, for long extraction times at higher temperatures, lower extraction yields would have been expected due to a possible compound degradation. More essays could be tried where the temperature and time, separately or/and together, could both be increased to see if at some point, there is a decrease of the extraction yields due to degradation.

The MAE extractions using ChCl:CA (2:1) have not the same behaviour of the other two (D)ESs. In fact, ChCl:CA (2:1) demonstrated a few problems to work with in MAE due to the high viscosity. The (D)ES is so viscous that the equipment itself returned warnings expressing the difficulty to control the temperature. This lack of control could be the reason why the results do not show the same conduct as the proline-based (D)ESs. At 60 ºC,

the behaviour of ChCl:CA (2:1), Figure 40 (c), is similar to the behaviour seen with the proline-based (D)ESs but for higher times, at 6, 9 and 12 minutes (instead of only 3, 6 and 9 minutes). This could be correlated with the higher viscosity that probably needs more time to reach the stablished temperature. Viscosity is actually a crucial mass transfer influencer and, temperature is variable that has a significant effect in the viscosity itself as can be seen by the viscosity measurements present in Supporting Information A.2. [148].

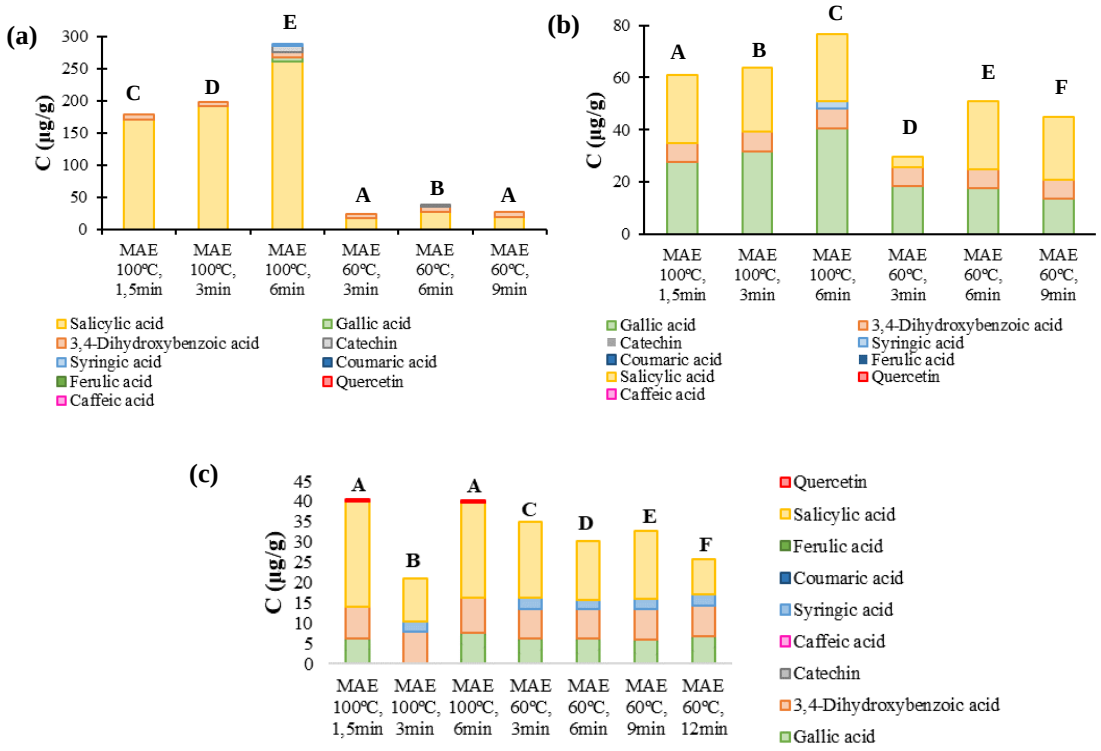



Figure 40. HPLC results for the MAE for the following (D)ES that have 30 %(v/v) of water: (a) Pro:PPG (1:4), (b) Pro:1,4-But (1:4), (c) ChCl: CA (2:1). The results not sharing the same letter are considering significantly different.

At 100 ºC for ChCl:CA (2:1), an unexpected behaviour occurred where with 3 min of extraction had a lower yield when compared with 1.5 min, Figure 40 (c), and also there is no evidence of gallic acid to be extracted. Additionally, at 100 ºC, 1.5 min or 6 min of extraction, the yield is practically equal, agreeing with the indications provided by Tukey's test. Once again, more experiments could be performed for ChCl:CA (2:1), for example to evaluate if longer times at 100 ºC would improve the extraction, as the viscosity of (D)ES could be imposing mass transfer limitations. Even though the results of ChCl:CA (2:1) create some doubts towards the trend of the extraction yield, it is clearly that the phenomena of the selectivity's switch observed in the UAE also can be seen with MAE, comparing Figure 25 with Figure 40 (c). Somehow, the US waves and the microwave energy that can cause a dipolar rotation of polar solvents are causing changes in the ChCl:CA (2:1), that is making this (D)ES to improve the extraction towards the salicylic acid and decreasing it towards the gallic acid.

The response surface models for the MAE were plotted in a similar way that for UAE results, with the difference that for MAE results a cubic model had to be used in order to fit better the experimental data, Figure 41. For the three (D)ES, the response surface models for MAE results give the same information, more time and higher temperature should return higher yields. However, once again, this surface models do not take into account the possible degradation of PCs when are exposing to high temperatures for long periods of time.

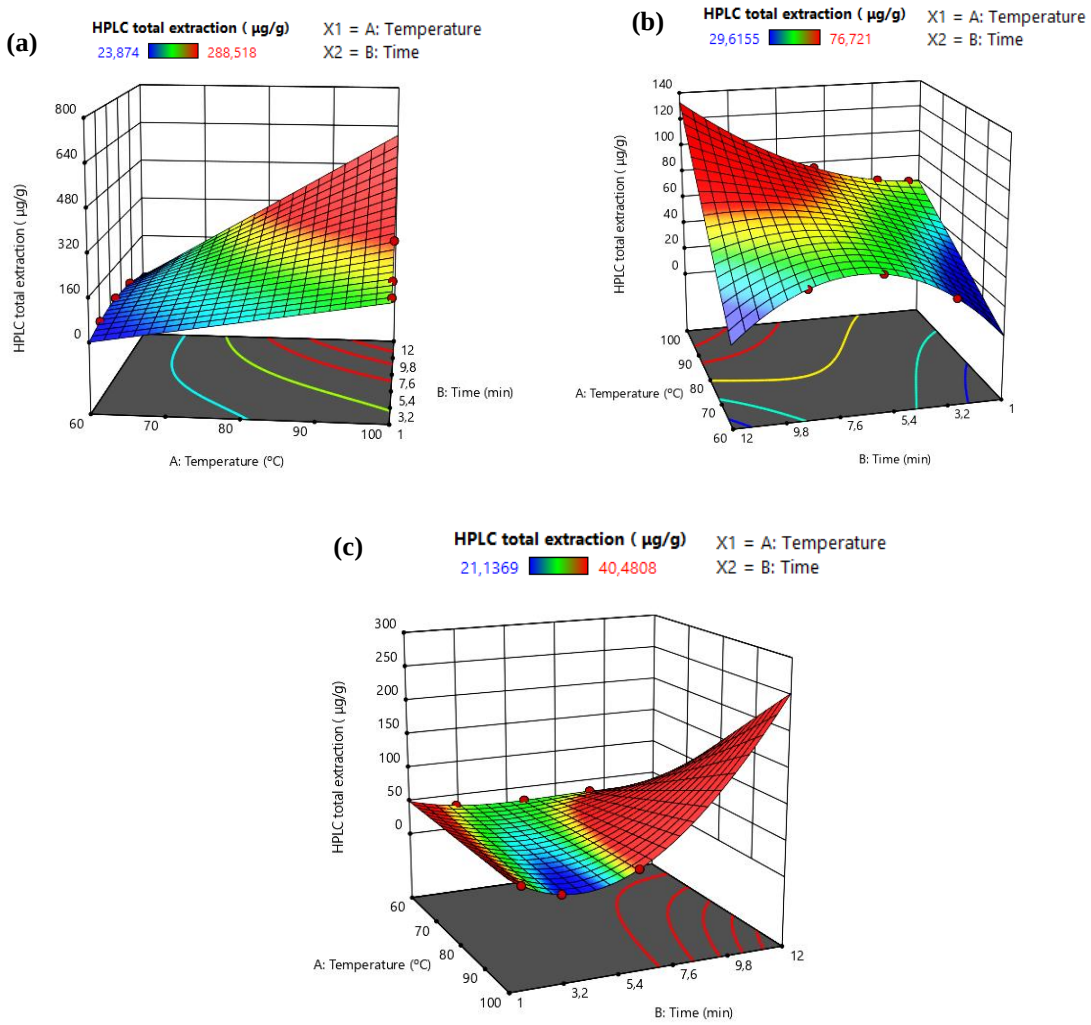



Figure 41. Response surface model for the total HPLC results for MAE performed for different times and temperatures: (a) Pro:PPG (1:4), (b) Pro:1,2-But (1:4) and (c) ChCl:CA (2:1). The red dots represent the experimental data

### 4.1.4. Overall comparison

Performing an overall comparison with the best results of each extraction technique all with 30 % (v/v) of water content, Figure 42, it is clear that using Pro:PPG (1:4) in combination with MAE, improves the extraction in terms of time and yield. Although, for Pro:1.2-But (1:4) the intensifications methods were not improving the extraction capacity in comparison with the orbital shaker, comparing the 100 minutes with the 9 and 6 minutes for the UAE and MAE, respectively, that can be considered an optimization because the amount of energy needed to supply is much lower. The situation of ChCl:CA (2:1) is similar to Pro:1.2-But (1:4), with the difference that in this (D)ES the selectivity changes with the intensification methods as mentioned before, this is a situation that should be evaluated more carefully in future work.

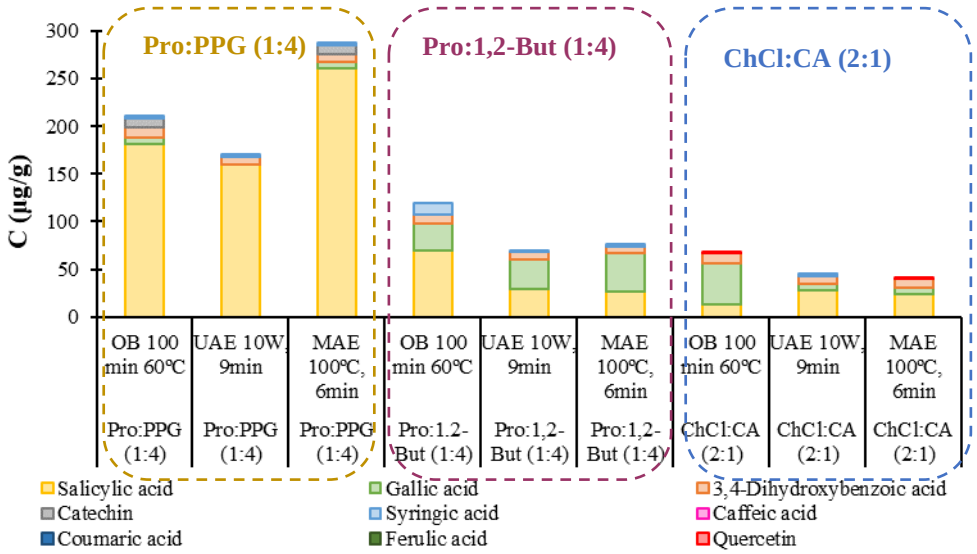



Figure 42. Overall comparison of the best conditions for the different technique used for the best (D)ESs with 30 %(v/v) of water.

### 4.1.5. Extraction with cycles

In order to enrich the extract using Pro:PPG (1:4), subsequent extraction cycles were performed using the best MAE conditions (6 min, 100 ºC), where the extract of the first extraction was used as extraction solvent for the second, and the same between the second and third extraction. Between each extraction a solvent loss was observed. Therefore, in order to have enough solvent to the following extraction, a small scale-up was made with 1.3 g, 0.8 g and finally 0.46 g for first, second and third cycle respectively, always maintaining the S:L ratio in 1:10.

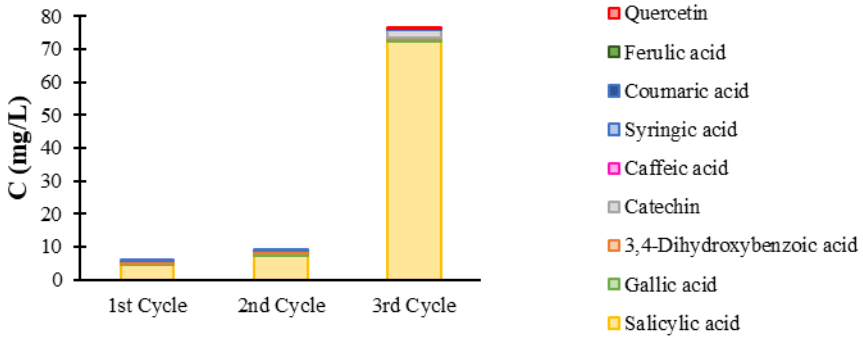



Figure 43. HPLC results for the extraction with cycles with an attempted of a scale-up.

It was immediately noticed that the extraction yield for the first cycle in Figure 43, was much lower than the extraction made for the optimization of the MAE conditions, as can be observed in Figure 46. The reason for this could be that a higher quantity of mass and solvent will probably need more time to reach the same extraction capacity. Given this, a combination of extractions with mass and solvent approximately to the quantity used for the first extraction performed for the optimization of MAE conditions. These combinations are schematized in Figure 44, and the results are presented Figure 45.

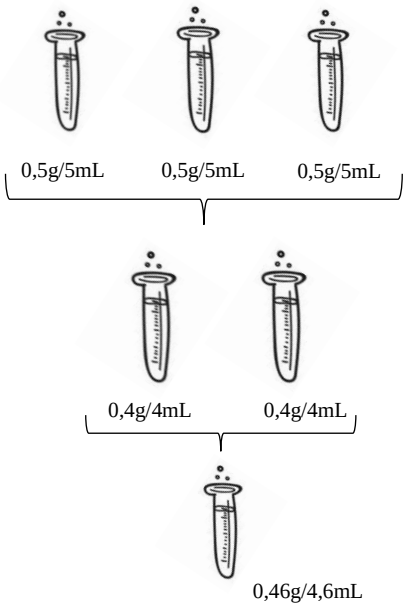



Figure 44. Schematic illustration of the extraction with cycles performed with the combinations of equal extractions.

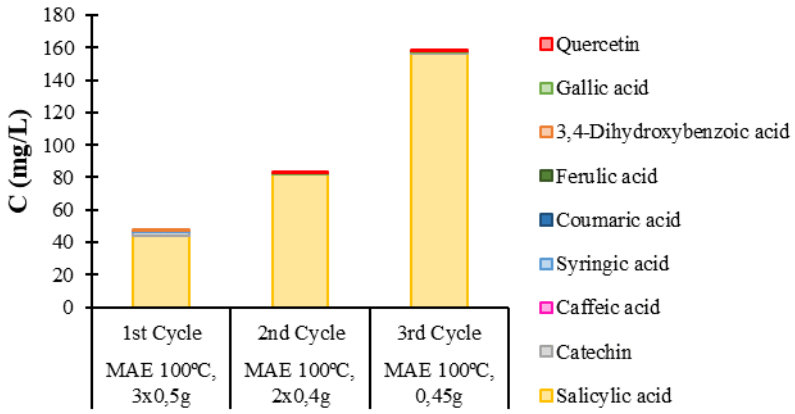



Figure 45. HPLC results for the extraction with cycles with the combinations of equal extracts.

Comparing the results from the first extraction performed with MAE at 100 ºC and 6 minutes, with the small scale-up and the combination of 3 extractions, Figure 46, it is notable that the difference between the first extraction performed with 0.5 g /5 mL and the small scale-up 1.3 g/13 mL. The combination of the three extractions showed a higher yield, even though only 0.5 g/5 mL was used for each, it is important to be aware that the *S. muticum* particles are not homogenous, the shape and size, and for a few also the tone of colour differ depending on the sample due to the heterogeneity of particles. The size and shape influence the surface area of a certain particle which influences the diffusion phenomena, and this, will have an impact in the extraction yield.

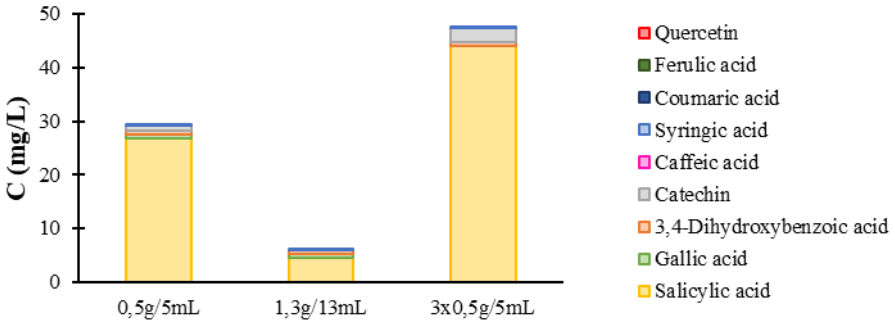



Figure 46. Comparison of the first extraction performed with MAE at the best conditions with the attempted of scale-up and with the combinations of three equal extracts.

## 4.2. Fucoxanthin, Fx

### 4.2.1. COSMO-RS

As previously mentioned, lower values of γ means higher solubilities, the explanation for this is present in the supporting information B.1. In terms of logarithmic values of **γ,** means that the more negative the values for ln (γ), the better the solubility is going to be according to COSMO-RS. The γ for different conventional and bio-solvents were calculated using COSMO-RS, Figure 47, and it is notable that the two solvents that should return a higher extraction of fucoxanthin are DMSO and 2-MeTHF, followed by EtAc, CPME and γ-Val [151]. Some of the solvents simulated in COSMO-RS have been already tested in previous studies for fucoxanthin extraction such as EtOH, MeOH, Isopropyl alcohol (IPA), 2-methyl tetrahydrofuran (2-MeTHF), ethyl lactate (EtLac), ethyl acetate (EtAc) and limonene [121–123]. Notwithstanding to this, a few new solvents were added to the screening of solvents, such as γ-valerolactone (γ-Val), dimethyl carbonate (DMC), dimethyl isorbide (DMI), cyrene, despite their used has not been reported for fucoxanthin extraction, they have been employed for other the extraction of other compounds [152,153].

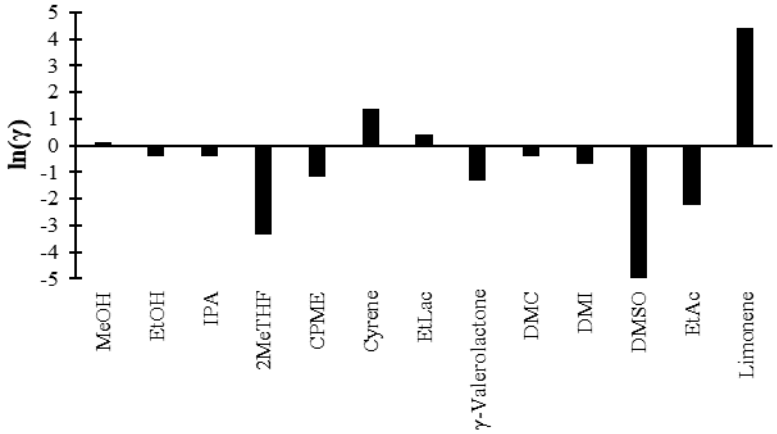



Figure 47. Neperian logarithm of the activity coefficients of fucoxanthin in different solvents.

The σ-profile of fucoxanthin, Figure 48, indicates an apolar behaviour with some weakly deviation to the HBD zone; accordingly, the solvents that should present better extraction results are 2-MeTHF and CPME that show also an apolar behaviour according to their σ-profiles, Figure 48. As was previously observed, the 2-MeTHF should offer a higher extraction yield than the CPME based on the activity coefficients; however, it is crucial to

keep in mind that this is merely a prediction made by the COSMO-RS calculations, providing a starting point for the experimental investigation.

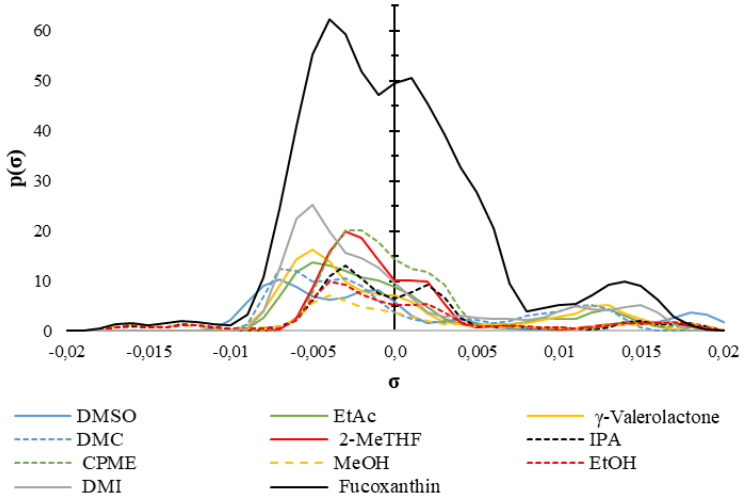



Figure 48. The σ-profile of fucoxanthin (black) and of the conventional and biosolvents that would present better results according with the activity coefficients: MeOH, EtOH, 2-MeTHF, EtAc, IPA, DMC, DMI, DMSO, CPME, γ-valerolactone.

### 4.2.2. SLE of fucoxanthin

The first attempted to extract fucoxanthin was performed using pure EtOH for 100 min in the orbital shaker at 50 ºC using a 1:10 (w/v) solid:liquid ratio; EtOH is one of the most used conventional solvents and according to the activity coefficients, Figure 47, fucoxanthin present some solubility in EtOH [66]. However, nothing was detected in the HPLC after this extraction.

According to the calculated γ, Figure 47, EtOH shows a higher solubility towards fucoxanthin when comparing with MeOH; nonetheless, a few studies already published reported that MeOH returned more prosperous results [66,76,154–156]. Given this, a change of the solvent from EtOH to MeOH was made as an attempt to extract some detectable quantity of fucoxanthin but again, nothing was detected in the HPLC. Another solvent exchange was made from EtOH to Ethyl acetate (EtAc), since in studies already published showed a good extraction capacity and COSMO-RS also predicted a higher solubility of fucoxanthin in EtAc compared with MeOH or EtOH [121–123]. However, once again, nothing was observed in HPLC using EtAc.

At this point the S:L ratio was changed to 1:5 and was possible to extract 1.86±0.22 μg/g of dry weight (0.38 ppm) using EtAc. At this point, the concerns if the solvent was green or not were not taken into account, as the focus was to try to quantify fucoxanthin and so, DMSO was tested as extraction solvent, and returned an extraction of 3.06±0.11 μg/g of dry weight (0.61 ppm). Until now, DMSO was the only solvent able to extract a significant amount of fucoxanthin whose yield is found to be in the calibration curve range which starts at 0.5 ppm, this calibration curve is present in the Supporting Information B.

Acknowledging this, a new calibration curve was performed for lower concentrations beginning at 0.12 ppm where higher injection volumes were inputted, Supporting Information B, to allow the detection of lower fucoxanthin concentrations. Employing this calibration curve, the screening of all the solvents simulated in COSMO-RS, Figure 47, was performed to evaluate if somehow there was a solvent that could extract a significant amount of fucoxanthin, with results presented in Table 7.

Table 7. HPLC results for all the solvents tested to extract fucoxanthin as the target compound.

| **Solvent** | **Extraction Yield µg/g of dry weight (ppm)** |
|---|---|
| MeOH | BD |
| Isopropyl alcohol | BD |
| DMSO | 0.95±0.59 (0.19) |
| EtAc | BD |
| EtLac | BD |
| DMC | BD |
| DMI | BD |
| ϒ - Val | 0.57±0.28 (0.11) |
| Limonene | BD |
| 2-MeTHF | BD |
| CPME | BD |
| Cyrene | 0.49±0.75 (0.0997) |

Looking at the results present in Table 7 it is possible to conclude that the algae does not possess much of fucoxanthin, almost inexistent. This could be due to the age of the algae which has already 7 years, even if the storage was made properly, as mentioned before, fucoxanthin is a really sensitive compound. However, *S. muticum* could be under an inappropriate storage, since the algae has been stored in the fridge and could be preferred to be in the freezer; in fact, SIGMA-ALDRICH company recommends that the fucoxanthin purchased from them should be stored at -20 ºC.

Other conclusions that were taken from the results in Table 7 is that the same extraction conditions using the same solvent, could not return the same extraction yield and that can be seen with the extraction using DMSO. For extractions with DMSO, the yield value of 3.06±0.11 µg/g of dry weight was obtained for the first extraction, while the yield value of 0.95±0.59 µg/g dry weight was obtained for the second extraction. This difference in the extraction yields using the same solvent and conditions could be related with the fact that the distribution of size and shape of the algae particles are not uniform, as previously mentioned. Also, having in mind that the two results were obtained from different calibration curves, since they are very small quantities, an experimental error performing the calibration curves can also be associated.

# 5. Conclusions

Several choline-based, proline-based and betaine-based (D)ESs were tested as green extraction solvents with the objective to replace conventional solvents that tend to be toxic and not biocompatible in the extraction of phenolic compounds. This is particularly important for application in the cosmetic and food industry. Some of the (D)ESs tested not only matched the extraction yields of the two conventional solvents used, water and ethanol, but also improved the extraction yield, especially the Pro:PPG (1:4), followed by the Pro:1,4-But (1:4) and ChCl:CA (2:1). An interesting phenomena was seen respecting the selectivity, since depending on the (D)ES used, the selectivity can change towards a certain compound. The (D)ES Pro:PPG (1:4) showed a high selectivity towards salicylic acid, and ChCl:CA (2:1) towards the gallic acid. The Pro:1,4-But (1:4) (D)ES, despite the good extraction yield, it is not a very selective (D)ES when extracting PCs.

The screening was followed by an optimization of two experimental variables, the water content present in the (D)ES and the temperature for the three most performant (D)ESs. It was concluded that 30 % (v/v) of water content at 60 ºC were the best conditions for 100 min of extraction. In order to reduce this time, intensification techniques such as MAE and UAE were also tested and clearly, a reduction of the extraction time by at least 10 times was accomplished, while decreasing the energy consumption. In the case of MAE, not only a decrease of the extraction time was observed but also an improvement of the extraction yield for Pro:PPG (1:4). It should be noted that in UAE the optimized parameters were the time and power, while in MAE was the time and temperature. A response surface model was plotted for both techniques, concluding that long times, high power in the UAE and high temperature in the MAE should return the best results. However, the surface model does not take into account the degradation of the compounds that tends to occur when they are exposed for a long period of time to high temperatures. This is especially notable in the UAE, where the control of temperature is extremely hard since the probe tend to reach high temperatures (above 100 ºC), and this needs attention when setting up the extraction conditions.

With the best conditions of MAE, since it was the technique returning the higher extraction yields, subsequent cycles of extraction were performed to enrich the extract. However, a few problems during scale up were noticed, since it was required an increased amount of solvent and biomass to address the loss of solvent during the extraction, as some quantity of solvent tend to be absorbed in the algae.

*S. muticum* has numerous added value compounds, not only phenolic compounds, but also fucoxanthin, which is the pigment of brown algae. Fucoxanthin is an expensive compound that possesses several important biological properties, which draws huge interest from the industrial point of view. However, the *S. muticum* studied in this project showed to have only trace amounts of fucoxanthin when using DMSO and other bio-solvents for extraction.

# 6. Future Work

As seen in the extraction with cycles, the scale-up needs to be addressed for the extraction of PCs, and hence it is important to understand how the quantity of biomass and solvent affects the amount of time required to achieve the same performance as on a small scale. Different applications of the extracts of PCs in the (D)ES could be tested out in cosmetics, food, pharmaceutical, etc., in order to evaluate the success of the practical application of this extract.

The composition of the algae suffers variations throughout the different seasons. An interesting study to perform might be to collect *S. muticum* from strategic times of the year and analyse the impact of the different seasons on *S. muticum* composition, not only for the PCs but also for fucoxanthin. Ideally, to have better results in the extraction of fucoxanthin, the algae should be fresh and caught right after the winter season.

Even though the algae was stored properly, some degradation of compounds present in *S. muticum* can occur. Thus, it would be relevant to evaluate the rate of this degradation for the different compounds, PCs and fucoxanthin.

After the optimization of the seasonal collection time to enhance the presence of fucoxanthin, hydrophobic (D)ES should be valuable in its extraction. Finally, the process of purification of fucoxanthin should also be included in the study, ideally using only green solvents.

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# Supporting Information

## A. Phenolic compounds

### A.1. Calibration Curves

***Folin – Ciocalteu assay***

The Folin-Ciocalteu assay allows to quantify the total phenolic content in mg of equivalents of gallic acid, where several standard solutions with known concentration of gallic acid is evaluated with the Folin method. The absorbance measure with the Folin methods is measure and it is plotted in function of the concentration of gallic acid, due to this, the TPC is expressed in mg of equivalents of gallic acid per liter.

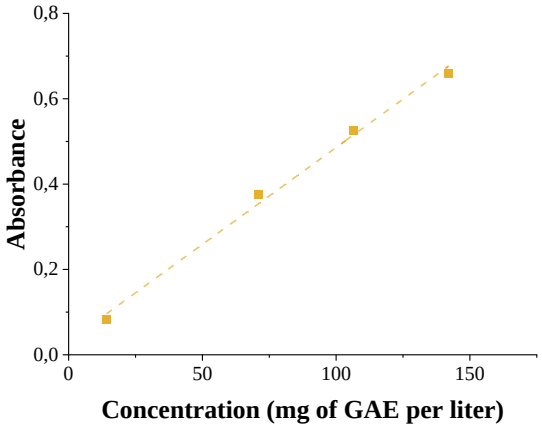



Figure S.1. Calibration curve for the Folin-Ciocalteu method with the absorbance in function of the concentration of gallic acid: $y = (4.54\pm1.02)x10^{-3}\ x + (-3.17\pm9.78)\ x10^{-2}$ with $R^2=0.992$.

***Christ-Müllers assay***

The Christ-Müllers assay is analogous to the Folin assay, standard concentrations with a known quercetin concentration are evaluated with the Christ-Müllers method in order to evaluate the total flavonoids content. After this the calibration curve can be made with the absorbance varying with the concentration of quercetin, therefore, the results are expressed in mg of equivalents of quercetin per liter.

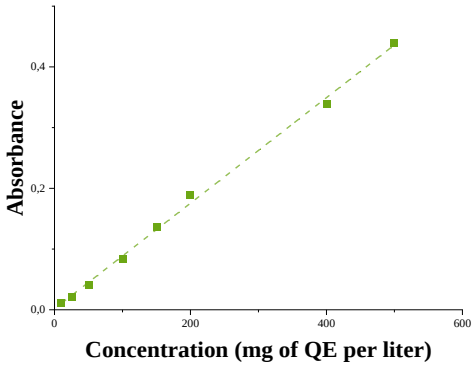



Figure S.2. Calibration curve for the Christ-Müllers method with the absorbance in function of the concentration of quercetin: $y = (86.90\pm4.01)x10^{-5}\ x + (1.71\pm9.88)\ x10^{-3}$ with $R^2=0.998$.

***DPPH free radical scavenging***

The DPPH free radical scavenging assay allows to know the percentage of inhibition the radical DPPH•. This percentage of inhibition was measure for several standard solutions of Trolox with known concentration.

Therefore, was possible to plot a calibration curve between the Trolox concentration and the percentage of inhibition allowing the antioxidant activity to be expressed in mg of Trolox equivalents per g of dry weight.

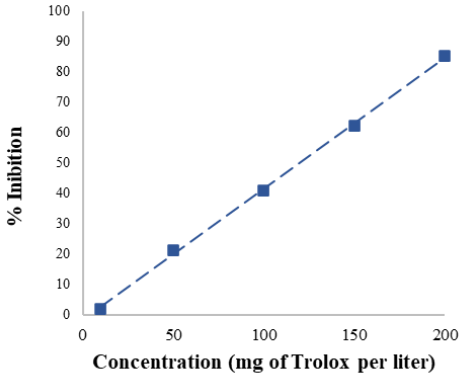


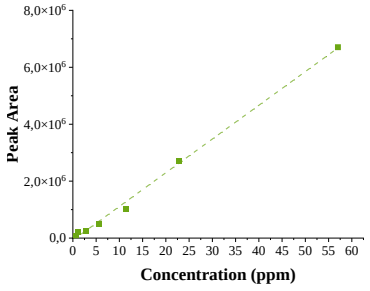

Figure S.3. Calibration curve for the DPPH free radical scavenging with the inhibition percentage in function of the concentration of Trolox: y = (437.21±8.95)x10-3 x + (-2.72±1.10) with $R^2$=0.998.

***Calibration curves of phenolic compound in HPLC***

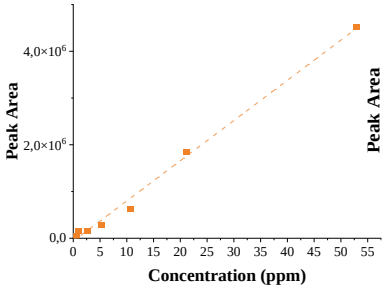
Table S.1. Retention times of the different phenolic compounds characterized in HPLC and the respective wavelength where they are measured.

| **Phenolic compound** | Gallic acid | 3,4-Dihydroxybenzoic acid | Catechin | Caffeic acid | Syringic acid | Coumaric acid | Ferulic acid | Salicylic acid | Quercetin |
|---|---|---|---|---|---|---|---|---|---|
| **Retention Time (min)** | 10.45 | 16.49 | 18.76 | 22.837 | 23.22 | 31.943 | 34.393 | 41.08 | 41.473 |
| **Wavelength of the channel** | 270 | 270 | 270 | 323 | 270 | 323 | 323 | 270 | 370 |

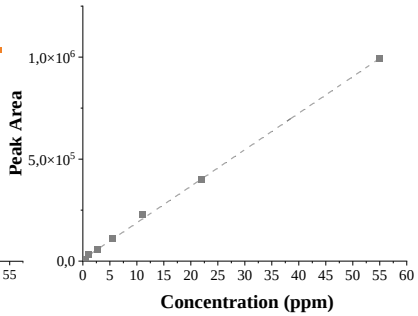
After the HPLC method is concluded each compound that was going to be characterized was injected into the HPLC with different known concentrations. All the peaks, for each concentration and phenolic compound, were integrated, this allowed the construction of calibrations curves for each compound.



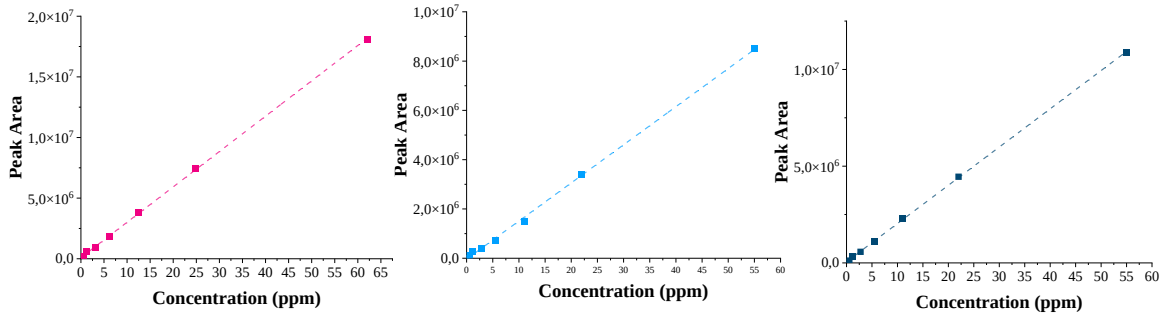

(a) Gallic Acid: $y$ = (118.41±6.36)x$10^3$ $x$ + (-0.72±1.41)x$10^5$ with $R^2$=0.997




(b) 3,4-Dihydroxybenzoic Acid: $y$ = (86.01±6.34)x$10^3$ $x$ + (-0.61±1.31)x$10^5$ with $R^2$=0.995



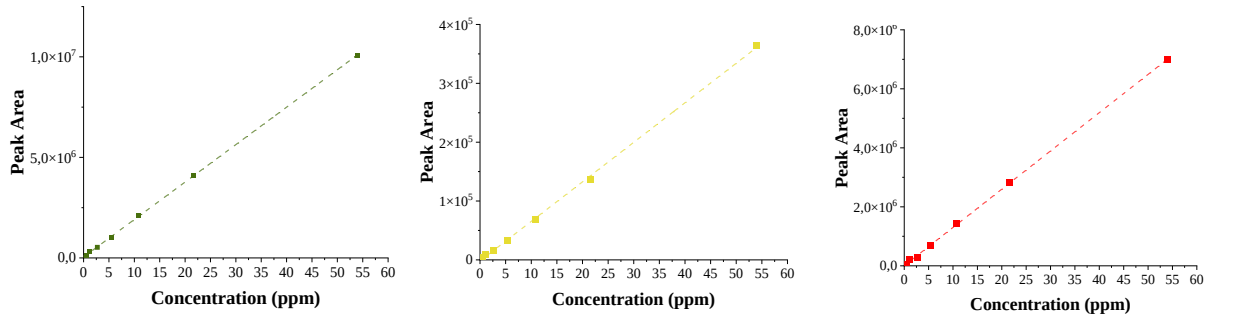

(c) Catechin: $y$ = (179.05±5.36)x$10^2$ $x$ + (0.93±1.15)x$10^4$ with $R^2$=0.995




(d) Caffeic Acid: $y = (292.16\pm4.72)x10^3\ x + (0.97\pm1.14)x10^5$ with $R^2=0.9997$

(e) Syringic Acid: $y = (154.68\pm5.33)x10^3\ x + (-0.36\pm1.14)x10^5$ with $R^2=0.998$

(f) Coumaric Acid: $y = (198.66\pm3.10)x10^3\ x + (5.82\pm6.63)x10^4$ with $R^2=0.9998$




(g) Ferulic Acid: $y = (186.23\pm2.68)x10^3\ x + (4.87\pm5.63)x10^4$ with $R^2=0.9998$

(h) Salicylic Acid: $y = (67.18\pm2.04)x10^2\ x + (-1.75\pm4.30)x10^3$ with $R^2=0.999$

(i) Quercetin: $y = (129.78\pm2.04)x10^3\ x + (0.46\pm4.29)x10^4$ with $R^2=0.9998$

Figure S.4. Calibration curves for the different phenolic compound characterized in the HPLC: (a) gallic acid (light green), (b) 3,4-Dihydroxybenzoic Acid (orange), (c) catechin (grey), (d) caffeic acid (pink), (e) Syringic acid (light blue), (f) Coumaric acid (dark blue), (g) ferulic acid (dark green), (h) salicylic acid (yellow), (i) quercetin (red).

## A.2. (D)ES densities & viscosities

***Pro:PPG (1:4)***

The densities of the (D)ES made with proline and propylene glycol with a 1:4 ratio were measured at different temperatures in order to have the variation of the density with the temperature since the extractions were performed between 50 and 70 ºC, it is important to understand

Table S.2. Densities measure at different temperatures for the pure (D)ES Pro:PPG (1:4).

| T (ºC) | Density (g/cm$^3$) |
|---|---|
| 20 | 1.0994±0.0053 |
| 25 | 1.0957±0.0053 |
| 30 | 1.0921±0.0053 |
| 35 | 1.0886±0.0053 |
| 40 | 1.0854±0.0053 |

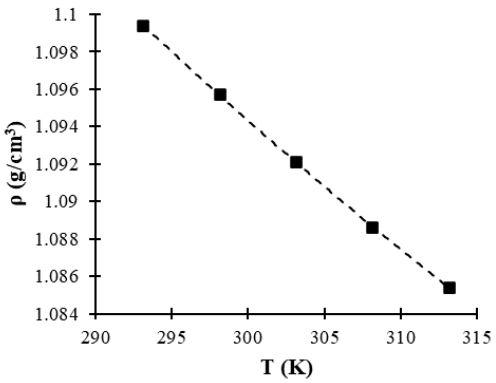



Figure S.5. Densities at different temperature from 20 to 40 ºC for the pure (D)ES: $y = (3.14\pm1.63)\text{x}10^{-6}\,x^2 + (-89.06\pm9.81)\text{x}10^{-5}\,x + (1159.71\pm1.41)\text{x}10^{-3}$ with $R^2=0.9999$

Table S.3. Densities measured for different contents of water at 25 ºC for Pro:PPG (1:4).

| Water content (%(v/v)) | Density (g/cm³) |
|---|---|
| 10.08 | 1.0942±0.0053 |
| 20.10 | 1.0911±0.0053 |
| 30.11 | 1.0856±0.0053 |
| 40.17 | 1.0776±0.0054 |
| 50.24 | 1.0670±0.0054 |

Table S.4. Viscosities measure at different temperatures for the pure (D)ES Pro:PPG (1:4) and with 30% (v/v) of water.

| T (ºC) | η pure (D)ES (mPa.s) | η (D)ES 30% (v/v) water (mPa.s) |
|---|---|---|
| 20 | -- | 21.30±0.40 |
| 25 | 175.98±0.77 | 16.65±0.34 |
| 30 | 128.11±0.77 | 13.26±0.28 |
| 35 | 95.19±0.81 | 10.73±0.21 |
| 40 | 72.09±0.86 | 8.84±0.15 |
| 45 | 55.57±0.90 | 7.34±0.10 |
| 50 | 43.54±0.96 | 6.18±0.06 |
| 55 | 34.63±1.07 | -- |
| 60 | 27.93±1.17 | -- |
| 65 | 22.81±1.24 | -- |
| 70 | 18.84±1.24 | -- |
| 75 | 15.73±1.18 | -- |
| 80 | 13.25±1.09 | -- |

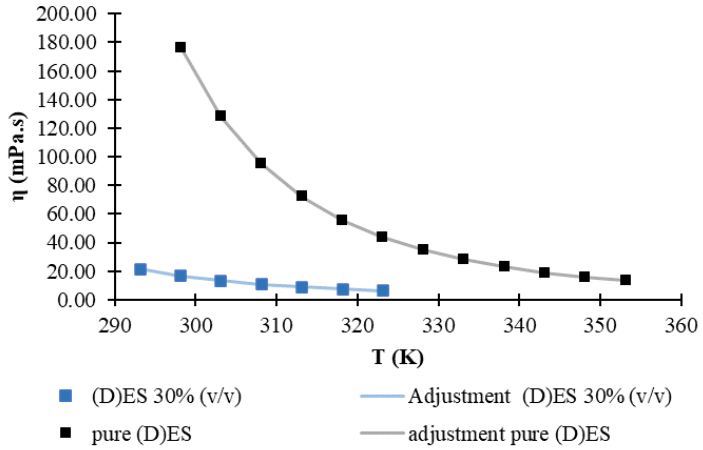



Figure S.6.Viscosities at different temperature for the pure (D)ES Pro:PPG (1:4) (black) and with 30% (v/v) of water (blue).

Table S.5. VFT fitting parameters for the viscosity adjustment for Pro:PPG (1:4) without water and with 30 %(v/v).

| | **pure Pro:PPG (1:4)** | **Pro:PPG (1:4) with 30 %(v/v) of water** |
|---|---|---|
| $A_\eta$ | -10.662 | -3.371 |
| $B_\eta$ | 1201.973 | 809.636 |
| $C_\eta$ | 163.483 | 167.218 |
| $R^2$ | 0.99999 | 0.99997 |

***Pro:1.2-But (1:4)***

Table S.6. Densities measure at different temperatures for the pure (D)ES Pro:1,4-But (1:4).

| **T (°C)** | **Density (g/cm$^3$)** |
|---|---|
| 20 | 1.0626±0.0054 |
| 25 | 1.0589±0.0055 |
| 30 | 1.0552±0.0055 |
| 35 | 1.0517±0.0055 |
| 40 | 1.0484±0.0055 |

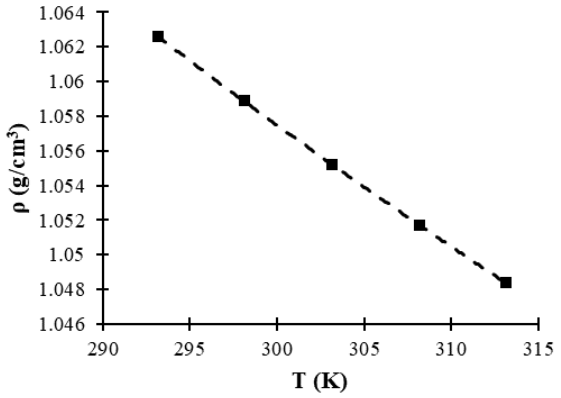



Figure S.7. Pure (D)ES Pro:1,2-But (1:4) densities variations with temperature: $y = (31.43\pm5.75)\text{x}10^{-7}\ x^2 + (-89.86\pm3.47)\text{x}10^{-5}\ x + (10793.11\pm5.00)\text{x}10^{-4}$ with $R^2$=0,99999

Table S.7. Densities measured for different contents of water at 25 ºC for Pro:1,4-But (1:4).

| Water contente (%(v/v)) | Density (g/cm$^3$) |
|---|---|
| 10.18 | 1.0603±0.0054 |
| 21.12 | 1.0597±0.0055 |
| 30.45 | 1.0573±0.0055 |
| 40.27 | 1.0538±0.0055 |
| 50.27 | 1.0482±0.0055 |

Table S.8. Viscosities measure at different temperatures for the pure (D)ES Pro:1,4-But (1:4) and with 30% (v/v) of water.

| T (ºC) | η pure (D)ES (mPa.s) | η (D)ES 30% (v/v) water (mPa.s) |
|---|---|---|
| 20 | -- | 22.57±0.06 |
| 25 | 172.05±1.26 | 17.58±0.12 |
| 30 | 124.02±1.05 | 13.96±0.15 |
| 35 | 91.31±0.88 | 11.26±0.25 |
| 40 | 68.52±0.72 | 9.24±0.28 |
| 45 | 52.36±0.64 | 7.65±0.32 |
| 50 | 40.68±0.55 | 6.42±0.35 |
| 55 | 32.21±0.10 | -- |
| 60 | 25.78±0.09 | -- |
| 65 | 20.91±0.10 | -- |
| 70 | 17.17±0.11 | -- |
| 75 | 14.28±0.29 | -- |
| 80 | 11.98±0.20 | -- |

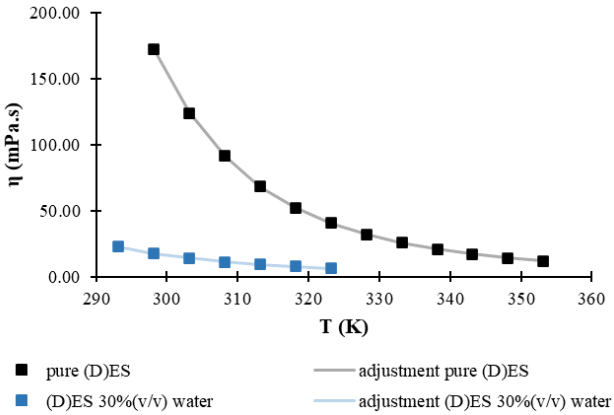



Figure S.8. Viscosities at different temperature for the pure (D)ES Pro:1,2-But (1:4) (black) and with 30% (v/v) of water (blue).

Table S.9. Parameters obtained with the VFT adjustment for viscosity for the pure Pro:1,2-But (1:4) and with 30 %(v/v) of water.

| | **pure Pro:1,2-But (1:4)** | **Pro:1,2-But (1:4) with 30 %(v/v) of water** |
|---|---|---|
| $A_\eta$ | -10.997 | -10.368 |
| $B_\eta$ | 1250.875 | 835.455 |
| $C_\eta$ | 162.724 | 166.120 |
| $R^2$ | 0.999996 | 0.999997 |

***ChCl:CA (2:1)***

The (D)ES composed by ChCl and CA in a ratio of 2:1 was so viscous that It was nearly impossible to handle it without adding a little amount of water. Also, the densimeter used only presented good measurements until 1.3 g/cm$^3$, therefore, water was added in order to reduce the density that was above 1.3 g/cm$^3$. An attempt to add the smallest amount of water was made to have the (D)ES the nearest possible from the pure (D)ES. The amount of water added to perform the measurements in was 6.4 %(w/w).

Table S.10. Densities measure at different temperatures for the (D)ES ChCl:CA (2:1) with 6.4 %(w/w).

| **T (°C)** | **Density (g/cm$^3$)** |
|---|---|
| 20 | 1.2545±0.0092 |
| 22 | 1.2529±0.0092 |
| 24 | 1.2513±0.0092 |
| 26 | 1.2497±0.0092 |
| 28 | 1.2481±0.0093 |
| 30 | 1.246±0.0046 |
| 32 | 1.2443±0.0046 |
| 34 | 1.2423±0.0047 |
| 36 | 1.2405±0.0093 |
| 38 | 1.2389±0.0047 |
| 40 | 1.2373±0.0047 |

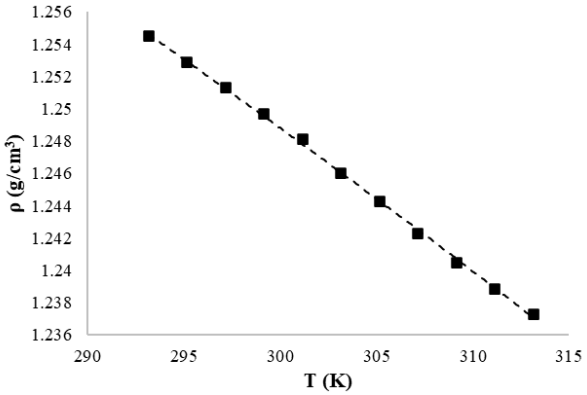



Figure S.9. Densities at different temperature from 20 to 40 ºC for the pure (D)ES ChCl:CA (2:1): $y = (-2.38\pm5.08)x10^{-6}\ x^2 + (--7.34\pm3.06)x10^{-5}\ x + (1270.25\pm4.46)x10^{-3}$ with $R^2 = 0.99897$

Table S.11. Viscosities values ofr ChCl:CA (2:1) at different temperatures with only 6,4 %(w/w) of water and with 30 %(v/v).

| T(ºC) | η (D)ES 6.4 %(w/w) (mPa.s) | η (D)ES 30% (v/v) water (mPa.s) |
|---|---|---|
| 20 | -- | 62.48±0.03 |
| 25 | 8877.27±3.40 | 49.11±0.03 |
| 30 | 5706.73±3.48 | 39.22±0.02 |
| 35 | 3767.40±3.51 | 31.77±0.02 |
| 40 | 2551.90±3.52 | 26.09±0.02 |
| 45 | 1769.03±3.58 | 21.67±0.02 |
| 50 | 1253.10±3.64 | 18.21±0.02 |
| 55 | 906.11±3.76 | -- |
| 60 | 666.44±3.56 | -- |
| 65 | 499.34±3.54 | -- |
| 70 | 379.83±3.35 | -- |
| 75 | 292.74±2.83 | -- |
| 80 | 228.89±2.39 | -- |

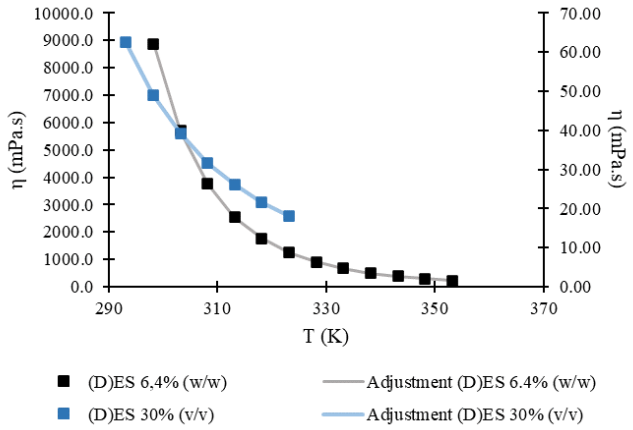



Figure S.10. Variation of viscosity with temperature for the (D)ES ChCl:CA (2:1) with only 6,4 %(w/w) (black-left axis) and with 30% (v/v) of water (blue-right axis).

Table S.12. Parameters for the VFT model adjusting the viscosity variation with temperature of ChCl:CA (2:1) with 6.4 %(w/w) and with 30 %(v/v) of water.

| | **ChCl:CA (2:1) with 6.4 %(w/w) of water** | **ChCl:CA (2:1) with 30 %(v/v) of water** |
|---|---|---|
| $\boldsymbol{A_\eta}$ | -11.034 | -9.684 |
| $\boldsymbol{B_\eta}$ | 1898.62216 | 954.377 |
| $\boldsymbol{C_\eta}$ | 154.528 | 155.073 |
| $\mathbf{R^2}$ | 0.999999 | 1.0000 |

## A.3. Excess volumes

An excess property ($X^E$) is given by the different of the real value and the value corresponding to ideality, equation (S.1) [157].

$$X^E = \Delta_{mixure} X^{Real} - \Delta_{mixture} X^{Ideal} \quad \text{(S.1)}$$

The molar volume ($V_m$) of a pure compound i can be determined by expression (S.2) where MW is the molecular weight (g/mol) and $\rho$ is the density (g/cm$^3$). The ideal $V_m$ of a mixture of two compounds is given the sum of $V_m$ of each one.

$$V_{m_i} = \frac{MW_i}{\rho_i} \quad \text{(S.2)}$$

To calculate the real $V_m$ of a mixture of compound i and j, it is necessary to be aware of the exact composition and have the value of the density for the mixture. Further the real $V_m$ can be easily calculated using expression (S.3). Note that one of the compounds if the (D)ES and the other one is water, so there is a need to determine the exact MW of the (D)ES.

$$V_m = \frac{x_i MW_i + x_j MW_j}{\rho_{mixture}} \quad \text{(S.3)}$$

## A.4. Polarity essay - Solvatochromic probes

Table S.13. Polarity results from the solvatochromic probes for the betaine scale ($E_T N$)a nd fot Kamlet-Taft scale (α, β, π*).

| | | α | β | π* | $E_T(33)$ (kcal/mol) | $E_T N$ |
|---|---|---|---|---|---|---|
| **Pro:PPG (1:4)** | 0% | 0.478 | 0.729 | 0.873 | 56.709 | 0.544 |
| | 10% | 0.432 | 0.686 | 0.959 | 56.954 | 0.552 |
| | 20% | 0.393 | 0.599 | 1.034 | 57.182 | 0.559 |
| | 30% | 0.387 | 0.574 | 1.102 | 57.857 | 0.579 |
| | 40% | 0.300 | 0.470 | 1.162 | 57.182 | 0.559 |
| | 50% | 0.328 | 0.438 | 1.205 | 58.092 | 0.587 |
| **Pro:1,2-But (1:4)** | 0% | 0.613 | 0.799 | 0.776 | 57.721 | 0.575 |
| | 10% | 0.540 | 0.749 | 0.889 | 57.837 | 0.579 |
| | 20% | 0.493 | 0.695 | 0.959 | 57.896 | 0.581 |
| | 30% | 0.427 | 0.541 | 1.052 | 57.916 | 0.581 |
| | 40% | 0.446 | 0.621 | 1.034 | 58.014 | 0.584 |
| | 50% | 0.392 | 0.555 | 1.134 | 58.290 | 0.593 |
| **ChCl:CA (2:1)** | 20% | 2.454 | 0.032 | 1.409 | 93.282 | 1.668 |
| | 30% | 2.603 | 0.411 | 1.202 | 93.282 | 1.668 |
| | 40% | 2.592 | 0.346 | 1.236 | 93.486 | 1.674 |
| | 50% | 2.612 | 0.318 | 1.264 | 94.101 | 1.693 |

## A.5. (D)ES screening

Table S.14. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for all the solvents tested as extraction solvents: water, ethanol and other 26 (D)ES.

| Solvent | TPC (mg/g) | TFC (mg/g) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|
| **Water** | 5.64±0.03 | 10.17±0.14 | 20.28±1.70 | 0.50±1.50 |
| **Ethanol** | 3.70±0.13 | 7.40±0.20 | 78.57±0.19 | 1.86±0.18 |
| **Bet:Gly (1:4)** | 9.38±0.63 | 9.50±0.14 | 74.46±0.12 | 1.76±0.11 |
| **Bet:PPG (1:4)** | 7.33±0.18 | 10.57±0.31 | 78.23±0.12 | 1.85±0.12 |
| **Bet:1.2-But (1:3)** | 5.29±1.36 | 13.89±0.06 | 54.39±0.39 | 1.30±0.37 |
| **Bet:1.2-But (1:4)** | 5.60±0.99 | 11.09±0.17 | 78.47±0.10 | 1.86±0.10 |
| **Bet:1.3-But (1:4)** | 2.92±1.32 | 7.36±0.17 | 70.18±0.35 | 1.67±0.34 |
| **Bet:LA (1:1)** | 2.90±1.26 | 4.75±0.09 | 71.86±0.26 | 1.69±0.25 |
| **Bet:LA (1:2)** | 2.29±1.42 | 2.40±0.15 | 56.58±2.93 | 1.34±2.79 |
| **Bet:LA (1:3)** | 8.22±0.38 | 6.58±0.01 | 50.81±1.12 | 1.22±1.06 |
| **Pro:Gly (1:4)** | 14.14±0.03 | 9.72±0.85 | 56.24±0.88 | 1.34±0.84 |
| **Pro:PPG (1:4)** | 15.26±0.08 | 13.66±0.22 | 25.04±0.61 | 0.63±0.55 |
| **Pro:1.2-But (1:3)** | 15.30±0.06 | 15.26±0.40 | 31.33±0.87 | 0.78±0.80 |
| **Pro:1.2-But (1:4)** | 14.84±0.10 | 20.61±0.02 | 0 | 0 |
| **Pro:1.3-But (1:4)** | 8.30±0.07 | 12.68±0.01 | 0 | 0 |
| **Pro:LA (1:1)** | 11.48±0.01 | 7.32±0.03 | 5.36±66.76 | 0.18±4.50 |
| **Pro:LA (1:2)** | 9.75±0.03 | 6.09±0.17 | 25.75±5.44 | 0.65±3.50 |
| **Pro:LA (1:3)** | 10.99±0.05 | 5.55±0.02 | 28.28±3.26 | 0.70±2.298 |
| **ChCl:Gly (1:4)** | 10.88±1.26 | 11.88±0.09 | 90.45±0.22 | 2.12±0.21 |

| | | | | |
|---|---|---|---|---|
| **ChCl:PPG (1:4)** | 0 | 9.15±0.09 | 84.56±0.08 | 1.99±0.07 |
| **ChCl:1.2-But (1:3)** | 0.20±7.39 | 11.64±0.02 | 87.30±0.10 | 2.05±0.10 |
| **ChCl:1.3-But (1:4)** | 3.71±0.43 | 7.49±0.05 | 74.10±0.22 | 1.75±0.21 |
| **ChCl:LA (1:1)** | 0 | 6.10±0.07 | 23.73±2.18 | 0.60±1.95 |
| **ChCl:LA (1:2)** | 0 | 3.72±0.05 | 18.86±2.70 | 0.49±2.36 |
| **ChCl:LA (1:3)** | 0.23±1.46 | 3.00±0.16 | 0 | 0 |
| **ChCl:CA (2:1)** | 0.49±4.54 | 2.46±0.69 | 12.41±3.07 | 0.35±2.52 |
| **ChCl:CA (1:1)** | 0.93±6.69 | 1.78±0.42 | 24.98±1.26 | 0.62±1.14 |
| **ChCl:CA (1:2)** | 0.33±2.66 | 1.85±0.06 | 22.34±0.58 | 0.57±0.52 |

Table S.15. HPLC results in μg/g of dry weight for each of the (D)ESs with 30% (v/v) of water tested on the screening. BD: Bellow detection

| | **Gallic acid** | **3,4-Dihydroxybenzoic acid** | **Catechin** | **Caffeic acid** | **Syringic acid** | **Coumaric acid** | **Ferulic acid** | **Salicylic acid** | **Quercetin** |
|---|---|---|---|---|---|---|---|---|---|
| **Water** | 6.14±0.09 | 7.32±0.10 | BD | BD | 2.52±0.23 | BD | BD | 7.01±3.78 | 0.36±0.55 |
| **Ethanol** | 6.26±0.22 | 8.10±0.33 | BD | BD | BD | BD | BD | 5.14±2.40 | BD |
| **Bet:Gly (1:4)** | 6.07±0.01 | 9.53±0.81 | BD | BD | 2.46±0.12 | BD | BD | 4.98±5.07 | BD |
| **Bet:PPG (1:4)** | 6.07±0.04 | 8.23±0.33 | BD | BD | BD | BD | BD | 3.07±0.29 | BD |
| **Bet:1.2-But (1:3)** | 8.74±0.07 | 8.06±0.45 | BD | BD | 2.33±0.07 | BD | BD | 13.63±0.31 | BD |
| **Bet:1.2-But (1:4)** | 17.73±0.30 | 8.20±0.26 | BD | BD | 2.39±0.11 | BD | BD | 24.73±9.15 | BD |
| **Bet:1.3-But (1:4)** | 6.21±0.13 | 8.26±0.05 | BD | BD | 2.42±0.28 | BD | BD | 27.16±3.18 | BD |
| **Bet:LA (1:1)** | 6.20±0.04 | 10.45±0.28 | BD | BD | 2.53±0.21 | BD | BD | 6.94±0.31 | 0.75±0.09 |
| **Bet:LA (1:2)** | 6.09±0.01 | 10.10±0.11 | BD | BD | 2.48±0.10 | BD | BD | 14.41±0.20 | 0.90±0.54 |
| **Bet:LA (1:3)** | 6.08±0.02 | 11.20±1.28 | BD | BD | 2.80±0.69 | BD | BD | 27.25±1.14 | 0.54±0.74 |
| **Pro:Gly (1:4)** | 6.11±0.03 | 8.68±0.28 | BD | BD | 2.36±0.43 | BD | BD | 12.17±0.79 | BD |
| **Pro:PPG (1:4)** | 6.17±0.20 | 10.85±0.31 | 9.48±0.89 | BD | 2.30±0.06 | BD | BD | 181.67±1.46 | BD |
| **Pro:1.2-But (1:3)** | 15.21±0.38 | 8.22±0.41 | BD | BD | BD | BD | BD | 17.06±0.58 | BD |
| **Pro:1.2-But (1:4)** | 28.98±0.30 | 8.63±0.17 | BD | BD | 12.91±0.74 | BD | BD | 69.29±0.04 | BD |
| **Pro:1.3-But (1:4)** | 6.07±0.07 | 7.94±0.39 | BD | BD | 2.37±0.21 | BD | BD | 26.30±6.85 | 0.51±0.29 |
| **Pro:LA (1:1)** | 9.65±0.35 | 8.49±1.76 | BD | BD | 2.43±0.80 | BD | BD | 17.35±1.24 | 0.04±7.99 |
| **Pro:LA (1:2)** | 9.64±1.20 | 8.31±0.40 | BD | BD | 2.59±0.13 | BD | BD | 18.44±1.30 | 0.02±10.29 |
| **Pro:LA (1:3)** | 9.16±1.30 | 8.84±0.37 | BD | BD | 2.65±0.71 | BD | BD | 27.51±1.98 | 1.56±1.11 |
| **ChCl:Gly (1:4)** | BD | 8.23±0.27 | BD | BD | BD | BD | BD | 4.59±7.87 | BD |
| **ChCl:PPG (1:4)** | 6.18±0.02 | 8.38±0.39 | BD | BD | 2.40±0.08 | BD | BD | 5.31±1.25 | BD |
| **ChCl:1.2-But (1:3)** | 19.39±0.70 | 8.22±0.32 | BD | BD | BD | BD | BD | 17.24±0.31 | BD |
| **ChCl:1.3-But (1:4)** | 6.31±0.05 | 8.00±0.35 | BD | BD | 2.33±0.61 | BD | BD | 15.44±2.43 | 0.61±4.28 |
| **ChCl:LA (1:1)** | BD | 10.57±1.29 | BD | BD | 2.44±0.36 | BD | BD | 26.35±0.55 | 0.18±1.38 |
| **ChCl:LA (1:2)** | 6.17±0.14 | 9.64±1.92 | BD | BD | 2.61±0.12 | BD | BD | 36.41±10.37 | 0.67±0.65 |
| **ChCl:LA (1:3)** | 6.08±0.01 | 11.42±1.92 | BD | BD | 2.78±0.18 | BD | BD | 21.12±0.85 | 1.69±2.44 |
| **ChCl:CA (2:1)** | 43.17±0.20 | 9.76±0.08 | BD | BD | BD | BD | BD | 13.65±0.15 | 1.52±065 |
| **ChCl:CA (1:1)** | 29.52±0.62 | 9.68±0.43 | BD | BD | 2.26±0.33 | BD | BD | 9.07±1.20 | 19.47±0.82 |
| **ChCl:CA (1:2)** | 28.76±0.23 | 9.623±0.74 | BD | BD | 2.32±0.70 | BD | BD | 10.01±3.98 | 4.81±0.22 |

## A.6. Optimization

***Water optimization***

Table S.16. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for the optimization of water content.

| (D)ES | %H2O (v/v) | TPC (mg/g) | TFC (mg/g) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|---|
| **Pro:PPG (1:4)** | 10 | 4.80±0.08 | 1.36±1.41 | 0 | 1.03±0.37 |
| | 20 | 10.46±0.05 | 10.69±0.20 | 0 | 0.82±0.88 |
| | 40 | 12.33±0.01 | 14.77±0.35 | 0 | 0.39±1.45 |
| | 50 | 13.25±0.08 | 14.76±0.05 | 0 | 0 |
| **Pro:1,2-But (1:4)** | 10 | 5.74±0.50 | 5.51±0.23 | 42.26±0.40 | 0 |
| | 20 | 7.96±0.07 | 10.79±0.45 | 33.14±0.95 | 0 |
| | 40 | 13.72±0.10 | 15.38±0.40 | 14.36±1.72 | 0 |
| | 50 | 12.45±0.07 | 20.31±0.04 | 0 | 0 |
| **ChCl:CA (2:1)** | 10 | 3.14±2.13 | 2.33±0.16 | 14.49±6.43 | 0.39±5.41 |
| | 20 | 6.04±1.65 | 2.00±0.15 | 32.76±3.51 | 0.81±3.24 |
| | 40 | 8.50±0.26 | 5.62±0.16 | 8.04±4.74 | 0.25±3.54 |
| | 50 | 7.44±2.26 | 7.82±0.05 | 23.61±3.43 | 0.59±3.08 |

Table S.17. HPLC results in μg/g of dry weight for the water content optimization for the three best (D)ES chosen.

| **Water content (%(v/v))** | 10% | 20% | 40% | 50% |
|---|---|---|---|---|
| | **Pro:PPG (1:4)** | | | |
| **Gallic acid** | BD | BD | 6.09±0.08 | 6.07±0.01 |
| **3,4-Dihydroxybenzoic acid** | 7.58±0.22 | 8.99±0.19 | 9.97±0.07 | 10.21±0.24 |
| **Catechin** | BD | BD | 32.51±0.45 | 30.12±0.29 |
| **Caffeic acid** | BD | BD | BD | BD |
| **Syringic acid** | BD | BD | 2.45±0.14 | 2.48±0.48 |
| **Coumaric acid** | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD |
| **Salicylic acid** | 77.09±0.22 | 69.33±0.60 | 48.14±0.63 | 41.08±0.98 |
| **Quercetin** | BD | BD | BD | BD |
| | **Pro:1.2-But (1:4)** | | | |
| **Gallic acid** | 23.68±0.74 | 19.42±0.37 | 9.11±0.40 | 8.88±1.68 |
| **3,4-Dihydroxybenzoic acid** | 7.90±0.14 | 8.52±0.03 | 9.20±0.56 | 9.51±0.76 |
| **Catechin** | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD |
| **Syringic acid** | BD | BD | 2.53±0.19 | 2.52±0.31 |
| **Coumaric acid** | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD |
| **Salicylic acid** | 30.57±1.00 | 80.76±0.14 | 16.76±1.27 | 53.11±2.46 |
| **Quercetin** | 0.07±22.27 | 0.31±0.65 | 0.85±0.73 | 0.46±3.01 |
| | **ChCl:CA (2:1)** | | | |
| **Gallic acid** | 9.72±0.58 | 9.17±0.37 | 9.92±0.66 | 8.38±0.70 |

| | | | | |
|---|---|---|---|---|
| **3,4-Dihydroxybenzoic acid** | 7.40±0.77 | 7.72±0.01 | 7.80±0.14 | 8.18±0.12 |
| **Catechin** | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD |
| **Syringic acid** | BD | 2.33±0.03 | BD | BD |
| **Coumaric acid** | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD |
| **Salicylic acid** | 6.31±4.50 | 9.59±4.10 | 5.73±5.05 | 6.16±8.71 |
| **Quercetin** | BD | BD | BD | BD |

***Temperature optimization***

Table S.18. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for the temperature optimization.

| (D)ES | T (ºC) | TPC (mg/g) | TFC (mg/g) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|---|
| **Pro:PPG (1:4)** | 50 | 10.18±0.10 | 11.67±0.09 | 17.91±4.92 | 23.40±5.58 |
| | 70 | 11.84±0.36 | 15.88±0.18 | 0 | 57.55±1.18 |
| **Pro:1,2-But (1:4)** | 50 | 5.93±0.06 | 12.25±0.13 | 7.51±7.60 | 47.18±4.27 |
| | 70 | 12.29±0.05 | 21.21±0.10 | 22.44±1.32 | 0 |
| **ChCl:CA (2:1)** | 50 | 3.20±2.83 | 4.32±0.06 | 0 | 0 |
| | 70 | 7.59±1.91 | 7.63±1.91 | 15.17±0.66 | 40.93±0.56 |

Table S.19. HPLC results in μg/g of dry weight for the temperature optimization

| **Temperature (ºC)** | 50 | 70 |
|---|---|---|
| | **Pro:PPG (1:4)** | |
| **Gallic acid** | BD | BD |
| **3,4-Dihydroxybenzoic acid** | 9.24±0.03 | 9.14±0.06 |
| **Catechin** | BD | BD |
| **Caffeic acid** | BD | BD |
| **Syringic acid** | BD | BD |
| **Coumaric acid** | BD | BD |
| **Ferulic acid** | BD | BD |
| **Salicylic acid** | 27.13±0.22 | 130.97±0.30 |
| **Quercetin** | BD | BD |
| | **Pro:1.2-But (1:4)** | |
| **Gallic acid** | 14.86±0.09 | 28.07±2.12 |
| **3,4-Dihydroxybenzoic acid** | 8.97±0.06 | 9.06±0.50 |
| **Catechin** | BD | BD |
| **Caffeic acid** | BD | BD |
| **Syringic acid** | BD | 2.51±0.07 |
| **Coumaric acid** | BD | BD |
| **Ferulic acid** | BD | BD |
| **Salicylic acid** | 59.45±1.94 | 69.64±0.89 |
| **Quercetin** | BD | BD |

| | ChCl:CA (2:1) | |
|---|---|---|
| **Gallic acid** | 10.16±0.31 | 8.68±0.68 |
| **3,4-Dihydroxybenzoic acid** | 7.62±0.26 | 8.00±0.23 |
| **Catechin** | BD | BD |
| **Caffeic acid** | BD | BD |
| **Syringic acid** | BD | 2.32±0.08 |
| **Coumaric acid** | BD | BD |
| **Ferulic acid** | BD | BD |
| **Salicylic acid** | 6.06±2.25 | 7.28±6.87 |
| **Quercetin** | BD | BD |

## A.7. Intensification techniques

***UAE***

Table S.20. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for the ultrasound assisted extractions.

| (D)ES | Conditions | TPC (mg/g) | TFC (mg/g) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|---|
| **Pro:PPG (1:4)** | UAE 20W, 1,5min | 10.85±6.98 | 10.11±0.94 | 0 | 0 |
| | UAE 20W, 3min | 18.30±3.76 | 16.35±0.19 | 0 | 0 |
| | UAE 10W, 3min | 14.33±4.61 | 11.22±1.01 | 0 | 0 |
| | UAE 10W, 6min | 22.94±3.03 | 16.76±0.18 | 0 | 0 |
| | UAE 10W, 9min | 27.94±5.65 | 20.57±0.30 | 0 | 0 |
| **Pro:1.2-But (1:4)** | UAE 20W, 1,5min | 13.86±4.07 | 12.57±0.24 | 0 | 0 |
| | UAE 20W, 3min | 20.20±2.83 | 17.62±0.17 | 0 | 0 |
| | UAE 10W, 3min | 16.98±3.44 | 14.98±0.13 | 0 | 0 |
| | UAE 10W, 6min | 20.11±2.90 | 19.81±0.28 | 0 | 0 |
| | UAE 10W, 9min | 38.12±1.51 | 23.98±0.11 | 0 | 0 |
| **ChCl:CA (2:1)** | UAE 20W, 1,5min | 8.73±6.12 | 4.90±0.23 | 8.50±2.58 | 0.26±1,95 |
| | UAE 20W, 3min | 4.62±8.96 | 4.26±0.27 | 0 | 0 |
| | UAE 10W, 3min | 12.27±1.43 | 5.41±0.31 | 0 | 0 |
| | UAE 10W, 6min | 8.99±5.17 | 6.57±0.78 | 0 | 0 |
| | UAE 10W, 9min | 8.74±5.16 | 9.38±0.10 | 0 | 0 |

Table S.21. HPLC results in µg/g of dry weight for the ultrasound assisted extraction for the three best (D)ESs containing 30 %(v/v) of water.

| **UAE conditions** | 1.5 min, 20W | 3 min , 20W | 3 min, 10W | 6 min, 10W | 9 min, 10W |
|---|---|---|---|---|---|
| | **Pro:PPG (1:4)** | | | | |
| **Gallic acid** | BD | #VALOR! | BD | BD | BD |
| **3,4-Dihydroxybenzoic acid** | 7.22±0.08 | 7.33±0.09 | 7.24±0.04 | 7.33±0.20 | 7.38±0.01 |
| **Catechin** | BD | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD | BD |
| **Syringic acid** | BD | 2.44±0.13 | BD | 2.37±0.12 | 2.38±1.76 |
| **Coumaric acid** | BD | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD | BD |

| | | | | | |
|---|---|---|---|---|---|
| **Salicylic acid** | 14.23±1.24 | 29.49±3.31 | 23.42±4.99 | 130.44±1.76 | 160.30±1.76 |
| **Quercetin** | BD | BD | BD | BD | BD |
| | **Pro:1.2-But (1:4)** | | | | |
| **Gallic acid** | 17.46±0.82 | 22.45±1.10 | 18.40±0.98 | 25.26±1.18 | 30.94±0.31 |
| **3,4-Dihydroxybenzoic acid** | 7.06±0.03 | 7.22±0.01 | 7.27±0.02 | 7.21±0.03 | 7.09±0.03 |
| **Catechin** | BD | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD | BD |
| **Syringic acid** | BD | BD | 2.33±0.06 | 2.32±0.07 | 2.30±0.06 |
| **Coumaric acid** | BD | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD | BD |
| **Salicylic acid** | 21.30±2.82 | 28.54±1.25 | 18.60±0.48 | 28.66±2.16 | 29.63±1.40 |
| **Quercetin** | BD | BD | BD | BD | BD |
| | **ChCl:CA (2:1)** | | | | |
| **Gallic acid** | 6.05±0.02 | 6.08±0.03 | 6.29±0.10 | 6.20±0.05 | 6.35±0.09 |
| **3,4-Dihydroxybenzoic acid** | 7.34±0.10 | 7.45±0.03 | 7.13±0.04 | 7.40±0.11 | 7.58±0.13 |
| **Catechin** | BD | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD | BD |
| **Syringic acid** | 2.43±0.10 | 2.52±0.12 | 2.34±0.04 | 2.49±0.12 | 2.67±0.27 |
| **Coumaric acid** | BD | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD | BD |
| **Salicylic acid** | 11.31±5.77 | 18.63±0.85 | 12.24±1.24 | 21.80±1.23 | 28.26±0.70 |
| **Quercetin** | BD | BD | BD | BD | BD |

***MAE***

Table S.22. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for the microwave assisted extractions.

| (D)ES | Conditions | TPC (mg/g) | TFC (mg/g) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|---|
| **Pro:PPG (1:4)** | MAE 100 ºC, 1,5min | 22.59±2.55 | 23.41±0.25 | 0 | 0 |
| | MAE 100 ºC, 3min | 20.86±2.86 | 18.78±0.03 | 0 | 0 |
| | MAE 100 ºC, 6min | 24.43±2.36 | 21.77±0.61 | 12.30±2.65 | 0.34±2.17 |
| | MAE 60 ºC, 3min | 9.89±5.78 | 8.41±0.33 | 0 | 0 |
| | MAE 60 ºC, 6min | 12.28±4.89 | 11.79±0.36 | 0 | 0 |
| | MAE 60 ºC, 9min | 13.29±4.30 | 9.07±0.14 | 0 | 0 |
| **Pro:1,2-But (1:4)** | MAE 100 ºC, 1,5min | 17.82±3.35 | 19.25±0.05 | 0 | 0 |
| | MAE 100 ºC, 3min | 23.86±2.43 | 19.88±0.56 | 0 | 0 |
| | MAE 100 ºC, 6min | 19.96±2.96 | 18.96±0.27 | 19.26±0.68 | 0.50±0.60 |
| | MAE 60 ºC, 3min | 12.91±4.43 | 9.02±0.17 | 0 | 0 |
| | MAE 60 ºC, 6min | 9.31±6.17 | 7.71±0.40 | 0 | 0 |
| | MAE 60 ºC, 9min | 8.70±6.80 | 11.29±0.22 | 0 | 0 |
| **ChCl:CA (2:1)** | MAE 100 ºC, 1,5min | 4.71±9.43 | 8.85±0.13 | 0.28±52.22 | 0.07±4.92 |
| | MAE 100 ºC, 3min | 6.79±7.30 | 8.65±0.16 | 0 | 0 |
| | MAE 100 ºC 6min | 2.71±0.80 | 11.02±0.18 | 31.33±0.21 | 0.77±0.19 |
| | MAE 60 ºC, 3min | 3.90±12.64 | 3.55±0.15 | 11.50±5.30 | 0.32±4.29 |

| | | | | | |
|---|---|---|---|---|---|
| | MAE 60 ºC, 6min | 4.24±11.14 | 3.56±0.11 | 14.85±3.03 | 0.40±2.56 |
| | MAE 60 ºC, 9min | 4.05±11.80 | 3.91±0.13 | 7.45±14.81 | 0.23±10.85 |
| | MAE 60 ºC, 12min | 1.66±26.17 | 3.99±0.20 | 28.54±3.97 | 0.70±3.62 |

Table S.23. HPLC results in μg/g of dry weight for the microwave assisted extractions for the three best (D)ESs.

| **MAE conditions** | 1.5 min, 100 ºC | 3 min , 100 ºC | 6 min, 100 ºC | 3 min, 60 ºC | 6 min, 60 ºC | 9 min, 60 ºC | 12 min, 60 ºC |
|---|---|---|---|---|---|---|---|
| | | | | **Pro:PPG (1:4)** | | | |
| **Gallic acid** | BD | BD | 6.60±0.18 | BD | BD | BD | -- |
| **3,4-Dihydroxybenzoic acid** | 7.75±0.05 | 7.52±0.04 | 7.58±0.21 | 7.18±0.08 | 7.32±0.14 | 7.17±0.06 | -- |
| **Catechin** | BD | BD | 10.09±1.40 | BD | 3.90±0.30 | BD | -- |
| **Caffeic acid** | BD | BD | BD | BD | BD | BD | -- |
| **Syringic acid** | BD | BD | BD | BD | BD | BD | -- |
| **Coumaric acid** | BD | BD | BD | BD | BD | BD | -- |
| **Ferulic acid** | BD | BD | BD | BD | BD | BD | -- |
| **Salicylic acid** | 170.92±2.28 | 190.87±3.25 | 261.14±0.63 | 16.69±0.30 | 26.94±0.42 | 19.57±0.61 | -- |
| **Quercetin** | BD | BD | BD | BD | BD | BD | -- |
| | | | | **Pro:1.2-But (1:4)** | | | |
| **Gallic acid** | 27.51±0.27 | 31.65±0.83 | 40.56±0.40 | 18.56±0.22 | 17.51±0.95 | 13.48±0.03 | -- |
| **3,4-Dihydroxybenzoic acid** | 7.55±0.18 | 7.44±0.10 | 7.59±2.61 | 7.08±0.02 | 7.17±0.06 | 7.22±0.02 | -- |
| **Catechin** | BD | BD | BD | BD | BD | BD | -- |
| **Caffeic acid** | BD | BD | BD | BD | BD | BD | -- |
| **Syringic acid** | BD | BD | 2.65±0.10 | BD | BD | BD | -- |
| **Coumaric acid** | BD | BD | BD | BD | BD | BD | -- |
| **Ferulic acid** | BD | BD | BD | BD | BD | BD | -- |
| **Salicylic acid** | 25.84±3.39 | 24.59±0.51 | 25.92±2.57 | 3.98±3.29 | 26.29±0.25 | 24.37±1.27 | -- |
| **Quercetin** | BD | BD | BD | BD | BD | BD | -- |
| | | | | **ChCl:CA (2:1)** | | | |
| **Gallic acid** | 6.25±0.05 | BD | 7.68±0.74 | 6.08±0.17 | 6.09±0.10 | 6.06±0.03 | 6.64±0.13 |
| **3,4-Dihydroxybenzoic acid** | 7.70±0.19 | 7.89±0.06 | 8.68±0.19 | 7.46±0.14 | 7.35±0.20 | 7.46±0.16 | 7.70±0.12 |
| **Catechin** | BD | BD | BD | BD | BD | BD | BD |
| **Caffeic acid** | BD | BD | BD | BD | BD | BD | BD |
| **Syringic acid** | BD | 2.57±0.07 | BD | 2.86±0.39 | 2.33±0.09 | 2.41±0.47 | 2.66±0.28 |
| **Coumaric acid** | BD | BD | BD | BD | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD | BD | BD | BD | BD |
| **Salicylic acid** | 26.19±2.40 | 10.67±2.39 | 23.44±1.20 | 18.47±2.17 | 14.56±0.40 | 16.74±0.99 | 8.77±2.38 |
| **Quercetin** | 0.34±1.77 | BD | 0.38±4.33 | BD | BD | BD | BD |

## A.8. Extraction with cycles

Table S.24. Results of the total phenolic content (TPC), total flavonoids content (TFC) and the antioxidant activity in inhibition percentage for the extractions performed with cycles.

| 3rd Cycle | TPC (mg/L) | TFC (mg/L) | %Inhibition | TEAC (mg/g) |
|---|---|---|---|---|
| **w/out combination** | 5398.66±1.29 | 5469.09±0.04 | 13.53±0.98 | 37.18±0.82 |
| **Combination w/ cycles** | 6649.71±1.03 | 6012.84±0.09 | 0 | 0 |

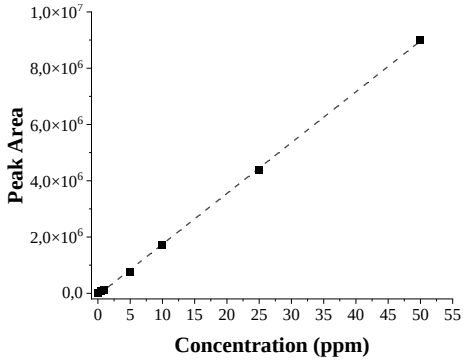
Table S.25. HPLC results in mg/L of the extraction cycles performed without combination of extracts between the cycles.

| **Cycle** | 1 | 2 | 3 |
|---|---|---|---|
| **S:L** | 1.3 g/13 mL | 0.8 g/8 mL | 0.45 g/4.5 mL |
| **Gallic acid** | 0.63±0.14 | 0.64±0.20 | 0.79±0.85 |
| **3,4-Dihydroxybenzoic acid** | 0.76±0.22 | 0.80±0.18 | 0.72±0.26 |
| **Catechin** | BD | BD | 2.29±1.06 |
| **Caffeic acid** | BD | BD | BD |
| **Syringic acid** | 0.23±0.22 | 0.24±0.17 | 0.31±0.57 |
| **Coumaric acid** | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD |
| **Salicylic acid** | 4.45±2.92 | 7.49±0.64 | 72.16±0.59 |
| **Quercetin** | BD | #VALOR! | 0.02±28.77 |

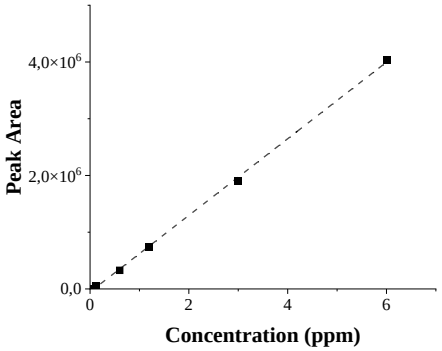
Table S.26. HPLC results in mg/L of the extraction cycles performed with the combination of extracts between the cycles.

| **Cycle** | 1 | 2 | 3 |
|---|---|---|---|
| **S:L** | 3x0,5g/5mL | 2x0,4g/4mL | 0,46g/4.6mL |
| **Gallic acid** | **BD** | 0.62±0.11 | 0.73±1.68 |
| **3,4-Dihydroxybenzoic acid** | 0.75±0.21 | **BD** | **BD** |
| **Catechin** | **2.59**±2.21 | BD | 0.43±6.66 |
| **Caffeic acid** | BD | BD | BD |
| **Syringic acid** | 0.25±0.53 | 0.26±0.50 | 0.59±1.08 |
| **Coumaric acid** | BD | BD | BD |
| **Ferulic acid** | BD | BD | BD |
| **Salicylic acid** | 44.11±1.09 | 81.93±0.29 | 156.30±0.39 |
| **Quercetin** | BD | 0.02±8-85 | 0.06±12.36 |

## B. Fucoxanthin




(a) For higher concentrations: $y = (180.57 \pm 3.71) \times 10^3 x + (-6.17 \pm 8.00) \times 10^4$ with $R^2 = 0.9997$




(b) For lower concentrations: $y = (67.50 \pm 2.76) \times 10^4 x + (-5.31 \pm 87.71) \times 10^4$ with $R^2 = 0.998$

Figure S.11. Calibration curves for fucoxanthin in HPLC.

### B.1. COSMO-RS

To understand the determination of the activity coefficients it is important to have an idea about the chemical potential of a certain compound B where its chemical potential is $\mu_B$ and can be determined with expression (S.4), ${\mu_B}^o$ is the standard chemical potential of the gas.

$$\underbrace{\mu_B(l)}_{\text{Liquid}} = \underbrace{{\mu_B}^o(g) + RTln(p_B)}_{\text{Vapour}} \tag{S.4}$$

The chemical potential of an ideal solution given by equation (S.5) can be then determined by implying the Raoult's law, equation (S.6), where ${\mu_B}^*$ is the chemical potential of the pure compound B and $x_B$ is the molar fraction of compound B.

$$\underbrace{\mu_B(l)}_{\text{Liquid}} = \underbrace{{\mu_B}^*(l) + RTln(x_B)}_{\text{Vapor}} \tag{S.5}$$

$$p_B = x_B \cdot {p_B}^* \tag{S.6}$$

The calculation of the activity coefficient ($\gamma_i$) is possible through the pseudo-chemical potential of a certain compound in in the liquid phase, equation (S.7) where ${\mu_B}^{solv}$ is the pseudo-chemical potential of compound B in a liquid solution, ${\mu_B}^{pure}$ is the pseudo-chemical potential of the pure compound B in the liquid phase, R is the gas constant and T the temperature [129].

$$\ln(\gamma_B) = \frac{{\mu_B}^{solv} - {\mu_B}^{pure}}{RT} \tag{S.7}$$

According to Henry's law, given by expression (S.8) the vapor pressure of the solute B ($p_B$) is directly proportional to content of the solute in solution ($x_B$). Note that the Henry's law is for ideal-dilute solutions, so the Henry's law is valid for $x_B \to 0$. Rearranging the equation (S.8) in order to $x_B$ can be seen that $H_B$ and $x_B$ are inversely proportional to each other, this means that a lower henry's constant returns a higher solubility of compound B.

$$p_B = H_B \cdot x_B \tag{S.8}$$

The values of Henry's law constant ($H_B$) depends on the temperature and can be derived from the equality of equations (S.4) and (S.5), arriving to (S.9).

$$H_B = exp\left(\frac{{\mu_B}^* - {\mu_B}^o}{RT}\right) \quad \text{(S.9)}$$

Comparing equation (S.7) and (S.9) they are similar and acknowledging that the solubility of a certain compound in a certain solvent can be predicted in COSMO-RS by knowing the $\gamma_i$. Concluding, lower activity coefficient means a higher solubility or the more negative the ln ($\gamma_B$) higher the solubility.

# C. Statistical treatment of results

## C.1. (D)ES screening

The Shapiro-Wilk test allows to evaluate the distribution if it is normal or not. For the data to be normally distributed the sigma has to be higher than 0.05, only in ChCl:1,3-But (1:4) and in ChCl:LA (1:3) the sigma is bellow of 0.05, so this means that only to these two (D)ES the data is not normal distributed [158,159].

Table S.27. Shapiro-Wilk test to evaluate the distribution of the data.
*non-normally distributed

| | df | Sig. |
|---|---|---|
| **Ethanol** | 3 | 0,975 |
| **ChCl:1.2-But (1:3)** | 3 | 0,289 |
| **ChCl:PPG (1:4)** | 3 | 0,206 |
| **ChCl:1.3-But (1:4)*** | 3 | 0,025 |
| **Pro:1.2-But (1:4)** | 3 | 0,331 |
| **Pro:1.3-But (1:4)** | 3 | 0,486 |
| **Bet:1.2-But (1:3)** | 3 | 0,137 |
| **Bet:1.3-But (1:4)** | 3 | 0,405 |
| **ChCl:LA (1:1)** | 3 | 0,801 |
| **ChCl:LA (1:2)** | 3 | 0,882 |
| **ChCl:LA (1:3)*** | 3 | 0,006 |
| **Bet:Gli (1:4)** | 3 | 0,991 |
| **ChCl:CA (2:1)** | 3 | 0,634 |
| **ChCl:CA (1:1)** | 3 | 0,301 |
| **ChCl:CA (1:2)** | 3 | 0,204 |
| **Bet:LA (1:1)** | 3 | 0,298 |
| **Bet:LA (1:2)** | 3 | 0,429 |
| **Bet:LA (1:3)** | 3 | 0,992 |
| **Pro:LA (1:1)** | 3 | 0,432 |
| **Pro:LA (1:2)** | 3 | 0,717 |
| **Pro:LA (1:3)** | 3 | 0,896 |
| **Bet:1.2-But (1:4)** | 3 | 0,967 |
| **Water** | 3 | 0,041 |
| **Bet:PPG (1:4)** | 3 | 0,961 |
| **Pro:Gli (1:4)** | 3 | 0,369 |

| | | |
|---|---|---|
| **Pro:1.2-But (1:3)** | 3 | 0,422 |
| **Pro:PPG (1:4)** | 3 | 0,202 |
| **ChCl:Gli (1:4)** | 3 | 0,179 |

Levene's test evaluates the homoscedasticity of a certain dataset, more in concrete if the data has equal variances or not. Equal variances means that the data is homogenous in case they have different variances they are considered heterogeneous. According with Levene's test if sigma is bigger than 0.05 the data are homogeneous, on the other hand, if sigma is lower than 0.05 the data is considered heterogeneous. It is notable with TABLE S that the data is not homogeneous [160].

Table S.28. Levene's Test of Equality of Error Variances[a,b]

| | | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| **Extraction yield** | Based on Mean | 4,823 | 27 | 56 | 0,000 |
| | Based on Median | 1,970 | 27 | 56 | 0,016 |
| | Based on Median and with adjusted df | 1,970 | 27 | 7,573 | 0,168 |
| | Based on trimmed mean | 4,607 | 27 | 56 | 0,000 |

Tests the null hypothesis that the error variance of the dependent variable is equal across groups.

a. Dependent variable: extraction yield

b. Design: Intercept + solvent

Table S. 29. Tukey HSD test for (D)ES screening.

| | A | B | C | D | E | F | G | H | I | J | K | L | M | N |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ChCl:Gli (1:4)** | 12,82 | | | | | | | | | | | | | |
| **Bet:PPG (1:4)** | | 17,37 | | | | | | | | | | | | |
| **ChCl:PPG (1:4)** | | 17,49 | | | | | | | | | | | | |
| **Ethanol** | | 19,50 | | | | | | | | | | | | |
| **Bet:Gli (1:4)** | | | 23,05 | | | | | | | | | | | |
| **Water** | | | 23,36 | | | | | | | | | | | |
| **Bet:LA (1:1)** | | | | 26,88 | | | | | | | | | | |
| **Pro:Gli (1:4)** | | | | 29,31 | | | | | | | | | | |
| **Bet:1.2-But (1:3)** | | | | | 32,75 | | | | | | | | | |
| **ChCl:1.3-But (1:4)** | | | | | 33,08 | | | | | | | | | |
| **Bet:LA (1:2)** | | | | | 33,97 | | | | | | | | | |
| **Pro:LA (1:1)** | | | | | | 37,97 | | | | | | | | |
| **Pro:LA (1:2)** | | | | | | 38,99 | | | | | | | | |
| **ChCl:LA (1:1)** | | | | | | 39,54 | | | | | | | | |
| **Pro:1.2-But (1:3)** | | | | | | 40,48 | 40,48 | | | | | | | |
| **ChCl:LA (1:3)** | | | | | | | 43,09 | 43,09 | | | | | | |
| **Pro:1.3-But (1:4)** | | | | | | | 43,18 | 43,18 | | | | | | |
| **Bet:1.3-But (1:4)** | | | | | | | | 44,05 | | | | | | |
| **ChCl:1.2-But (1:3)** | | | | | | | | 44,85 | 44,85 | | | | | |
| **Bet:LA (1:3)** | | | | | | | | | 47,87 | 47,87 | | | | |
| **Pro:LA (1:3)** | | | | | | | | | | 49,71 | | | | |
| **Bet:1.2-But (1:4)** | | | | | | | | | | | 53,05 | | | |
| **ChCl:LA (1:2)** | | | | | | | | | | | 55,50 | | | |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **ChCl:CA (1:2)** | | | | | | | | | | | 55,53 | | | |
| **ChCl:CA (2:1)** | | | | | | | | | | | | 68,11 | | |
| **ChCl:CA (1:1)** | | | | | | | | | | | | 70,01 | | |
| **Pro:1.2-But (1:4)** | | | | | | | | | | | | | 119,81 | |
| **Pro:PPG (1:4)** | | | | | | | | | | | | | | 210.48 |
| **Sig.** | 1 | 0.73 | 1 | 0.48 | 1.00 | 0.41 | 0.28 | 0.94 | 0.12 | 0.91 | 0.44 | 0.88 | 1 | 1 |

Table S.30. Post hoc test proposed by Herberich et al. [140]. Only are present the pairwise comparison that are not significantly different.

| | | **Sig.** |
|---|---|---|
| ChCl:1.2-But (1:3) | Pro:1.3-But (1:4) | 0,99 |
| ChCl:1.2-But (1:3) | Bet:1.3-But (1:4) | 0,99 |
| Pro:1.3-But (1:4) | Bet:1.3-But (1:4) | 1 |
| Pro:1.3-But (1:4) | ChCl:LA (1:1) | 0,32 |
| Pro:1.3-But (1:4) | ChCl:LA (1:3) | 1 |
| Pro:1.3-But (1:4) | Pro:LA (1:2) | 0,12 |
| ChCl:LA (1:2) | ChCl:CA (1:2) | 1 |
| ChCl:LA (1:2) | Bet:LA (1:3) | 0,27 |
| ChCl:LA (1:2) | Pro:LA (1:3) | 0,78 |
| Bet:Gli (1:4) | Water | 1 |
| Pro:1.2-But (1:3) | Pro:1.3-But (1:4) | 0,84 |
| Bet:1.2-But (1:4) | ChCl:LA (1:2) | 1 |
| Bet:1.2-But (1:4) | ChCl:CA (1:2) | 0,99 |
| Bet:1.2-But (1:4) | Bet:LA (1:3) | 0,14 |
| Bet:1.2-But (1:4) | Pro:LA (1:3) | 0,89 |
| ChCl:1.3-But (1:4) | Bet:1.2-But (1:3) | 0,99 |
| Bet:1.3-But (1:4) | ChCl:LA (1:3) | 0,99 |
| ChCl:LA (1:1) | Pro:LA (1:2) | 0,16 |

## C.2. UAE

Table S.31. Tukey HSD test for UAE using Pro:PPG (1:4).

| **UAE** | **N** | **Subset for alpha = 0.05** | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| **20W, 1,5min** | 3 | 21.4507 | | | | |
| **20W, 3min** | 3 | | | 39.2617 | | |
| **10W, 3min** | 3 | | 30.6597 | | | |
| **10W, 6min** | 3 | | | | 140.1407 | |
| **10W, 9min** | 3 | | | | | 170.0563 |
| **Sig.** | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000; b. Alpha = .05.

Table S.32. Tukey HSD test for UAE using Pro:1,2-But (1:4).

| UAE | N | Subset | | | |
|---|---|---|---|---|---|
| | | A | B | C | D |
| **20W, 1,5min** | 3 | 45.8200 | | | |
| **20W, 3min** | 3 | | 58.2137 | | |
| **10W, 3min** | 3 | 46.5990 | | | |
| **10W, 6min** | 3 | | | 63.4497 | |
| **10W, 9min** | 3 | | | | 69.9553 |
| **Sig.** | | 0.459 | 1.000 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000; b. Alpha = .05.

Table S.33. Tukey HSD test for UAE using ChCl:CA (2:1).

| UAE | N | Subset | | |
|---|---|---|---|---|
| | | A | B | D |
| **20W, 1,5min** | 3 | 27.1100 | | |
| **20W, 3min** | 3 | | 34.6827 | |
| **10W, 3min** | 3 | 27.9957 | | |
| **10W, 6min** | 3 | | | |
| **10W, 9min** | 3 | | | 44.8553 |
| **Sig.** | | 0.101 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000;. b. Alpha = .05

## C.3. MAE

Table S.34. Tukey HSD test for MAE using Pro:PPG (1:4).

| MAE | N | Subset | | | | |
|---|---|---|---|---|---|---|
| | | A | B | C | D | E |
| **60 ºC, 3min** | 3 | 23.8740 | | | | |
| **60 ºC, 9min** | 3 | 26.7358 | | | | |
| **60 ºC, 6min** | 3 | | 38.1610 | | | |
| **100 ºC, 1,5min** | 3 | | | 178.6694 | | |
| **100 ºC, 3min** | 3 | | | | 198.3911 | |
| **100 ºC, 6min** | 3 | | | | | 288.3076 |
| **Sig.** | | 0.852 | 1.000 | 1.000 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000; b. Alpha = .05.

Table S. 35. Tukey HSD test for MAE using Pro:1,2-But (1:4).

| MAE | N | Subset | | | | | |
|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F |
| **100 ºC, 1,5min** | 3 | 60.8991 | | | | | |
| **100 ºC, 3min** | 3 | | 63.6823 | | | | |
| **100 ºC, 6min** | 3 | | | 76.7210 | | | |
| **60 ºC, 3min** | 3 | | | | 29.6155 | | |
| **60 ºC, 6min** | 3 | | | | | 50.9728 | |
| **60 ºC, 9min** | 3 | | | | | | 45.0595 |
| **Sig.** | | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000. b. Alpha = .05.

Table S.36. Tukey HSD test for MAE using ChCl:CA (2:1).

| MAE | N | Subset | | | | | |
|---|---|---|---|---|---|---|---|
| | | A | B | C | D | E | F |
| **100 ºC, 1,5min** | 3 | 40.1427 | | | | | |
| **100 ºC, 3min** | 3 | | 21.1369 | | | | |
| **100 ºC, 6min** | 3 | 39.7978 | | | | | |
| **60 ºC, 3min** | 3 | | | 34.8743 | | | |
| **60 ºC, 6min** | 3 | | | | 30.3256 | | |
| **60 ºC, 9min** | 3 | | | | | 32.6631 | |
| **60 ºC, 12min** | 3 | | | | | | 25.7728 |
| **Sig.** | | 0.858 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

a. Uses Harmonic Mean Sample Size = 3.000. b. Alpha = .05.