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# Red Seaweeds as a Source of Nutrients and Bioactive Compounds: Optimization of the Extraction

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**Abstract:** The present work aimed to determine the nutritional composition (ash, protein, fat, carbohydrate content and energy value), phenolic compounds, pigments and organic acids content of three typical red algae from the Northwest of Spain: *Chondrus crispus*, *Mastocarpus stellatus*, and *Gigartina pistillata*; as well as their antioxidant and antimicrobial activities. Furthermore, the present work compared two extraction techniques: conventional heat assisted extraction (HAE) and high pressure assisted extraction (HPAE) to maximize the yield and the concentration of target compounds. Different independent variables were considered for the response study. Time (*t*) and percentage of ethanol of the solvent (*S*) were chosen for both techniques and temperature (*T*) and pressure (*P*) were used for HAE and HPAE, respectively. The experiments were designed following a response surface methodology (*RSM*) approach. The obtained results showed a similar nutritional composition between algae samples: low-fat content and high content of proteins, carbohydrates and energy. All tested algae showed good antioxidant and antimicrobial properties. Finally, HEA demonstrated to be the most efficient extraction technique. This study confirms the potential of red algae to be part of the human diet as a source of non-animal protein, due to its nutritional content, phenolic profile, pigments concentration and bioactive properties, which proves that HAE is the optimum technique for the extraction maximization.

**Keywords:** red algae; nutritional value; chemical characterization; bioactivities



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## 1. Introduction

Marine algae, macroalgae or seaweeds, are pluricellular photosynthetic organisms responsible for the primary production in marine ecosystems They provide oxygen and they are considered as food, substrate and shelter sources for other species, constituting an important basis of marine biodiversity [1].

Algae have been consumed as food since ancient times, especially in Asian regions such as China, Japan or Korea [2]. From a nutritional point of view, they are characterized by a high content of carbohydrates (<60%) and proteins (17–44%), a low percentage of lipids (<4.5%) and a high presence of other micronutrients, such as vitamins, pigments and minerals [3]. Their nutritional composition, together with recent studies about their health beneficial properties, has justified the growing demand for incorporating algae into the human diet [4–7]. Likewise, different studies justify algae potential application as additives for the food (both animal or human), pharmaceutic and cosmetic industry

due to the presence of bioactive compounds with different beneficial properties [4,8–10]. Among them, sulfated polysaccharides (SP) such as agar, alginate or carrageenan, are highlighted since they are frequently used as food additives and they are currently the most valuable compounds derived from algae [4,9]. However, even though they own their importance to their stabilizing, thickening or jellifying properties, it has been also addressed that SP extracted from algae possess biological properties such as antioxidant, anti-inflammatory, anticoagulant or anti-tumor, among others. Algae are also a source of polyunsaturated fatty acids (PUFAs) and omega 3 (ω-3), which are imperative for the diet and beneficial for consumers' health due to their anti-inflammatory properties [11,12]. Besides the contribution of algae to the diet and their richness in bioactive compounds, they have shown other applications. Nowadays, some of the aforementioned compounds have been included in pharmaceutical or cosmetic products [2], and in aquaculture sector, these matrices can be used in feed formulations as bioactive ingredients [12].

In the last decades, marine algae production has experienced a noticeable increase due to their wide diversity of applications. Algae production has increased during the period of 2000 to 2018, from $10.6 \times 10^6$ to $32.4 \times 10^6$ tons, reaching a primary sell value estimated on $13.3 \times 10^9$ USD [2]. This increment is mostly associated to the cultivation of red algae (RA) to produce carrageenans. While the production of other algae has remained quite stable. In respect of the main producers, it is estimated that China and Indonesia produced the 86.6% of the world volume in 2016 [2].

Marine algae are usually classified into three groups according to their main pigments in green, brown and red algae (phyla Chlorophyta, Phaeophyta and Rhodophyta, respectively) [12–14]. Rhodophyta represents the largest phylum among algae in terms of number and diversity of species [5,15]. These organisms contain high amounts of pigments such as chlorophyll (*a* and *d*), carotenoids (β-carotene, fucoxanthin, astaxanthin, xanthophyll) and other pigments such as phycoerythrin, phycocyanin and allophycocyanin [5]. In respect of their nutritional interest, RA usually present higher content of proteins, carbohydrates and minerals than green and brown algae [13,16,17]. Their incorporation into the diet is mainly due to their contribution of micronutrients (iron, calcium, iodine, potassium, selenium), vitamins (A, C and B-12) and their content in natural long chain fatty acids such as ω-3 [2]. On the other hand, numerous studies have confirmed that RA present high contents of bioactive compounds (~1600 described) [18]. Regarding their properties, antioxidant, anti-inflammatory, anti-tumor, antiviral, anti-hyperlipidemic, anticancer and immunomodulatory activities have been described both on in vitro and in vivo experiments [5,19–21]. In Galicia (Spain), some of the most representative species are *Chondrus crispus*, *Gigartina pistillata* and *Mastocarpus stellatus*, commonly known as Irish mosh, alginate and cats' puff or false Irish mosh, respectively [22]. The three species belong to the Gigartinales order and are in rocky areas, sub- or infra-littoral, forming grass in areas protected from light. The chemical composition of these algae has been previously studied by different authors [19,23–26], as well as their biological properties including anti-tumor [27], antioxidant [25,28], antimicrobial [29,30] or antiviral [31].

Heat assisted extraction (HAE) is a conventional solid-used extraction method that has been used to obtain biological compounds from plant matrices. The procedure consists of subjecting the sample together with the solvent to stirring, under specific conditions of time and temperature. It is a relatively simple technique, with few requirements in terms of equipment, however it can entail high energy costs. On the other hand, advances in green technologies are currently offering a wide spectrum of solid-liquid procedures useful for the extraction of compounds of interest. Some of these emerging technologies include microwave assisted extraction, supercritical assisted extraction, ultrasound assisted extraction, high pressure assisted extraction (HPAE) or pressurized liquid assisted extraction [32–34]. In this case, HPAE was selected as an alternative technique, since it has been reported as a suitable technique for the extraction of compounds of interest (e.g., phenolic compounds or anthocyanins) due to its ability to induce structural changes (e.g., cell walls and membranes rupture) and, therefore, facilitate the diffusion of secondary metabolites.

HPAE has been recognized as a less energy-demanding technique. Also, high pressure can increase the solubility of the compounds allowing to work at room temperature thus avoiding the possible degradation of heat-sensitive compounds. In addition, it allows to reduce extraction time and provides higher extraction yields [35–37].

The present work attempted to study three of the most representative and commercially valued edible RA species from the Galician coast (*Chondrus crispus*, *Gigartina pistillata* and *Mastocarpus stellatus*) to evaluate their nutritional and chemical composition, namely, phenolic compounds (PC) and pigments. Subsequently, an optimization process was carried out to maximize the obtaining of extracts rich in bioactive compounds from the target species, using two extraction techniques: HAE and HPAE. For this purpose, a response surface methodology (RSM) was applied using the circumscribed central composite design (CCCD). Furthermore, the antioxidant and antimicrobial activity of the extracts were analyzed. This work aims to provide a better understanding of the potential of the extraction techniques and the potential of these three RA species as a source of bioactive compounds.

## 2. Materials and Methods

### *2.1. Preparation of the Samples*

Wild samples of *Chondrus crispus* (CC), *Mastocarpus stellatus* (ME) and *Gigartina pistillata* (GP) were collected from the natural environment along the Pontevedra coasts (June 2019, Galicia, Spain). The algae were then manually washed with distilled water to separate other algae and animals that may have remained adhere to their surface. Samples were lyophilized (LyoAlfa10/15, Telstar, Thermo Fisher Scientific, Madrid, Spain, reduced to fine dried homogeneous powder (~20 mesh) and stored (−20 °C) protected from light, until further analysis.

### *2.2. Nutritional and Chemical Characterization*

According to the AOAC methods [38], protein, fat, carbohydrates and ash contents were determined on the three selected macroalgae species. Total carbohydrates were calculated by difference and energetic value was calculated using Equation (1):

$$Energy\ (kcal) = 4 \times (g\ protein + g\ carbohydrates) + 9 \times (g\ fat) \quad (1)$$

The organic acids content of the three macroalgae samples was determined using an Ultra-Fast Liquid Chromatography (UFLC, Shimadzu 20A series, Kyoto, Japan) coupled to a photodiode array detector [39]. A SphereClone reverse phase C18 column (5 µm, 250 mm × 4.6 mm i.d., Phenomenex, Torrance, CA, USA) was used for separation at 35 °C. The mobile phase was sulfuric acid 3.6 mM at a flow rate of 0.8 mL/min. To quantify the compounds, calibration curves plotted using commercial standards (L-(+)-ascorbic acid, citric acid, malic acid, oxalic acid, shikimic acid, succinic acid, fumaric acid and quinic acid) procured from Sigma-Aldrich (St. Louis, MO, USA) were used. All tests were carried out in duplicate, expressed in terms of mean ± standard deviation (SD) and expressed in g per 100 g of dry weight (dw).

### *2.3. Optimization and Comparison of Extractive Techniques (HAE and HPAE)*

#### 2.3.1. Extraction Techniques

Heat Assisted Extraction (HAE)

For this assay, 600 mg dw of each sample were weighed and placed in flasks with 20 mL of solvent (acidified with 0.05% HCl), to obtain a solid/liquid ratio (*S/L*, grams of algae/liter of solvent, constituted by an hydroalcoholic mixture) of 30 g/L. They were placed in a water bath and stirred (at 500 rpm), using a magnetic stirrer. The variables and ranges evaluated were time ($t$ or $X_1$, 19.5 to 120.5 min), temperature ($T$ or $X_2$, 21.4 to 88.6 °C) and percentage of ethanol of the solvent ($S$ or $X_3$, 0 to 100%) (Table 1).

**Table 1.** Experimental domains and coding of the independent variables in the CCCD factorial design with five levels of values.

| Coded Values | Natural Values | | | | | |
|---|---|---|---|---|---|---|
| | HAE | | | HPAE | | |
| | *t* (min) | *T* (°C) | *S* (%) | *t* (min) | *P* (MPa) | *S* (%) |
| **−1.68** | 19.5 | 21,4 | 0 | 10 | 100 | 0 |
| **−1** | 40 | 35 | 20.3 | 30.3 | 201.3 | 20.3 |
| **0** | 70 | 55 | 50 | 60 | 350 | 50 |
| **+1** | 100 | 75 | 79.8 | 89.7 | 498.7 | 79.8 |
| **+1.68** | 120.5 | 88,6 | 100 | 110 | 600 | 100 |

High Pressure Assisted Extraction (HPAE)

This extraction was carried out in a 'FOOD-LAB' model S-FL-850-9-W high hydrostatic pressure research device (Stansted, Thermo Fisher Scientific, Madrid, Spain). In this case, 1.5 g dw of each sample were extracted in 50 mL of solvent (acidified with 0.05% HCl), to maintain the same *S/L* ratio of 30 g/L. In this case, the independent variables were time (*t* or $X_1$, 10–110 min), pressure (*P* or $X_2$, 100–600 MPa) and percentage of ethanol of the solvent (*S* or $X_3$, 0–110%) (Table 1) [40].

2.3.2. Experimental Design, Analysis Model and Statistic Evaluation

Experimental Design

To design the experiment, a series of single-variable experiments and a literature review were first conducted. Then, the most relevant variables for each extraction technique were selected along with their appropriate ranges. Table 1 contains a detailed description of the ranges analyzed for each variable and extraction technique. Considering that the *S/L* remained constant (30 g/L) in both cases. To obtain the conditions that would maximize the obtaining of extracts rich in bioactive compounds, an RSM methodology was used with a CCCD of three variables [41]. The interaction between the different variables generates a total of 28 combinations of responses. Among these, six are replicas at the central point of the experiment while the others are independent experimental points located around the center in a spherical arrangement. This central point is assumed to be close to the optimal position for the response; therefore, it is repeated to maximize the prediction. The experimental trials were randomized to minimize unpredictable effects on the observed responses. Table 1 shows the experimental domains tested and the coding of the variables tested to calculate the design distribution.

Maximized Response

Responses were measured in terms of the extraction yields of each alga under study ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$) for each of the extraction techniques used (HAE and HPAE) through the study of dw. To calculate the weight, 30 mL were placed in crucibles in an oven at 104 °C, 1–2 h, they were cooled and weighed gravimetrically. Next, 5 mL of the extracted solution were added and they were dried in an oven at 104 °C for 24 h. After that time, they were dried in the desiccator, allowed to cool and weighed. The value of dw was obtained by difference of the two weight values. Finally, by proportionality, the value of dw was calculated for the total sample (mg extract/g dried seaweed).

Mathematical Model

The RSM models were fitted by calculating least-squares using the following second-order polynomial model from Equation (2):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ij} {X_i}^2 \quad (2)$$

where $Y$ defines the dependent variable (response variable), $X_i$ and $X_j$ are the independent variables, $b_0$ is the constant coefficient, $b_i$ is the linear effect coefficient, $b_{ij}$ is the interaction effect coefficient, $b_{ii}$ the quadratic effect coefficients and $n$ is the number of variables.

Procedure to Optimize the Variables to Their Maximum Response

For the optimization of the extraction, the responses produced by the model were maximized. A simplex method was used to solve nonlinear problems [42]. Coded values were limited to avoid unnatural conditions (e.g., $t > 0$).

### *2.4. Bioactive Evaluation*

#### 2.4.1. Extract Preparation

Considering the data obtained for the extraction optimization of the three red algae, the HAE method was chosen with the optimal global conditions that maximized the response to obtain the extracts (*36.6 min, *88.6 °C and *50.0% acidified ethanol). So, 1 g of each dried macroalgae sample was stirred at these conditions. The alcoholic fraction of the extracts was removed in a rotary evaporator at 40 °C. The resultant aqueous phase was frozen, lyophilized and stored to obtain a dry extract and further evaluate its composition and biological properties.

#### 2.4.2. Identification and Quantification of Phenolic Compounds

10 mg of lyophilized sample were dissolved in 2 mL of water/methanol (80:20, *v*/*v*) to obtain a 5 mg/mL concentration solution. This solution was filtered through 0.22 µm nylon for chromatographic analysis [43]. The analysis of the PC was carried out using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS, 1260 Series, Agilent). The results were expressed in mg/g of dw.

#### 2.4.3. Identification and Quantification of Main Pigments

For this assay, 1 g of lyophilized sample were dissolved in 20 mL of ethanol/water (50:50 v:v) to obtain a concentration of 50 mg/mL. The solution was transferred to an amber vial and subsequently kept stirring at 50 °C and 200 rpm. After 24 h of extraction, the supernatant was removed by centrifugation and the sample was re-extracted with 10 mL of fresh solvent and kept in agitation for 3 h under the same conditions. After the extraction was complete, the remaining solvent was recovered by centrifugation (8 min, 4800 rpm) and the supernatant was removed. The solutions obtained were stored in a refrigerator at −20 °C until rotary evaporated at 40 °C. After evaporation of the solvent, the extracts were suspended in 10 mL of ethanol/water, (80:20, *v*/*v*) and filtered through 0.22 µm nylon and an aliquot was transferred to opaque vials for analysis. The pigments were separated, identified and quantified using an Agilent 1260 Infinity HPLC system (company, Thermo Fisher Scientific, Madrid, Spain) equipped with a 2690 separation module and a 996 DAD (Waters, Thermo Fisher Scientific, Madrid, Spain) and a Waters Symmetry C8 column (100 Å, 150 mm × 4.6 mm i.d.). Mobile phase A was a mixture of methanol:acetonitrile:aqueous pyridine solution (0.25 M pyridine, see below) (50:25:25 v:v:v) while phase B was methanol:acetonitrile:acetone (20:60:20 v:v:v). The flow rate was set at 1 mL/min. The program of the gradient elution carried out by a mixture of mobile phase A and B was set as follow: 100% A (0 to 22 min); 60% A—40% B (22 to 28 min); 5% A—95% B (28 to 38 min); 100% A (38 to 40 min). A Waters 474 scanning fluorescence detector (FLD) programmed for excitation at λ = 440 nm and emission at λ = 650 nm was used for detection. Standard curves were plotted and the results were expressed in mg/g of dw.

#### 2.4.4. Evaluation of Antioxidant Activity

Dried extracts were re-dissolved (2.5 mg/mL) in an ethanol/water (80:20 *v*/*v*) solution and in succession diluted to figure out their respectively inhibitory concentration ($EC_{50}$ value, µg/mL) to evaluate the antioxidant activity. The inhibition of lipid peroxidation in

porcine (*Sus scrofa*) brain homogenates is showed by the decrease in thiobarbituric acid reactive substances (TBARS) [39]. The oxidative hemolysis inhibition assay (OxHLIA) was performed using sheep blood samples [44]. In this assay, results were also expressed giving the $EC_{50}$ value (μg/mL) as the concentration with the ability to produce a $\Delta t$ hemolysis delay of 30 min. For this purpose, Trolox was utilized as positive control in both assays.

#### 2.4.5. Evaluation of Antimicrobial Activity

The dried extracts of the three algae samples were dissolved in water (10 mg/mL) and the antibacterial potential was evaluated [45]. *Bacillus cereus* (human isolate), *Micrococcus flavus* (ATCC 10240), *Staphylococcus aureus* (ATCC 11632) were selected as Gram-positive bacteria, and *Proteus mirabilis* (ATCC 7002) and *Salmonella Typhimurium* (ATCC 13311) were used as Gram-negative bacteria, to determine the potential antimicrobial activity. The minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations were determined and results were expressed in mg/mL. Streptomycin was used as positive control.

The antifungal activity was assessed following a previous procedure [46] and three yeast species: *Candida albicans* (clinical isolate), *Candida tropicalis* (ATCC 750) and *Candida krusei* (clinical isolate) were tested for their susceptibility. The MIC and minimum fungicidal concentration (MFC) were determined and results were expressed in mg/mL. In this case, ketoconazole was used as positive control.

The bacterial strains were cultured on solid tryptic soy agar (TSA) and yeasts were sustained on Sabouraud dextrose agar (SDA) medium. The cultures were submitted to subculture once a month and stored for further utilization (4 °C). All the tested microorganisms are deposited at the Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Siniša Stankovic", University of Belgrade.

### *2.5. Numerical Methods, Statistical Analysis, and Graphic Illustrations*

All statistical calculations, fitting procedures and coefficient estimates were performed using a *Microsoft Excel* spreadsheet. The statistical analysis of the experimental results was carried out in four phases. Firstly, the coefficients were obtained by minimizing the sum of the quadratic differences between the obtained and predicted values, using the least-squares method (quasi-Newton) by the "Solver" macro in Microsoft Excel, which allows the rapid analysis of a hypothesis and its consequences [47]. Then, to obtain the significance of these values, the confidence intervals of the parameters were calculated using "SolverAid" [48]. The model was simplified by discarding the non-statistically significant terms for the $p$ value ($p > 0.05$). It was carried out through Fisher's $F$ test ($\alpha = 0.05$), to determine if the constructed models were adequate to describe the data obtained and evaluate the consistency of the model. To re-verify the uniformity of the model, the following criteria was applied: "SolverStat" was used to evaluate the prediction uncertainties of parameters and models [49]; as well as the $R^2$ value, interpreted as the proportion of versatility of each dependent variable explained by the model.

All the assays were performed in triplicate and the results were expressed in the mean ± standard deviation (SD) format. ANOVA test was used to analyze data collected, to determine the significant differences between the samples, with $p$-value = 0.05 (SPSS v. 23.0; IBM Corp., Armonk, NY, USA).

## 3. Results and Discussion

### *3.1. Nutritional Characterization and Chemical Composition*

The composition of algae is highly variable, as it depends on many factors: species, time of collection, growth conditions or habitat, among others [2,12]. Currently, the legislation regarding the food industry is strictly regulated and both food and additives must be well nutritionally and chemically characterized, besides accomplishing the pertinent toxicological controls [50].

Nutritional and chemical composition results are shown in Table 2. The three species presented similar nutritional composition values. The inorganic content presented sig-

nificant differences: lower percentages for *Mastocarpus stellatus* (~21%), while for *Chondrus crispus* and *Gigartina pistillata* values of ~30% were obtained. Previous results had reported lower inorganic content both in *C. crispus* (21.4 g/100 g dw) and in *M. stellatus* (15.6 g/100 g dw) than in this study (29.46% and 20.81%, respectively) [51–53]. Another study, carried out in Spain with samples provided by a local supplier from A Coruña (Galicia), obtained an inorganic content for *M. stellatus* and *G. pistillata* of 24.99 and 34.56 g/100 g dw, respectively, similar to those obtained in this work (20.8% and 31.8%) [24]. Obtained data were also compatible with other red seaweeds, which ash values ranges from 15–46 g/100 g dw. To our knowledge, there are not previous studies of ash content in *G. pistillata* [12,54,55].

**Table 2.** Nutritional and chemical composition of dried macroalgae species (mean ± standard deviation (SD)) ($\bar{x} \pm \sigma$).

| | ME | CC | GP |
|---|---|---|---|
| **Nutritional Composition and Energetic Value** | | | |
| Ash | 20.81 $^{a}$ ± 0.61 | 29.46 $^{b}$ ± 0.26 | 31.82 $^{c}$ ± 0.54 |
| Proteins | 18.62 $^{a}$ ± 0.04 | 17.00 $^{b}$ ± 0.26 | 14.35 $^{c}$ ± 0.26 |
| Lipids | 0.14 $^{a}$ ± 0.03 | 0.12 $^{a}$ ± 0.01 | 0.11 $^{a}$ ± 0.04 |
| Carbohydrates | 60.43 $^{a}$ ± 0.59 | 53.43 $^{b}$ ± 0.01 | 53.72 $^{b}$ ± 0.75 |
| Energy (kcal/100 g dw) | 317.50 $^{a}$ ± 2.26 | 282.75 $^{b}$ ± 1.01 | 273.26 $^{c}$ ± 2.35 |
| Energy (kJ/100 g dw) | 1329.3 $^{a}$ ± 9.45 | 1183.83 $^{b}$ ± 4.21 | 1144.07 $^{c}$ ± 9.82 |
| **Organic Acids** | | | |
| Oxalic acid | 0.52 $^{a}$ ± 0.08 | 0.30 $^{b}$ ± 0.09 | 0.89 $^{c}$ ± 0.02 |
| Quinic acid | 2.01 $^{a}$ ± 0.27 | nd | 0.57 $^{b}$ ± 0.15 |
| Malic acid | 9.45 $^{a}$ ± 1.05 | 4.28 $^{b}$ ± 1.00 | 4.29 $^{b}$ ± 0.37 |
| Shikimic acid | nd | nd | 0.07 $^{a}$ ± 0.02 |
| Citric acid | 1.28 $^{a}$ ± 0.12 | 1.85 $^{b}$ ± 0.42 | 2.52 $^{c}$ ± 0.16 |
| **Total** | 13.26 $^{a}$ ± 1.51 | 6.13 $^{b}$ ± 1.42 | 7.45 $^{b}$ ± 0.03 |

**ME**: *Mastocarpus stellatus*; **CC**: *Chondrus crispus*; **GP**: *Gigartina pistillata.* The results of ash, proteins, lipids and carbohydrates were expressed in g/100 g of dry weight (dw). Carbohydrates were calculated by difference and energetic value was calculated using Equation (2). Different letters in each line correspond to significant differences ($p < 0.05$) between samples.

Regarding the protein content, the three algae showed high values, similar to some legumes (20–30%), cereals (10–15%) or nuts (20–30%) [56]. *M. stellatus* and *C. crispus* have a higher protein concentration compared to *G. pistillata*. It is important to note that RA are the group with the highest protein content among marine algae, followed by green algae [13,17]. Protein content in marine algae usually varies from 5–20% of dw (brown algae) while in green and red ones may fluctuate between 10–47% [5,19,57]. In any case, there are some species as those from *Gracilaria* genus with very low protein levels, under 5% and other species like *Porphyra tenera* which content rises to almost 37% of dw. Particularly, other studies have shown a protein content from 20.1–27% dw for *C. crispus*; 25.4% of dw for *M. stellatus* and 15.59% for *G. pistillata* [24,51,53,57]. These data could be analogous to those obtained in this study, which revealed a content of 18.62%, 17.00% and 14.35% for *M. stellatus*, *C. crispus* and *G. pistillata*, respectively. It should be noted that these values are highly variable depending on the collection area, the method of extraction employed and the season. For example, in the case of *C. crispus*, protein content ranged from 6 to 29 g/100 g dw [11].

The lipid content evaluation revealed low values. Generally, marine algae do not have high lipid content, it usually ranges between 1–5% dw, however these species possess a high level of PUFAs [13,57]. In this study, the results were very similar between the three species and around 0.1–0.15 g/100 g dw, similar to previous results [13,57]. This is a lower value compared with other studies in *C. crispus* (1–3%) [58] or in *M. stellatus* (3%) [51], as well as in other RA species such as *Acanthophora spicifera* and *Gracilaria edulis* which had 0.48% and 0.72%, respectively [59].

In respect of carbohydrates content of the three samples of algae, results were very similar for *C. crispus* and *G. pistillata* showing non-significant differences, but higher levels were found in *M. stellatus*. Generally, marine algae have a higher concentration of carbohydrates than terrestrial plants [57]. They have similar values to *Gelidium amansii* or *Gracilaria verrucosa* species. The three samples evaluated in this study showed high carbohydrates content (50–60%), analogous to the content in other foods such as chestnuts, chocolate and flour or bread. Previously, some studies had quantified carbohydrates in the same species, with a content of 64 g/100 g dw for *C. crispus* and around 50 g/100 g dw for *M. stellatus* and *G. pistillata* [60,61].

In respect of caloric content, *M. stellatus* was the algae with the highest value while *G. pistillata* was the one with the lowest content. When comparing the three species with two widely used and commercialized edible algae, i.e., *Porphyra tenera* (nori) and *Gracilaria verrucosa* (ogonori), all showed similar values regarding their nutritional composition [62].

Different organic acids were found in the three algae samples. *M. stellatus* presented the highest content in organic acids (13.26 ± 1.51 g/100 g dw), doubling the value of the other two species, mainly due to the content in malic acid (94.5 ± 10.5 g/100 g dw). Besides, malic, citric and oxalic acids were also detected in the three algae samples. In this case, *G. pistillata* showed higher contents in both acids (2.52 and 0.89 g/100 g dw, respectively). Moreover, citric acid concentration must also be highlighted as it was present in a range between 1.28–2.52 g/100 g dw and it is known for being a great natural antioxidant, chelator and synergist for other antioxidants [63–65]. Quinic acid was only identified in *M. stellatus* and *G. pistillata* whereas shikimic acid was only found in *G. pistillata*. According to the revised literature, organic acids determination in marine algae is not very common. Nevertheless, some have been found in green species like *Caulerpa scalpelliformis*, in which lactic and oxalic acid have been reported [66].

### *3.2. Optimization and Comparison of Extraction Techniques (HAE and HPAE) to Obtain Extracts Rich in Biological Compounds from Red Algae*

#### 3.2.1. Selection of Relevant Variables and Instrumental Parameters to Focus Their Experimental Domains before Applying the RSM

Different studies have tried to develop and compare different extraction techniques to improve production yields [67]. However, it is difficult to directly compare direct the results of different extraction techniques, mainly because of the wide variety of biological matrices, their nature and composition and the respective variations between them. Therefore, to provide a better understanding of the potential of extraction techniques, two solid-liquid techniques (HAE and HPAE) were chosen and compared, by applying a similar set of ranges for the main extraction conditions.

For both techniques, several factors have been associated with the chemical changes of the extraction process that affect extraction yield. The most frequently reported in the literature are the combination of $T$ and $t$ for HAE, and of $P$ and $t$ for HPAE. Thus, ranges of $t$ ($X_1$, 19.5 to 120.5 min), of T ($X_2$, 21.4 to 88.6 °C) and $S$ ($X_3$, 0 to 100%) for HAE and $t$ ($X_1$, 10–110 min), $P$ ($X_2$, 100–600 MPa) and $S$ ($X_3$, 0 to 100%) for HPAE were selected (Table 1).

In summary, the extraction processes of biological compounds from marine algae by two techniques (HAE and HPAE) was carried out by applying a RSM of three variables ($t$, $T$ and $S$ for HAE, and $t$, $P$ and $S$ for HPAE) in a CCCD with five levels of values for each variable. This multivariable approach provides a reliable tool that minimize experimental errors with a reduced number of tests, while optimizing the extraction conditions of the variables, according to the mathematical empirical models that predict the maximum extraction performance. Once the optimal conditions of the analyzed variables had been determined, the antioxidant and antimicrobial properties of the extract were studied.

### 3.2.2. Analysis of the Optimization by RSM of the Three Variables

#### Mathematical Models Derived from the RSM for a CCCD with Three Variables, and Statistical Evaluation

Table 3 shows the results obtained by the statistical CCCD for the two extraction techniques (HAE and HPAE).

**Table 3.** Experimental results of RSM of CCCD for the optimization of the three implied variables ($X_1$, $X_2$ y $X_3$) in HAE and HPAE, for the evaluation of the response in terms of yield (mg of extract per f of alga, mg/g) in the three red algae (ME, CC and GP). The employed variables, natural values and coded ranges are collected in Table 1.

| | **Experimental Response** | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **Coded Values** | | | **Natural Values** | | | | | | **HAE** | | | **HPAE** | | |
| | | | | **HAE** | | | **HPAE** | | | **ME** | **CC** | **GP** | **ME** | **CC** | **GP** |
| | $X_1$ | $X_2$ | $X_3$ | $X_1$: $t$ | $X_2$: $T$ | $X_3$: $S$ | $X_1$: $t$ | $X_2$: $P$ | $X_3$: $S$ | $Y_{ME}$ | $Y_{CC}$ | $Y_{GP}$ | $Y_{ME}$ | $Y_{CC}$ | $Y_{GP}$ |
| | | | | **min** | **°C** | **%** | **min** | **MPa** | **%** | **mg/g** | **mg/g** | **mg/g** | **mg/g** | **mg/g** | **mg/g** |
| **1** | −1 | −1 | −1 | 40 | 34.2 | 20.3 | 30.3 | 201.3 | 20.3 | 196.4 | 392.2 | 305.5 | 236.0 | 121.4 | 358.3 |
| **2** | −1 | −1 | 1 | 100 | 34.2 | 79.7 | 30.3 | 201.3 | 79.7 | 163.5 | 351.3 | 287.4 | 94.8 | 200.7 | 186.7 |
| **3** | −1 | 1 | −1 | 40 | 75.8 | 20.3 | 30.3 | 498.7 | 20.3 | 254.9 | 443.8 | 355.2 | 255.6 | 140.9 | 323.3 |
| **4** | −1 | 1 | 1 | 100 | 75.8 | 79.7 | 30.3 | 498.7 | 79.7 | 230.6 | 384.7 | 254.6 | 92.2 | 204.5 | 151.7 |
| **5** | 1 | −1 | −1 | 40 | 34.2 | 20.3 | 89.7 | 201.3 | 20.3 | 147.1 | 419.4 | 234.2 | 258.0 | 124.0 | 360.0 |
| **6** | 1 | −1 | 1 | 100 | 34.2 | 79.7 | 89.7 | 201.3 | 79.7 | 126.4 | 353.4 | 269.2 | 116.8 | 108.3 | 151.7 |
| **7** | 1 | 1 | −1 | 40 | 75.8 | 20.3 | 89.7 | 498.7 | 20.3 | 95.0 | 463.9 | 375.9 | 245.8 | 95.5 | 333.3 |
| **8** | 1 | 1 | 1 | 100 | 75.8 | 79.7 | 89.7 | 498.7 | 79.7 | 82.9 | 386.1 | 300.7 | 82.5 | 64.2 | 150.0 |
| **9** | −1.68 | 0 | 0 | 19.5 | 55 | 50 | 10 | 350 | 50 | 198.2 | 344.1 | 236.6 | 184.8 | 193.5 | 258.3 |
| **10** | 1.68 | 0 | 0 | 120.5 | 55 | 50 | 110 | 350 | 50 | 126.7 | 436.9 | 313.0 | 195.1 | 77.6 | 271.7 |
| **11** | 0 | −1.68 | 0 | 70 | 20 | 50 | 60 | 100 | 50 | 163.2 | 364.7 | 274.7 | 197.1 | 149.9 | 256.7 |
| **12** | 0 | 1.68 | 0 | 70 | 90 | 50 | 60 | 600 | 50 | 169.7 | 438.9 | 350.3 | 184.7 | 129.2 | 221.7 |
| **13** | 0 | 0 | −1.68 | 70 | 55 | 0 | 60 | 350 | 0 | 283.8 | 442.4 | 344.7 | 272.1 | 112.8 | 431.7 |
| **14** | 0 | 0 | 1.68 | 70 | 55 | 100 | 60 | 350 | 100 | 80.4 | 141.2 | 173.0 | 16.0 | 153.1 | 28.3 |
| **15** | −1.68 | −1.68 | −1.68 | 19.5 | 20 | 0 | 10 | 100 | 0 | 272.8 | 157.9 | 283.7 | 191.9 | 35.0 | 365.0 |
| **16** | −1.68 | −1.68 | 1.68 | 19.5 | 20 | 100 | 10 | 100 | 100 | 162.0 | 62.9 | 307.0 | 16.7 | 151.2 | 25.0 |
| **17** | −1.68 | 1.68 | −1.68 | 120.5 | 90 | 0 | 10 | 600 | 0 | 429.6 | 401.5 | 352.5 | 273.3 | 23.7 | 456.7 |
| **18** | −1.68 | 1.68 | 1.68 | 120.5 | 90 | 100 | 10 | 600 | 100 | 381.8 | 103.4 | 41.1 | 3.3 | 176.1 | 33.3 |
| **19** | 1.68 | −1.68 | −1.68 | 19.5 | 20 | 0 | 110 | 100 | 0 | 173.4 | 439.6 | 112.2 | 356.7 | 40.6 | 458.3 |
| **20** | 1.68 | −1.68 | 1.68 | 19.5 | 20 | 100 | 110 | 100 | 100 | 182.4 | 135.9 | 283.0 | 119.1 | 40.0 | 16.7 |
| **21** | 1.68 | 1.68 | −1.68 | 120.5 | 90 | 0 | 110 | 600 | 0 | 45.1 | 494.1 | 522.2 | 286.7 | 45.0 | 425.0 |
| **22** | 1.68 | 1.68 | 1.68 | 120.5 | 90 | 100 | 110 | 600 | 100 | 27.5 | 275.5 | 339.7 | 5.0 | 56.0 | 41.7 |
| **23** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 167.6 | 427.5 | 291.7 | 188.3 | 173.3 | 286.7 |
| **24** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 178.3 | 416.9 | 285.0 | 193.3 | 191.7 | 291.7 |
| **25** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 113.6 | 382.2 | 270.2 | 210.0 | 168.3 | 270.0 |
| **26** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 147.9 | 392.2 | 288.9 | 200.0 | 186.7 | 276.7 |
| **27** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 120.3 | 420.9 | 292.5 | 178.3 | 143.3 | 201.7 |
| **28** | 0 | 0 | 0 | 70 | 55 | 50 | 60 | 350 | 50 | 123.6 | 403.5 | 292.5 | 193.3 | 138.3 | 273.7 |

The responses obtained were adjusted to the second order polynomial model expressed in Equation (2), using nonlinear least squares estimates, to obtain the parametric values that are presented in Table 4.

Those coefficients whose confidence interval value ($\alpha = 0.05$) were higher than the value of the parameter, were considered not significant and were not used for the development of the model. The resulting models (Equations (3)–(8)) for each response ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$) and each extraction technique (HAE and HPAE) are shown in Table 5.

**Table 4.** Parametric results of the polynomic model of second order of Equation (2) for the extraction techniques HAE and HPAE, in terms of the extraction performance for the response value ($Y$, yield), for each one of the algae ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$) according to the CCCD with 5 range levels (Table 1). Also, statistic information about the adaptation process of the model is shown.

| Parameters | | HAE | | | HPAE | | |
|---|---|---|---|---|---|---|---|
| | | **ME** | **CC** | **GP** | **ME** | **CC** | **GP** |
| Origin | $b_0$ | $148.2 \pm 15.4$ | $419.0 \pm 20.9$ | $290.1 \pm 10.2$ | $190.3 \pm 6.2$ | $167.1 \pm 9.7$ | $264.5 \pm 9.3$ |
| Lineal effect | $b_1$ | $-52.1 \pm 9.2$ | $34.4 \pm 12.5$ | $15.6 \pm 5.7$ | $14.2 \pm 3.7$ | $-22.4 \pm 5.3$ | $13.8 \pm 5.2$ |
| | $b_2$ | ns | $30.1 \pm 12.5$ | $21.2 \pm 5.7$ | $-6.8 \pm 3.7$ | ns | ns |
| | $b_3$ | $-19.7 \pm 9.2$ | $-63.1 \pm 12.5$ | $-26.2 \pm 5.7$ | $-73.4 \pm 3.7$ | $17.4 \pm 5.3$ | $-112.6 \pm 5.2$ |
| Quadratic effect | $b_{11}$ | ns | ns | ns | ns | $-11.5 \pm 7.0$ | ns |
| | $b_{22}$ | ns | ns | $8.3 \pm 7.0$ | ns | $-10.1 \pm 7.0$ | $-18.3 \pm 6.4$ |
| | $b_{33}$ | $19.2 \pm 8.9$ | $-51.2 \pm 12.0$ | $-10.7 \pm 7.0$ | $-13.2 \pm 3.6$ | $-12.4 \pm 7.0$ | $-8.5 \pm 6.4$ |
| Interactiveeffect | $b_{12}$ | $-29.0 \pm 6.6$ | ns | $28.3 \pm 4.1$ | $-10.8 \pm 2.7$ | ns | $-6.7 \pm 3.8$ |
| | $b_{13}$ | ns | ns | $11.9 \pm 4.1$ | $-2.9 \pm 2.7$ | $-12.8 \pm 3.8$ | ns |
| | $b_{23}$ | ns | ns | $-29.7 \pm 4.1$ | $-6.1 \pm 2.7$ | ns | ns |
| Statistics ($R^2$) | | 0.8623 | 0.8560 | 0.9451 | 0.9796 | 0.8878 | 0.9403 |

**Table 5.** Mathematical models of the extraction processes derived from the polynomial model of Equation (2).

| | | | |
|---|---|---|---|
| **HAE** | *ME:* | $Y_{ME}^{EAC} = 148.2 - 52.1t - 19.7S + 19.2S^2 - 29.0t$ | Equation (3) |
| | *CC:* | $Y_{CC}^{EAC} = 419.0 + 34.4t + 30.1T - 63.1S - 51.2S^2$ | Equation (4) |
| | *GP:* | $Y_{GP}^{EAC} = 290.1 + 15.6t + 21.2T - 26.2S + 8.3T^2 - 10.7S^2 + 28.3tT + 11.9tS - 29.7TS$ | Equation (5) |
| **HPAE** | *ME:* | $Y_{ME}^{EAAP} = 190.3 + 14.2t - 6.8P - 73.4S - 13.2S^2 - 10.8tP - 2.9tS - 6.1PS$ | Equation (6) |
| | *CC:* | $Y_{CC}^{EAAP} = 167.1 - 22.4t + 17.4S - 11.5t^2 - 10.1P^2 - 12.4S^2 - 12.8tS$ | Equation (7) |
| | *GP:* | $Y_{GP}^{EAAP} = 264.5 + 13.8t - 112.6S - 18.3P^2 - 8.5S^2 - 6.7tP$ | Equation (8) |

In statistical terms, the tests carried out to evaluate the competence of the obtained models showed that the non-significant parameters for the two techniques studied by RSM (Table 4) did not improve the reached solution. However, all significant parameters were highly consistent ($p < 0.01$). In addition, the high $R^2$ values also confirmed this hypothesis by indicating the percentage of variability calculated by the model.

Finally, the agreement between the experimental and predicted values indicates that the results obtained can be explained by means of the independent variables used. Therefore, Equations (3)–(8) develop functional and suitable models for the prediction and optimization of the process.

Response Patterns

Table 4 shows the parametric values derived from the RSM models. These values provide a global vision, as well as relevant information for each of the responses and the suitability of the method. In respect of the parameters obtained for each technique, linear and quadratic effects played an important and significant role. The presence of both effects implied that all responses showed non-linear patterns and, therefore, that these patterns are characteristic of the variables involved in the HAE and HPAE techniques. Regarding the interactive effects between the different variables (Table 4), both the HAE and the HPAE showed more influential interactions in the case of $tT$ and $tP$. The interactions $tS$, $TS$ and $PS$ did not present clear interactions since they were only significant in some cases. In conclusion, a non-linear multivariate analysis was needed to fully adjust to the experimental results since all the responses presented components of linear and quadratic or interactive effects.

Figure 1 shows the results for HAE and HPAE in terms of the extraction performance of each alga ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$). Each figure is divided into two columns, which show the

results obtained for each alga by HAE and HPAE. Furthermore, each column is divided into two parts (A and B). Section A shows the 3D surface plots for the three possible combinations through the binary action between variables produced by Equations (3)–(8) excluding the third variable at its individual optimal predicted value (part A of Table 6). Section B shows the adjustment between the obtained and the predicted results, i.e., it reveals the predictive capacity of the model and the distribution of the residual points based on each variable. Considering the distribution of the residuals (section B, Figure 1) as an example of all the obtained responses, it was possible to distinguish an arbitrary distribution around zero and no groups of values or autocorrelations were observed.

Individual and Global Numerical Conditions That Maximize Extraction

The absolute or relative optimal conditions (marked with *) that maximized the individual and global response criteria of the results, and therefore, the extraction yields are graphically presented in Figure 1, and numerically in Table 6. These values were obtained by the application of restrictions to the experimental ranges.

Part A of Table 6 shows the individual optimal conditions for the variables by HAE and HPAE for each alga (ME, CC and GP). The highest responses were obtained by HAE, achieving 405.0 ± 40.5 mg extract/g dried alga obtained at *19.5 min, *88.6 °C and *0% of acidified ethanol for ME, 547.0 ± 16.4 mg/g obtained at *120.5 min, *88.6 °C and *31.7% of acidified ethanol for CC and 519.6 ± 10.4 mg/g obtained at *120.5 min, *88.6 °C and *0% of acidified ethanol for GP. The responses reached by HPAE were close but lower to those produced by the HAE. Combining the information produced by the response for each alga ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$), the complete behavior of each variable with influence on the responses was defined in global terms.

Part B of Table 6 shows the optimal global results that are also described below:

(1) For HAE: the optimal global conditions were *36.6 min, *88.6 °C and *50.0% of acidified ethanol, producing a response yield of 405.0 ± 12.2 mg/g for ME, 373.2 ± 22.4 mg/g for CC and 375.0 ± 3.8 mg/g for GP.

(2) For HPAE: the optimal global conditions were 49.0 min, 51.9 MPa and 50.0% of acidified ethanol, producing a response yield of 292.2 ± 14.6 mg/g for ME, 94.4 ± 4.7 mg/g for CC and 441.1 ± 4.4 mg/g for GP.

Once the conditions for obtaining maximum extraction values were obtained, both techniques were re-evaluated experimentally under these values to guarantee the rigor of the data obtained. A priori, HPAE should present better results in terms of extraction, given its theoretical advantages previously mentioned, however, the responses obtained for this technique were slightly less efficient than conventional HAE. This could be related to the nature of the applied energy. In addition, the results obtained are compatible with similar studies in other matrices [68,69]. Regarding the solvent used in the extractions, the literature shows that in most cases, hydroalcoholic mixtures are considered as safe and effective solvents that can achieve better extraction yields than pure alcohol or water [70].

In conclusion, it can be observed that HAE was the technique that allowed to maximize the extraction yield of compounds of interest from marine algae. Therefore, it was the selected as the technique for conducting studies on biological compounds and their bioactivity (antioxidant and antimicrobial).

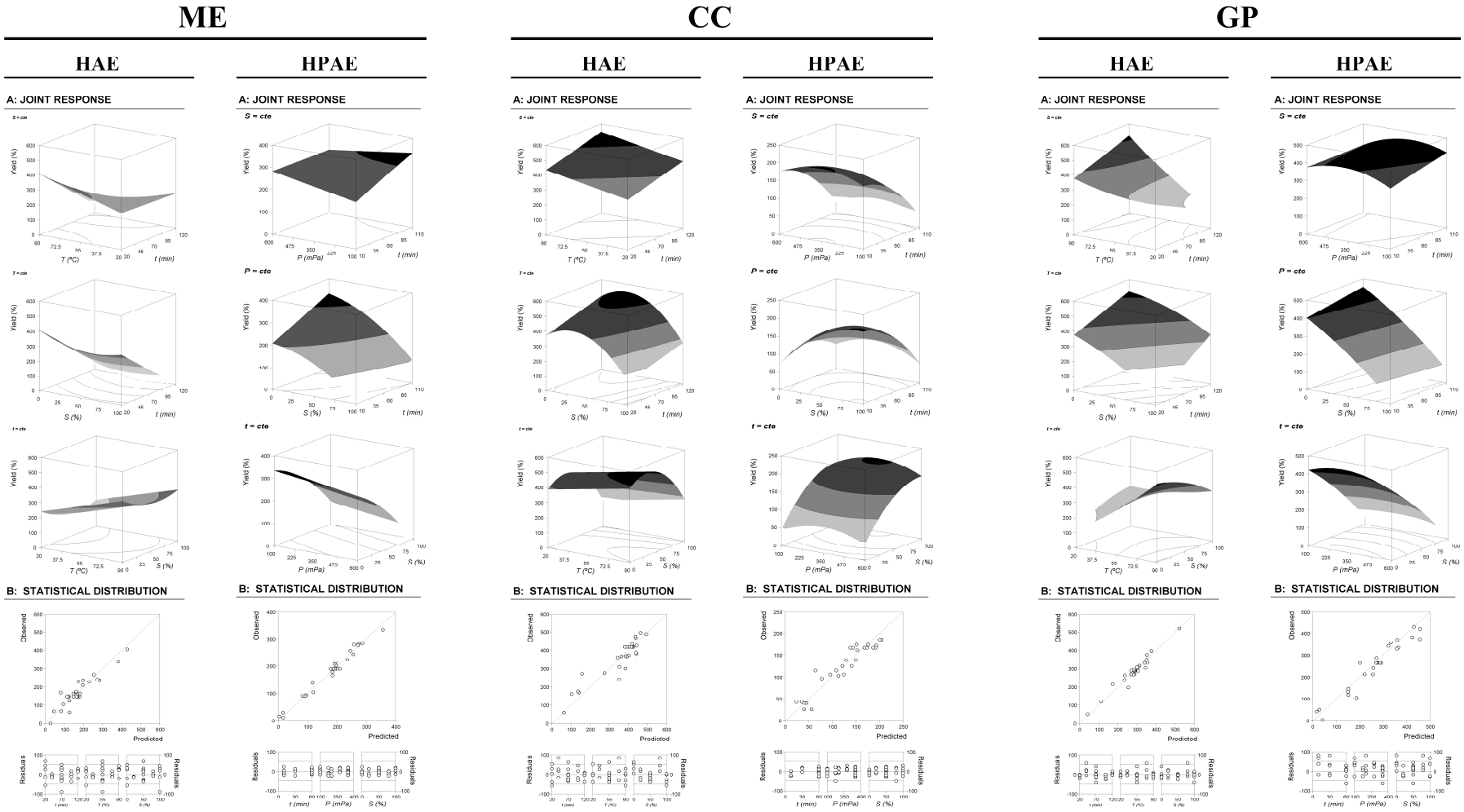


**Figure 1.** Graphical results in terms of the extraction yields of each alga ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$) for both HAE and HPAE. Each figure is divided into two columns: ***Part A:*** 3D graphs of the predicted response surface thanks to the second order polynomial of Equation (2) through the binary actions between two variables, when the excluded variable is placed in the individual optimal value (Table 6). ***Part B:*** To show the benefit of adjustment, two basic statistical criteria were used. On the one hand, the ability to simulate response changes between predicted and observed data; and on the other hand, the residual values distribution according to each one of the variables. Note to the values of the z axis corresponding to the yield (%) for HAE are constant (0–600), however, for the HPAE the axes vary according to the species of algae.

**Table 6.** Absolute or relative conditions (marked with *) of the variables in natural values that lead to optimal response values (*Y, yield*) for RSM using CCCD for each of the extraction techniques (HAE and HPAE) and for each of the alga ($Y_{ME}$, $Y_{CC}$ and $Y_{GP}$).

| Criteria | | Variables Optimal Conditions | | | Optimal Response | |
|---|---|---|---|---|---|---|
| | | $X_1$: *t* (min) | $X_2$: *T* (°C) or *P* (MPa) | $X_3$: *S* (%) | | |
| **(A) Optimal Individual Conditions of the Variables** | | | | | | |
| **HAE** | *ME:* | *19.5 ± 0.2 | *88.6 ± 8.9 | *0.0 ± 0.0 | 405.0 ± 40.5 | mg/g |
| | *CC:* | *120.5 ± 12.1 | *88.6 ± 8.9 | 31.7 ± 0.6 | 547.0 ± 16.4 | mg/g |
| | *GP:* | *120.5 ± 9.6 | *88.6 ± 8.0 | *0.0 ± 0.0 | 519.6 ± 10.4 | mg/g |
| **HPAE** | *ME:* | *110.0 ± 7.7 | *100.0 ± 8.0 | *0.0 ± 0.0 | 333.1 ± 13.3 | mg/g |
| | *CC:* | *10.0 ± 1.0 | 350.0 ± 28.0 | 96.7 ± 4.8 | 202.9 ± 18.3 | mg/g |
| | *GP:* | *110.0 ± 9.9 | 304.3 ± 24.3 | *0.0 ± 0.0 | 454.6 ± 4.5 | mg/g |
| **(B) Optimal Global Conditions of the Variables** | | | | | | |
| **HAE** | *ME:* | 36.6 ± 10.8 | | 50.0 ± 2.4 | 405.0 ± 12.2 | mg/g |
| | *CC:* | *88.6 ± 2.4 | | | 373.2 ± 22.4 | mg/g |
| | *GP:* | | | | 375.0 ± 3.8 | mg/g |
| **HPAE** | *ME:* | 49.0 ± 3.4 | | 50.0 ± 5.0 | 292.2 ± 14.6 | mg/g |
| | *CC:* | 51.9 ± 2.6 | | | 94.4 ± 4.7 | mg/g |
| | *GP:* | | | | 441.1 ± 4.4 | mg/g |

### *3.3. Bioactive Evaluation*

#### 3.3.1. Identification and Quantification of Phenolic Compounds and Fundamental Pigments

PC are considered bioactive compounds mainly due to their antioxidant activity and other effects such as antibiotic, antidiabetic or photoprotective functions. In this work a high PC content was obtained for each alga expressed as 9.11 mg/g dw for *G. pistillata*, 10.36 mg/g dw for *C. crispus* and 12.18 mg/g dw for *M. stellatus* (Table 7). Regarding the abundance of each compound, high variability was observed. Thus, *M. stellatus* and *G. pistillata*, presented oleuropein as the major PC whereas in the case of *C. crispus*, it was tyrosol. In general, algae PC content can vary from <1 to 14% of its dw. However, green and red algae have been reported to have lower PC content than brown algae [11]. PC content in red algae has been previously assessed, although many studies have been focused on the determination of the total phenolic content. For example, for *C. crispus*, a content of ~4 mg GAE (gallic acid equivalents)/g dw has been reported [71] while for *M. stellatus* and *G. pistillata* it ranged between 0–5 mg PGE (Phloroglucinol equivalents)/g dw [61].

However, these data can vary depending on different factors, for example, the percentage of the extraction solvent due to its polarity [23]. Regarding PC identification, other studies had reported the presence of catechin, rutin, hesperidin, epicatechin, caffeic acid, quercetin, *p*-coumaric acid, salicylic acid, vanillin, hypogalic acid or chlorogenic acid [72,73]. For example, catechin, hesperidin and rutin were found in *Porphyra dentata* [73] or catechin, rutin and quercetin in *Euchema cottonii* [74].

Regarding the pigment content, β-carotene, chlorophyll *a* and lutein were found in the three RA samples. The pigment content was 2.56 mg/g for *G. pistillata*, (similar to the value of *M. stellatus*, 2.34 mg/g) while *C. crispus* showed lower values (0.52 mg/g). Concerning the percentage of abundance, the major pigment for the three species studied was β-carotene followed by chlorophyll *a*. Previous studies had indicated the presence of chlorophyll *a* in RA. In respect of their carotenoids' composition, it had been indicated that they contained mainly α and β carotenes, lutein and zeaxanthin. Specifically, the β-carotene content could be of interest, both for its application as a natural colorant, as well as for its antioxidant properties [11].

**Table 7.** Total content (mg/g) and individual content (in % of abundance) of phenolic compounds and pigments in the marine algae.

| Compounds | | Red Algae | | |
|---|---|---|---|---|
| | | **ME** | **CC** | **GP** |
| **(A) Phenolic Compounds** | | | | |
| | Total (mg/g): | 12.18 | 10.36 | 9.11 |
| *(−) Epicatechin* | (2*R*,3*R*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol | 1.14 | 9.95 | 5.78 |
| *(+) Catechin* | (2*R*,3*S*)-2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-chromene-3,5,7-triol | 1.34 | 10.34 | - |
| *Protocatechuic acid* | 3,4-Dihydroxybenzoic acid | 9.10 | 17.72 | 7.14 |
| *Benzoic acid* | Benzoic acid | 0.56 | 0.97 | 0.40 |
| *Caffeic acid* | 3-(3,4-Dihydroxyphenyl)prop-2-enoic acid | 1.67 | 1.31 | 0.83 |
| *Caffeinic acid* | 3-(3,4-Dihidroxyphenyl)-2-propenoic acid | 0.31 | 1.04 | 0.38 |
| *Chlorogenic acid* | 3-(3,4-Dihydroxycinnamoyl)quinic acid | - | 0.31 | - |
| *Galagin* | 3,5,7-Trihydroxy-2-phenylchromen-4-one | 0.15 | - | 0.05 |
| *Hesperidin* | (2*S*)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one | 0.14 | - | 0.40 |
| *Kaempherol* | 3,5,7-Trihydroxy-2-(4-hydroxyphenyl)chromen-4-one | 0.19 | - | 0.03 |
| *Luteolin* | 2-(3,4-Dihidroxyphenyl)- 5,7-dihydroxy-4-chromenone | 0.22 | - | 0.03 |
| *Naringenin* | (2*S*)-5,7-Dihydroxy-2-(4-hydroxyphenyl)-2,3-dihydrochromen-4-one | - | 0.30 | - |
| *Oleuropein* | Methyl (4*S*,5*E*,6*S*)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]-5-ethylidene-6-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4*H*-pyran-3-carboxylate | 61.39 | - | 32.28 |
| *p-aminobenzoic acid* | 4-Aminobenzoic acid | 3.98 | - | 16.58 |
| *p-coumaric acid* | (*E*)-3-(4-Hydroxyphenyl)prop-2-enoic acid | 5.80 | 3.01 | 2.19 |
| *Pinocembrin* | (2*S*)-5,7-dihydroxy-2-phenyl-2,3-dihydrochromen-4-one | - | 0.53 | - |
| *Salicylic acid* | 2-Hydroxybenzoic acid | 12.44 | 4.14 | 10.12 |
| *Sinapic acid* | 3,5-Dimethoxy-4-hydroxycinnamic acid | | 0.64 | - |
| *Syringaldehyde* | 4-Hydroxy-3,5-dimethoxybenzaldehyde | 1.58 | 6.59 | 1.04 |
| *Tyrosol* | 2-(4-Hydroxyphenyl)ethanol | - | 43.01 | 22.75 |
| **(B) Pigments** | | | | |
| | Total (mg/g): | 2.34 | 0.52 | 2.56 |
| *β-Carotene* | 1,3,3-Trimethyl-2-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-3,7,12,16-tetramethyl-18-(2,6,6-trimethylcyclohexen-1-yl)octadeca-1,3,5,7,9,11,13,15,17-nonaenyl]cyclohexene | 73.19 | 73.76 | 84.08 |
| *Chlorophyll a* | magnesium;methyl (3*R*,21*S*,22*S*)-16-ethenyl-11-ethyl-12,17,21,26-tetramethyl -4-oxo-22-[3-oxo-3-[(*E*,7*R*,11*R*)-3,7,11,15-tetramethylhexadec-2-enoxy]propyl]-23,25-diaza-7,24-diazanidahexacyclo [1 8.2.1.15,8.110,13.115,18.02,6]hexacosa-1,5,8(26),9,11,13(25),14,16,18,20(23)-decaene-3-carboxylate | 26.45 | 26.17 | 15.72 |
| *Lutein* | (1*R*)-4-[(1*E*,3*E*,5*E*,7*E*,9*E*,11*E*,13*E*,15*E*,17*E*)-18-[(1*R*,4*R*)-4-hydroxy-2,6,6-trimethylcyclohex-2-en-1-yl]-3,7,12,16-tetramethyloctadeca-1,3,5,7,9,11,13,15,17-nonaenyl] -3,5,5-trimethylcyclohex-3-en-1-ol | 0.35 | 0.061 | 0.18 |

**ME**: *Mastocarpus stellatus;* **CC**: *Chondrus crispus;* **GP**: *Gigartina pistillata,* Underlined values correspond to the majority phenolic compounds of each algae species.

#### 3.3.2. Evaluation of Antioxidant Activity

All samples showed antioxidant activity (Table 8) in the two assays, presenting *C. crispus* the best result of antioxidant activity followed by *M. stellatus* and *G. pistillata* for TBARS assay. In the case of OxHLIA assay, the three algae presented similar results with no significant differences. When comparing both studies, it was observed that the extracts of the three algae had potential as water-soluble antioxidants, since the results for the OxHLIA assay were promising as they present antioxidant activity at low concentrations. When comparing the $EC_{50}$ values of the RA with the positive control (Trolox), OxHLIA assay showed higher antioxidant activity values compared with TBARS method.

**Table 8.** Antioxidant ($EC_{50}$; µg/mL; mean ± standard deviation (SD)) ($\bar{x} \pm \sigma$) and antimicrobial activity (mg/mL) of the extracts obtained from the studied marine algae.

| | | Red Algae | | | Reference Value | | |
|---|---|---|---|---|---|---|---|
| | | **ME** | **CC** | **GP** | **Trolox** | **Str** | **Ktz** |
| ***Antioxidant Activity*** | | | | | | | |
| **TBARS** | | 209 $^{a}$ ± 27 | 160 $^{a}$ ± 18 | 285 $^{b}$ ± 41 | 5.4 ± 0.3 | - | - |
| **OxHLIA (Δt = 30 min)** | | 1.0 $^{a}$ ± 0.1 | 1.4 $^{a}$ ± 0.2 | 1.5 $^{a}$ ± 0.3 | 46 ± 2 | - | - |
| ***Antibacterial Activity*** | | | | | | | |
| ***B. cereus*** | *MIC* | 0.06 | 0.045 | 0.045 | - | 0.1 | - |
| | *MBC* | 0.12 | 0.06 | 0.06 | - | 0.2 | - |
| ***S. aureus*** | *MIC* | 0.09 | 0.06 | 0.06 | - | 0.05 | - |
| | *MBC* | 0.12 | 0.1 | 0.12 | - | 0.1 | - |
| ***M. flavus*** | *MIC* | 0.09 | 0.09 | 0.09 | - | 0.2 | - |
| | *MBC* | 0.12 | 0.12 | 0.12 | - | 0.3 | - |
| ***P. miriabilis*** | *MIC* | 0.045 | 0.045 | 0.045 | - | 0.2 | - |
| | *MBC* | 0.06 | 0.06 | 0.06 | - | 0.3 | - |
| ***S. Typhimurium*** | *MIC* | 0.06 | 0.06 | 0.06 | - | 0.2 | - |
| | *MBC* | 0.12 | 0.12 | 0.12 | - | 0.3 | - |
| ***Antifungal Activity*** | | | | | | | |
| ***C. albicans*** | *MIC* | 0.045 | 0.045 | 0.06 | - | - | 0.5 |
| | *MFC* | 0.06 | 0.06 | 0.12 | - | - | 1 |
| ***C. tropicalis*** | *MIC* | 0.045 | 0.045 | 0.03 | - | - | 0.3 |
| | *MFC* | 0.06 | 0.06 | 0.06 | - | - | 0.5 |
| ***C. krusei*** | *MIC* | 0.06 | 0.06 | 0.06 | - | - | 0.5 |
| | *MFC* | 0.12 | 0.12 | 0.12 | - | - | 1 |

**ME**: *Mastocarpus stellatus*; **CC**: *Chondrus crispus*; **GP**: *Gigartina pistillata.* **Str**: Streptomycin; **Ktz**: Ketoconazole. MIC: minimum inhibitory concentration; MFC: minimum fungicidal concentration; MBC: minimum bactericidal concentration. Different letters in each line correspond to significant differences ($p < 0.05$) between samples.

Specifically, the antioxidant activity has been studied in *M. stellatus* and *C. crispus*. In *M. stellatus*, the antioxidant capacity of the aqueous fraction of its SP was verified and it was suggested that this activity would be directly related to the sulfate content [25]. In the case of *C. crispus*, its antioxidant activity was studied using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, in which the methanolic extract of this alga showed an $IC_{50}$ of 1.4 mg/mL, similar to the results obtained with OxHLIA [28]. Another study carried out different tests on the reducing potential in organic and aqueous extracts of *G. pistillata* and *M. stellatus*, obtaining better results in the aqueous extracts of both and better values for *G. pistillata* [61].

In addition, it should be mentioned that numerous studies in other RA species have shown that antioxidant activity can be associated with different compounds, such as polyphenols or SP and that is linked to the extraction solvent, molecular size, sulfate content and extraction methodologies that can be complemented with the use of enzymes [36,75]. Specifically, the antioxidant activity of SP has been confirmed in other RA such as *Gracilaria birdiae*, *Porphyra yezoensis* or *Pyropia yezoensis* [76–78]. In some species such as *Palmaria palmata*, antioxidant activity has been related to different substances such as mycosporin-type amino acids or polyphenols [79,80]. These latter have also demonstrated to have antioxidant potential in extracts obtained from RA such as *Kappaphycus alvarezii* or *Porphyra tenera* [72,81]. Considering all the above, antioxidant activity similar to other extracts reported in the bibliography can be confirmed.

### 3.3.3. Evaluation of Antimicrobial Activity

Finally, antimicrobial activity was also demonstrated for the three species against different bacteria and yeasts (Table 8). Regarding the antibacterial activity against Gram (-) species, the three algae showed the same results for MIC and MBC against *P. miriabilis* and *S. Typhimurium*. These values are lower than the concentration of the antibiotic used as positive control (0.20 and 0.30 mg/mL, respectively).

In the case of Gram (+) bacteria, the results showed activity against *B. cereus,* with better values in the case of *G. pistillata* and *C. crispus* whose MIC and MBC were half and a third part, respectively of the concentration necessary to produce the desired effect in the case of streptomycin. Regarding the potential against *S. aureus*, the results did not exceed the activity of the control antibiotic; however, it presented similar results, especially for the species *C. crispus*. In the case of *M. flavus*, the data obtained were identical for the three species. On the other hand, the activity against three yeasts of the genus Candida was also studied. *G. pistillata* showed greater activity against *C. tropicalis*, while *M. stellatus* and *C. crispus* showed a greater action potential against *C. albicans*. The results were the same for the three algae against *C. krusei*.

Other studies had studied the antimicrobial potential of *C. crispus*. These studies demonstrated its activity against the studied species in this work, but they also showed that antibacterial activity against *Escherichia coli*, *Enterococcus faecalis*, *Halomonas marina*, *Lactobacillus brevis*, *Listeria innocua*, *Marinobacter hydrocarbonoclasticus*, *Pseudomonas aeruginosa* and *Salmonella enteriditis* among others [30,82]. To our knowledge, there are no data about the antimicrobial activity of *G. pistillata*. *M. stellatus*, has been showed to have antimicrobial capacity against different species of vibrios and marine bacteria (*Pseudoalteromonas* sp., *Marinobacter* sp. or *Bacillus licheniformis*, among others) as well as against some fish pathogens with a similar efficacy to streptomycin [83].

## 4. Conclusions

Some of the most representative species of red algae in Galicia (NW Spain), are *Chondrus crispus, Gigartina pistillata* and *Mastocarpus stellatus*. These species have an as yet unexplored added value for their use as food and as a source of bioactive compounds in relevant applications in the food, cosmetic and/or pharmaceutical industries. This work concluded that the three algae selected constitute a suitable alternative to incorporate in the diet, for example, as a source of vegetable protein or minerals. In addition, its use as food additive could also be valued due to its PC and pigment content.

Regarding the optimization of the extraction from red algae by two extraction methods, HAE and HPAE, the joint effect of the variables of each technique was described through RSM models and the prediction of the extraction yield responses and the maximization of their conditions in both cases was performed. The most effective technique was HAE, applied under conditions of 36.6 min, 88.6 °C and 50.0% of acidified ethanol, obtaining a response yield of 405.0 ± 12.2 mg/g for *M. stellatus*, 373.2 ± 22.4 mg/g for *C. crispus* and 375.0 ± 3.8 mg/g for *G. pistillata*. So, even though HAE is not the most environmentally friendly technique, from an industrial approach it would allow to maximize the yield the most feasible in terms of profitability. In relation to the analysis of the biological properties, the three RA species showed potential as antioxidant and antimicrobial agents. These data open the door to future research such as the analysis of the stability of the extract when it comes to incorporating it into different food matrices, for example, as a preservative additive.

Finally, the obtained results suggest that the extracts of these three species would have potential industrial application as a source of PC and pigments. Moreover, the application of HPAE and other green extraction techniques at an industrial level would allow to reduce costs such as energy or solvent consumption. In this sense, the modernization of the protocols, as well as keeping an approach based on optimization would be essential to develop processes able to maximize responses. In this way, not only a suitable process could be achieved, but also a sustainable one, both from an ecological and economic point of view.

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