# FACTS ABOUT THE FORMATION OF NEW ANTIOXIDANTS IN NATURAL SAMPLES AFTER SUBCRITICAL WATER EXTRACTION

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**ABSTRACT.**

Subcritical water extraction (SWE) is a very promising technique for obtaining bioactives (mainly antioxidants) from natural sources; even if sometimes the high operation temperatures have been suggested as responsible for thermal degradation of bioactives, the fact is that this type of extraction processes may generate new bioactive (antioxidant) compounds. The present study involved the analysis of antioxidants either naturally found in raw samples and/or those formed during extraction *via* Maillard reaction and other chemical events. Samples of different nature like microalgae (*Chlorella vulgaris*), algae (*Sargassum vulgare, Porphyra spp., Cystoseira abies-marina, Sargassum muticum, Undaria pinnatifida, Halopitys incurvus*) and plants (rosemary, thyme and verbena) were studied. Amino acids availability, sugar content, fluorescence and absorbance at different wavelengths were determined to follow chemical changes due to reactions such as Maillard, caramelization and thermoxidation. Folin reaction also provided information related to total phenol content of the samples. $ABTS^{\bullet+}$, peroxyl as well as superoxide radical scavenging assays were used to measure the antioxidant capacity of the extracts. Results obtained from this study suggest that neoformed compounds derived from Maillard, caramelization and thermoxidation reactions affect the overall antioxidant capacity of water subcritical extracts depending on the nature of the sample. The brown algae *U. pinnatifida* was the sample in which these chemical events contributed to a higher extent to improve the antioxidant capacity (from 0.047 to 1.512 mmol/g and from 45.356 to 1522.692 μmol/g for the TEAC and $ORAC_{FL}$ methods, respectively) when the extraction temperature was raised from 100 to 200°C. To the best of our knowledge, this is the first work supporting the formation of neoantioxidants in natural complex matrices during subcritical water extraction.

**Keywords**: Antioxidant, caramelization, Maillard reaction, natural extracts, subcritical water extraction.

## 1. INTRODUCTION.

The use of water under high temperature and pressure below to supercritical conditions in extraction processes is generally termed as subcritical water extraction (SWE)". Under SWE conditions, water is maintained in the liquid state during the whole extraction procedure. This technique presents a series of important advantages over the traditional extraction techniques; is faster, generally produces higher yields and the use of solvents can be greatly reduced (Huie, 2002). These characteristics are mainly due to the increase in the solubility of compounds affected by the raise on the extraction temperature, increasing the mass transfer. Besides, under these conditions, the viscosity of the water is also reduced, allowing a better penetration of the solvent in the matrix, consequently favoring the extraction rate (Ong, Cheong & Goh, 2006). However, other important parameters have to be considered regarding SWE, such as the dielectric constant of water (ε, as a measure of its polarity) and the solubility parameter. Both properties are modified with the temperature, when the liquid state is maintained. Thus, although the value of dielectric constant of water at room temperature is nearly 80, this value can be decreased to ca. 30 at 250 ºC, being at these conditions a similar value to that presented by some organic solvents like ethanol or methanol. The same occurs with the solubility parameter, that decreases, approaching to the one obtained for less polar compounds (Sirinivas, King, Monrad, Howard & Hansen, 2009). Therefore, this technique has been proposed as an alternative to the use of relatively non-polar organic solvents for some applications. In this sense, it has been already observed that in spite of the use of high temperatures, SWE has been successfully applied to the extraction of different antioxidant and functional compounds from natural matrices (Herrero, Cifuentes & Ibáñez, 2006; Wiboonsirikul & Adachi, 2008; Rostagno, Villares, Guillamón, García-Lafuente & Martinez, 2009). For instance, SWE has been employed

to extract nutraceutical compounds from citrus pomace (Kim, Nagaoka, Ishida, Hasegawa, Kitagawa & Lee, 2009), oregano (Rodriguez-Meizoso, Marin, Herrero, Señorans, Reglero, Cifuentes, & Ibañez, 2006) or rosemary (Herrero, Plaza, Cifuentes & Ibañez, 2010), as well as phenolic antioxidants from canola meal (Hassas.Roudsari, Chang, Pegg & Tyler, 2009) and bitter melon (Budrat & Shotipruk, 2009), or even proanthocyanidins from wine-related products (Garcia-Marino, Rivas-Gonzalo, Ibañez & Garcia-Moreno, 2006), among others. However, during the extraction procedure, some components originally present in the sample can be released and may interact, even forming new compounds. Examples of this kind of interactions are Maillard reaction or caramelization, which could be favored under the SWE extraction conditions.

In a previous work, we studied the Maillard and caramelization reactions occurring in different glycation model systems extracted using SWE at different temperatures (Plaza, Amigo, Ibañez, Del Castillo & Herrero, 2010). Our findings indicated the formation of Maillard reaction (MRPs) and caramelization products possessing antioxidant capacity. However, these results can not be directly translated to real samples, considering that natural complex samples contain other compounds besides amino acids and sugars, that may significantly influence the occurrence, or not, of these reactions.

On the other hand, several SWE applications described in the literature have shown an enhancement on the antioxidant capacity of natural extracts when the temperature is increased (Herrero, Martin-Alvarez, Señorans, Cifuentes & Ibañez, 2005; Rodriguez-Meizoso, Jaime, Santoyo, Señorans, Cifuentes & Ibañez, 2010); a possible explanation could be the neoformation of antioxidants at high temperatures as a result of interaction between the compounds of the extract.

Thus, the aim of this contribution is to verify the neoformation of antioxidants during SWE extraction of different natural products, including microalgae (*Chlorella vulgaris*), algae (*Sargassum vulgare, Sargassum muticum, Porphyra spp., Cystoseira abies-marina, Undaria pinnatifida* and *Halopitys incurvus*) and plants (rosemary, *Rosmarinus officinalis* L.; thyme, *Thymus vulgaris*; and verbena, *Verbena officinalis*). To the best of our knowledge, this is the first time that this kind of study is directly performed to confirm the presence of newly form antioxidants in different extracts obtained from a variety of natural complex matrices during SWE.

## 2. MATERIALS AND METHODS

### 2.1. Chemical and reagents

All the chemicals were of analytical grade. Sodium fluorescein, microcrystalline cellulose and 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Fluka (Buchs, Switzerland). O-Phthaldialdehyde (OPA), 2,2´-Azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroma-2-carboxylic acid (Trolox), glucose, L-leucine, xanthine, xanthine oxidase from buttermilk, nitro blue tetrazolium (NBT), ethylenediamine-tetraacetic acid (EDTA), gallic acid and potassium persulfate were supplied by Sigma- Aldrich (Steinheim, Germany). Sodium dihydrogen orthophosphate dehydrate, Folin-Ciocalteu phenol reagent, sodium carbonate, and di-sodium hydrogen orthophosphate anhydrous were from Merck (Darmstadt, Germany). Ethanol was provided by Prolabo (Briare, France). The ultrapure water used was obtained from a Milli-Q (Millipore, Billerica, MA, USA) instrument.

## 2.2. Samples

Microalgae (*Chlorella vulgaris*) and macroalgae (*Cystoseira abies-marina*, *Porphyra spp.*, *Sargassum vulgare*, *Sargassum muticum*, *Undaria pinnatifida*, and *Halopitys incurvus*) samples consisted of freeze-dried algae supplied by Las Palmas de Gran Canaria University (Las Palmas, Spain), stored under dry and dark conditions until use. Rosemary samples (*Rosmarinus officinalis* L.) consisted of dried rosemary leaves obtained from Herboristeria Murciana Company (Murcia, Spain). Rosemary leaves were dried using a traditional method (Herrero, Plaza, Cifuentes, & Ibañez, 2010). Thyme (*Thymus vulgaris* L.) and Verbena (*Verbena officinalis* L.) leaves were purchased in a local herbal store. All plant samples were stored at −20 °C protected from light until use.

## 2.3. Subcritical water extraction (SWE)

Extractions were performed using an accelerated solvent extractor (ASE 200, Dionex, Sunnyvale, CA, USA), equipped with a solvent controller. Ultrapure water was used as solvent. At the beginning of the day, the water was sonicated for 10 min. Extractions were carried out at two different extraction temperatures (100 and 200 ºC) whereas the extraction time was 20 min. An extraction cell heat-up was carried out for a given time prior to any extraction. The warming-up time changed depending on the extraction temperature (i.e., 5 min if the extraction temperature was 100 ºC and 9 min if the extraction temperature was 200 ºC). All extractions were done using 11 mL extraction cells, containing 1 g of sample. Samples were prepared in duplicate.

Extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with solvent up to a pressure of 1500 psi (1 psi. = 6894.76 Pa), (iii) heat-up time was

applied, (iv) static extraction takes place (i.e. 20 min) in which all system valves are closed, (v) cell is rinsed (with 60 % cell volume using extraction solvent), (vi) solvent is purged from cell with $N_2$ gas and (vii) depressurization took place. Between extractions, a rinse of the complete system was made in order to overcome any carry-over. The extracts obtained were protected from light, freeze-dried and stored at 4ºC until analysis.

### 2.4. Sample fractionation

Samples were dissolved with Milli-Q water to a final concentration of 1 mg dry matter (d.m.)/mL. Two milliliters of these samples were ultrafiltrated by using a Microcon centrifugal filter (Millipore, Billerica, MA, USA) with 3000 Da cut off membrane and centrifuging at 9000 g for 60 min. Retentate and filtrate were separately collected, freeze dried and stored at -20ºC for antioxidant capacity determination.

### 2.5. Total protein content

The protein content of the samples was determined by the Kjeldahl method (AOAC Official Method, 2002). Nitrogen data were converted into protein values employing a conversion factor of 6.25 and were expressed as grams per 100 g of d.m.. Analysis was performed in duplicate.

### 2.6. Amino acids availability determination (OPA assay)

Available free amino groups were determined in the natural SWE extracts by OPA assay to estimate the level of blockage during extraction process. OPA assay was performed as described by Goodno, Swaisgood and Catignani (1981) adapted to a micro-well plate (Arribas-Lorenzo, & Morales, 2009). Briefly, OPA stock solution was

prepared by dissolving 100 mg of OPA in 1 mL of methanol, 0.5 mL of β-mercaptoethanol, and 8.5 mL of 0.1 M borate buffer (pH 10). OPA working solution was prepared dissolving 100-fold OPA stock solution. Samples were dissolved to an appropriate dilution with borate buffer. Twenty microliters of the sample, 180 µL of 0.1 M borate buffer (pH 10) and 50 µL of OPA solution were placed in a 96-well black microplate (Porvair, Leatherhead, UK). The plate was incubated at room temperature for 2 min and fluorescence was determined at $\lambda_{exc}$= 340 nm and $\lambda_{em}$= 455 nm using a Synergy HT (BioTek, Bad Friedrichshall, Germany) spectrofluorometer and Gen version 5.1.06 software. Blanks of samples in the same concentration than in the assay (20 µL of sample + 230 µL of borate buffer) were also analyzed in order to discard possible interferences due to the natural fluorescence of the samples.

Quantitative analysis was performed by the external standard method, employing the calibration curve of leucine ranging from 1.5 to 24 µM in the well final concentration. Data were the mean values (n=6) expressed as mmols Leu /g d.m.

### 2.7. Phenol-sulfuric acid method

Phenol-sulfuric acid method was carried out according to Dubois, Gilles, Hamilton, Rebers and Smith (1956) and modified by Masuko, Minami, Iwasaki, Majima, Nishimura and Lee (2005) in order to determine sugar content in the extract obtained by SWE. Briefly, the method consists of adding 100 µL of sample, 300 µL of concentrated sulfuric acid and 90 µL of 5% phenol in water to an eppendorf vial, shaking the mixture plus heating for 5 min at 90 ºC in a static water bath. After cooling to room temperature for 5 min, 100 µL of the mixture were transferred into a well of the microplate and the absorbance was measured at 490 nm in a microplate spectrophotometer reader (Bio Tek) and compared to a glucose calibration curve (0.25-2.0 mg/ml, final concentration)

elaborated in the same manner. The data were expressed as the mean of triplicate analyses.

### 2.8. Total phenols determination (Folin-Ciocalteu Assay)

Total phenols were estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g d.m. (Koşar, Dorman & Hiltunen, 2005). The total volume of reaction mixture was miniaturized to 1 mL. Six hundred microliters water and 10 μL of sample were mixed, to which 50 μL undiluted Folin-Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2 % (w/v) $Na_2CO_3$ were added and the volume was made up to1.0 mL with water. After 2 h of incubation at 25 ºC, 300 μL of the mixture were transferred into a well of the microplate, the absorbance was measured at 760 nm in a microplate spectrophotometer reader (BioTek) and compared to a gallic acid calibration curve (0.025 – 2 mg/mL) elaborated in the same manner. The data were presented as the average of triplicate analyses.

### 2.9. Fluorescent advanced glycated end-products (AGEs) determination

Three hundred microliters of the extracts obtained by SWE from the natural samples (1 mg/ml) were collected and placed in a 96-well microplate. Fluorescence analysis was obtained at $\lambda_{Ex}$=360/40 nm and $\lambda_{Em}$ of 460/40, 528/20 and 590/35 nm using a microplate spectrofluorometer Synergy HT model (Bio-Tek). Data were expressed as the mean values (n=4) in arbitrary fluorescence units (FU).

### 2.10. Final MRPs (melanoidins) determination

Melanoidins were estimated by means of browning intensity of the extracted samples. Browning intensity was measured at 360 and 420 nm wavelengths using a microplate

spectrophotometer reader PowerWave TM XS model (BioTek). When necessary, samples were diluted in order to obtain an absorbance reading less than 1.5 arbitrary units.

Changes in composition induced by heating were also followed by spectral analysis. Three hundred microliters of the extracted samples were collected and put in a 96-well microplate, and the visible spectral analysis (380-780 nm) recorded in a Power Wave XS microplate spectrophotometer (Bio-Tek). All the measurements were made in triplicate. Results were expressed as arbitrary absorbance units (a.u.).

## 2.11. Antioxidant capacity measurements

### 2.11.1 ABTS assay

$ABTS^{\bullet+}$ radical scavenging capacity was determined using the method described by Re, Pellegrini, Proteggente, Pannale, Yang and Rice-Evans (1999) with some modifications. $ABTS^{\bullet+}$ was produced by reacting 7 mM ABTS and 2.45 mM potassium persulfate in dark at room temperature during 16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.7 (± 0.02) at 734 nm. Ten microliters of sample (10 mg d.m./mL) and 1 mL of $ABTS^{\bullet+}$ solution were mixed in a plastic vial and 300 μL of the mixture were transferred into a well of the microplate. The absorbance was measured at 734 nm every 5 min during 30 min in a microplate spectrophotometer reader (BioTek). Trolox was used as reference standard and results were expressed as TEAC values (mmol of trolox/g d.m.). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20-80% of the blank absorbance. Moreover, all analyses were done in triplicate.

2.11.2. $ORAC_{FL}$ assay

$ORAC_{FL}$ assay was carried out according to Ou, Hampsch-Woodill and Prior (2001) following a slightly modified method (Davalos, Gomez-Cordoves & Bartolome, 2004). Briefly, 20 μL of sample (50-100 μg d.m./mL) and 120 μL of fluorescein (70 nM of fluorescein in 75 mM phosphate buffer, pH 7.4, final concentration) were placed in a 96 multi-well microplate and preincubated at 37ºC for 10 min. The oxidation reaction was initiated by adding 60 μL of AAPH solution in a final concentration of 12 mM. The fluorescence readings were recorded at $\lambda_{exc}$=493 nm and $\lambda_{em}$=515 nm every minute for 104 min. A blank consisting in fluorescein, AAPH and phosphate buffer was also analyzed in each run. Calibration curves of trolox in concentrations of 10-80 μM (final concentration) were constructed and employed for quantification of the antioxidant capacity against peroxyl radicals. $ORAC_{FL}$ values calculation was performed employing the Fluostar Galaxy software and Microsoft Excel. Normalized antioxidant curves (fluorescence versus time) were obtained and the area under the fluorescence decay curve (AUC) was calculated employing the equation:

$AUC=1+f_{104}/f_0+f_{103}/f_0+.....+f_0/f_0$;

Where $f_0$ is the initial fluorescence reading at 0 min and $f_i$ is the fluorescence reading at time i. The net AUC of each sample was calculated by subtracting the AUC to the blank. Regression equations between net AUC and antioxidant concentration were obtained and employed for calculation of the $ORAC_{FL}$ values that were expressed as μmols of trolox equivalents/g d.m.

2.11.3. Superoxide radical ($O2^{\bullet -}$) scavenging capacity

The scavenging capacity against superoxide radicals was measured using xanthine/xanthine oxidase system (X/XO) and detected using nitro-blue tetrazolium

(NBT) reduction method combining the conditions previously described by others (Sanz et al., 1994; Fernandes et al., 2007) with some modifications. In this assay, $O2^{\bullet -}$ is generated *in vitro* by the xantina - xanthine oxidase system, these radicals reduced the yellow dye ($NBT^{2+}$) to produce the blue formazan colour which is associated with an increase in the absorbance at 560 nm. When a scavenger compound is added, it competes with the NBT for the oxidation of the generated superoxide anions. Therefore, there is a decrease in the rate of the NBT reduction, avoiding the development of blue color as a function of the antioxidant concentration. The assay mixture (final volume = 250 µL) contained 100 µM of xanthine, 100 µM of NBT and 1 mM EDTA in phosphate buffer 50 mM, pH 7.4, final concentrations. One hundred microliters of different dilutions of the samples (1-10 mg d.m./mL) or trolox were added into the well. A blank was analyzed with every batch of sample as a quality control measure. The reaction was started by the addition of 30 µL of XO (0.5 U/mL). Absorbance readings at 560 nm were taken every 30 sec for 2 min. Each experiment was performed in triplicate and each concentration generated a time-dependent curve. The percentage of inhibition for each concentration was calculated as follows: [100 – (slope/slope control) * 100]. The $IC_{50}$ of each compound was defined as the concentration which inhibited 50 % of the NBT reduction by $O2^{\bullet -}$ produced in the X-XO system.

**2.12. Data analysis**

Microsoft Excel 2000 Program was employed for statistical analysis of the data with the level of significance set at 95 %. One-way analysis of variance (ANOVA) was used to assess statistical differences between extractions. Differences were considered as significant different at a value of $p < 0.05$.

## 3. RESULTS AND DISCUSSION

As mentioned in the introduction section, SWE has been suggested as a green alternative to obtain functional food ingredients from natural matrices. Usually, complex extracts are characterized considering their bioactive components but usually the occurrence of modifications and/or interactions between the extracted compounds during SWE is not considered, even though they may contribute to the whole bioactivity of the extract. An exhaustive knowledge of extract composition is needed in order to increase the benefit/risk ratio of the functional ingredients obtained using SWE.

Therefore, the present work attempts to describe newly formed antioxidant compounds in natural samples extracted using SWE. Based on previously published results, Maillard reaction products (MRPs) and caramelization products are expected to be produced since they occur in model systems (Plaza, Amigo, Ibañez, Del Castillo, & Herrero, 2010), but in this case our interest is to know how different samples (with different composition in terms of aminoacids, sugars and phenolics, among others) behave and how their content can influence or modulate the occurrence of such reactions. Several natural matrices including microalgae, macroalgae and plants were selected as representative samples commonly used for the extraction of functional compounds by SWE.

### 3.1. SWE extraction of natural matrices

The ten different selected samples were extracted by SWE using water at two different temperatures, 100 and 200 ºC. As it can be seen in Figure 1, the temperature directly influenced the extraction yield for all the studied samples. This value was significantly increased ($p < 0.05$) in all samples, especially for red alga *Porphyra spp.* in which the yield was raised from 12.8% at 100 ºC to 68% at 200 ºC. Most samples showed an

increment higher than 25% between the two temperatures. This effect has been extensively observed in SWE processes (Herrero, Plaza, Cifuentes, & Ibañez, 2010), and it is basically explained by the increase on the mass transfer according to the increase in the extraction temperature.

### 3.2. Chemical characterization of the extracts

#### 3.2.1. Total phenols

Concerning the total phenols extracted (Table 1), measured by Folin method, it can be observed that this value also increased with the temperature for almost all the samples, although the highest differences could be observed among samples of different nature (Table 1). The richest phenol content was found in rosemary and thyme with values above 150 mg gallic acid/g d.m. For these samples, the increase on the extraction temperature produced a further but small increase on the amount of total phenols determined, although for thyme no statistical differences between the phenol content at the two different temperatures were observed ($p < 0.05$). For the rest of the samples, the increase on the extraction temperature produced an enhancement of the total phenols of, at least, twice, reaching for some samples more than ten-fold increase (*U. pinnatifida*). From the data presented on Table 1, it can be deduced that plants were richer on phenols, followed by algae, whereas the only microalgae studied (*C. vulgaris*) was the sample with the lowest phenol's content. This trend was maintained for the extracts obtained at 200 ºC, although verbena values were relatively close to those presented by some algae (*S. vulgare, U. pinnatifida*) (Table 1). These data has to be considered since this kind of compounds *per se* will have a strong influence on the total antioxidant capacity of the different extracts. On the other hand, phenolic compounds could modulate the occurrence and intensity of Maillard and caramelization reactions

(Gugliucci, Markowicz-Bastos, Schulze & Ferreira-Souza, 2009; Wang, Sun, Cao & Tian, 2009; Peng et al., 2008; Wu & Yen, 2005), thus influencing the final bioactivity of the extracts.

3.2.2. Nitrogen compounds

Total nitrogen content of the raw samples and primary available amino groups of the extracts were determined (Table 1). As can be observed, the total nitrogen content was statistically significantly different ($p<0.05$) among the samples, being *C. vulgaris* and *Porphyra spp*. the samples which presented higher values. On the other hand, SW extracts of the three plants and the algae *S. vulgare* and *C. abies-marina* showed the lowest levels of primary amino groups among the samples obtained at 100ºC. In contrast, the extracts of *H. incurvus* and *Porphyra* obtained at 100ºC were the samples with highest content in free amino groups. Comparing the results for the two extraction temperatures, significant decreases in amino acids availability were observed for all samples ($p<0.05$), except for rosemary (Table 1). Interestingly, this plant presented a high level of total phenols. On the contrary, the samples which presented the most marked decreases in the amino acids contents were *U. pinnatifida*, *S. muticum* and verbena, with reductions above 75% at 200 ºC compared to the extracts obtained at 100 ºC.

3.2.3. Sugars availability

Another parameter to be considered to know the progression of Maillard reaction is the amount of sugars remaining in the sample after SWE (obtained using the phenol-sulfuric acid assay). As it can be observed in Table 1, the behavior of the different samples was not always similar. The three studied plants, together with the microalga

sample (*C. vulgaris*) were the samples which presented higher amounts of sugars after extraction at 100 ºC. However, except for *Porphyra*, the algae samples contained relatively low amounts of sugars in their chemical composition (see data on Table 1). The amount of sugars determined in the extracts obtained at 200 ºC decreased compared to the amount of sugars at 100 ºC only for *C. vulgaris*, *S. muticum* and the three plant samples. Since both carbohydrate and free amino group contents were affected by the extraction temperature applied to *C. vulgaris*, *S. muticum* and verbena, the results suggested the occurrence of Maillard reaction during SWE processing.

For the rest of algae samples a significant ($p<0.05$) increase in the sugars concentration was observed. Nevertheless, in this case, these results are not against the hypothesis of the Maillard and caramelization reactions taking place during SWE at high temperatures, since we can consider that the increase in sugars concentration in the extracts at 200ºC may be a consequence of cellulose and/or other complex polysaccharides hydrolysis produced under SWE conditions at very high temperature.

3.2.4. Changes on pH

If the Maillard reaction progresses and a decrease on the amino groups available is produced as a result of their interaction with reducing sugars, a decrease on the pH of the samples can also be observed (Liu, Yang, Jin, Hsu & Chen, 2008). For this reason, the measure of pH of the sample can be considered as a fast method to monitor the occurrence of this kind of reactions. In Figure 2, the pH values found in the different extracts obtained at 100 and 200 ºC by SWE are shown. From this data, it can be observed that for all samples, the increase on the extraction temperature produced a decrease on the pH in the extracts. These decreases ranged from a 26% drop in the pH value of *Porphyra* to 13% for *S. muticum*. These differences might indicate a loss of

amino groups as a consequence of the increase rate of Maillard reaction since similar behavior was previously observed for glycation model systems (Plaza, Amigo, Ibañez, Del Castillo, & Herrero, 2010). However, in this case, the decrease on pH can be also due to the extraction of different compounds (e.g., phenolic acids) at the highest temperature. Further experiments should be performed in order to estimate the degree of progress of the Maillard reaction and its contribution to the pH of the samples.

3.2.5. Fluorescent advanced MRPs

It has been demonstrated that the formation of new compounds presenting strong emission between 400 and 500 nm when excited at a wavelength of 370 nm occur as a result of the Maillard reaction (Dolhofer-Bliesener & Gerbitz, 1990; Tregoat, Brohee, Cordeiro & Van Hengel, 2009; Matiacevich, Satagapita & Buera, 2005). These compounds are commonly called fluorescent AGEs (advanced glycation end products). In order to check the formation of this kind of compounds in the SWE extracts, fluorescence at $\lambda_{exc}$ 360/40 and $\lambda_{em}$ 460/40 wavelengths was measured. The results obtained are summarized in Table 2. As it can be observed, the fluorescence values of all samples were significantly increased ($p < 0.05$) after SWE at 200 ºC. The increment was significant but smaller for the three plants as well for *H. incurvus* (less than 5 times), whereas *U. pinnatifida* presented the highest increase in the fluorescence values. Data on fluorescence seem to indicate that the formation of fluorescence Maillard products is favored at 200 ºC compared to 100 ºC, being *U. pinnatifida* the sample containing highest amount of this type of products. Besides, it was also observed a correlation between data on fluorescence and amino acids availability, as it can be appreciated for *U. pinnatifida* (Tables 1 and 2).

### 3.2.6. Browning

It is widely accepted that the development of brown color can be effectively used to monitor the occurrence of non enzymatic browning reactions, including Maillard reaction (Purlis, 2010; Delgado-Andrade, Seiquer, Haro, Castellano & Navarro, 2010). In fact, this color is the easiest way of measuring the existence of MRPs, through just a visual estimation. For this reason, this value has been often employed as an indicator of the extent of Maillard reaction advances in foods, as well as a marker of occurrence of caramelization. The increase on browning is directly associated to advanced phases of the reaction (Morales & Jimenez-Perez, 2001). The absorbance at 360 nm and 420 nm is commonly employed to monitor the formation of brown advanced MRPs. However, browning is also associated to caramelization processes (Benjakul, Lertittikul & Bauer, 2005). Table 2 shows the data collected on browning. Significant differences between the values obtained at 360 and 420 nm were found for most of algae samples, in which increments between the values obtained at 100ºC and 200ºC could be assessed. *S. vulgare* extracts obtained at 200ºC produced a significant increment of the absorbance at 360 nm, but no statistically differences were observed in the absorbance measured at 420 nm between the two extracts. Interestingly, *U. pinnatifida* was again the algae in which the highest relative differences were observed between the two extraction temperatures. Data supported the occurrence of Maillard reaction during SWE processing of the sample and are in agreement with those of fluorescence and amino acids. On the contrary, for the microalgae sample no formation of brown MRPs was observed. In fact, the absorbance at 420 nm was lower for the *C. vulgaris* extracts obtained at 200ºC compared to 100ºC. Concerning plant samples, the absorbance values at 360 nm were by far the highest of the studied samples. However, comparing both temperatures, no statistical difference were observed (i.e., Verbena) or lower values

were recorded for the highest temperature (rosemary and thyme). Increments on absorbance at 420 nm were observed in the case of thyme and verbena. In general and taking into account data on amino acids, sugars, fluorescence and browning it can be said that the rate of the Maillard reaction and caramelization was different among the samples. Thus, new and different chemical structures may be formed during SWE treatment. In agreement, the contribution of the compounds formed in each case to the overall antioxidant properties of the extracts may be different.

### 3.3. Antioxidant capacity of the SWE extracts

The antioxidant capacity of SWE extracts was measured by using three different *in-vitro* assays, i.e., TEAC, $ORAC_{FL}$ and superoxide radical scavenging test. This latter method provided the $IC_{50}$ value, thus, indicating that higher antioxidant capacity was presented by the extracts with lower $IC_{50}$ values. The methods provided the capacities of extracts against three different radicals with the aim to gain insight of their mechanism of action. Results obtained are summarized in Table 3.

All the extracts were able to act against $ABTS^{\bullet+}$, peroxyl and superoxide radicals. In all cases those samples obtained at 200ºC showed antioxidant capacity significantly higher than the corresponding extracted at 100ºC. Rosemary was the only sample for which no statistical differences were observed between the antioxidant capacity of the two extracts neither by using the TEAC nor the $ORAC_{FL}$ method, whereas thyme extracts presented also similar ($p>0.05$) capacity against peroxyl and superoxide radicals. Nevertheless, these two samples (rosemary and thyme) were those which presented, by far, the highest antioxidant capacity among all the studied samples. These two samples were also those which presented highest contents on total phenols (see data on Table 1). The increments on the antioxidant capacity for the rest of samples ranged from twice for

verbena and *S. vulgare* (see Table 3) to more than 30-fold increments recorded for *U. pinnatifida*. In fact, the extracts produced at 100ºC from this alga possessed the lowest values of antioxidant capacity among all the samples.

The formation of neoantioxidants derived from Maillard reaction in glycation model systems was previously demonstrated by us (Plaza, Amigo, Ibañez, Del Castillo, & Herrero, 2010). Other authors have also proposed the formation of antioxidants during Maillard reaction and caramelization (Bekedam, Schols, Cämmerer, Kroh, Van Boekel & Smit, 2008; Del Castillo, Ames & Gordon, 2002; Atrooz, 2008; Cämmerer & Kroh, 2006; Mesa, Silvan, Olza, Gil & Del Castillo, 2008; Michalska, Amigo-Benavent, Zielinski & Del Castillo, 2008; Sahin, Topuz, Pischetsrieder & Ozdemir, 2009; Xu, Tao & Ao, 2007).

Therefore, the antioxidant capacity data collected seems to indicate the occurrence of Maillard reaction in most samples (although in lesser extent for thyme and rosemary). This conclusion is supported by data on amino acids, sugars, fluorescence and browning (see Tables 1 and 2).

Antioxidants with different physicochemical properties may be also formed in the SWE extracts. Future work employing antioxidant methods based on the use of the Quencher approach very recently proposed by others (Amigo-Benavent, del Castillo & Fogliano, 2010; Gokmen, Serpen & Fogliano, 2009; Serpen, Capuano, Fogliano & Gokmen, 2007) may be undertaken.

### 3.4. Fractionation of the extracts

In order to gain insight on the identity of the antioxidants of the SWE extracts, fractionation of the extracts at 100 and 200 ºC was performed. To do that, ultrafiltration using a filter device with cut-off of 3 kDa was carried out. Thus, two fractions of each

extract were obtained and divided in low molecular weight fraction (LMW, < 3 KDa) and high molecular weight fraction (HMW, > 3 KDa). The aim of this experiment was to estimate the contribution of low (phenols, small peptides, caramels and Maillard reaction products) and high molecular weight (melanoidins, proteins) compounds to the overall antioxidant capacity of the SWE extracts. As can be observed in Figure 3, both LMW and HMW antioxidants are present in the SWE extracts. A relationship between temperature of extraction and level of antioxidants was observed in all cases. The only exception was rosemary that, considering the results obtained from the rest of experiments, seems to be mainly affected by the composition in terms of phenolic compounds. However, for the rest of samples, according to the above mentioned evidences, the enhancements on the antioxidant capacity may be related to the formation of new compounds with antioxidant capacity derived from several chemical events including Maillard and caramelization reactions. On the other hand, the LMW fractions may be formed by phenolic compounds beside those new generated compounds during processing of the samples such as type Maillard compounds and caramels. Some low molecular weight compounds derived from this reaction have been previously described as possessing some antioxidant capacity (Plaza, Amigo, Ibañez, Del Castillo, & Herrero, 2010).

## 4. CONCLUSIONS.

The data presented in this contribution evidence, for the first time, the occurrence of chemical events like Maillard and caramelization reactions during SWE of natural samples at high temperatures. The extent of each reaction depends on the chemical composition and nature of the particular sample being extracted. It has been shown how in samples richer on phenolic compounds (i.e., rosemary and thyme), the occurrence of

these reactions is more limited. *Undaria pinnatifida* was the sample in which these reactions progressed in higher extent. These observations are also in agreement with the tremendous difference between the antioxidant capacity shown by the two extracts obtained from this alga. Data supported the formation of new antioxidants during SWE processing. Further research is currently under development in our laboratory to identify the structure of newly formed antioxidants and their individual contribution to the overall antioxidant capacity of the SWE extracts.


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## ABBREVIATIONS USED

**ABTS**, 2,2´-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); **AGEs**, advanced glycation end products; **APPH**, 2,2´-Azobis (2-methylpropionamidine) dihydrochloride; **ASE**, accelerated solvent extractor; **AUC**, area under curve; **d.m.**, dry matter; **EDTA**, ethylenediamine-tetraacetic acid; **FU**, fluorescence units; **GAE**, gallic acid equivalents; **HMW**, high molecular weight; **LMW**, low molecular weight; **MRPs,** Maillard reaction

products; **NBT**, nitro blue tetrazolium; **OPA**, O-Phthaldialdehyde; **$ORAC_{FL}$**, oxygen radical absorbance capacity-fluorescein; **SWE**, subcritical water extraction; **TEAC**, trolox equivalent antioxidant capacity; **X/XO**, xanthine/xanthine oxidase system.

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## FIGURE LEGENDS

**Figure 1.** Yields (%) of dried extract obtained after SWE of the natural samples at 100 and 200ºC. All the values for each sample are statistically different ($p<0.05$).

**Figure 2.** pH values determined for each sample after SWE at 100 and 200 ºC. All the values for each sample are statistically different ($p<0.05$).

**Figure 3.** Antioxidant capacity determined using (A) $ORAC_{FL}$ (μmol trolox equivalents/g) and (B) TEAC (mmol trolox equivalents/g) *in-vitro* assays on the low molecular weight (LMW) and high molecular weight (HMW) fractions of the natural extracts obtained after SWE at 100 and 200 ºC.

**Table 1**. Contents on total phenols, total protein, amino acids and sugars in the natural extracts obtained after SWE at 100 and 200ºC. Pairs of extracts with values not statistically different ($p>0.05$) are marked with superscript letters. All measurements were done at least by triplicate.

| Sample | Total Phenols (mg gallic acid/g extract) | | Protein (%) | Amino acids (µmol/g d.m.) | | Glucose (g/100g) | |
|---|---|---|---|---|---|---|---|
| | 100 ºC | 200 ºC | | 100 ºC | 200 ºC | 100 ºC | 200 ºC |
| ***Microalgae*** | | | | | | | |
| *Chlorella vulgaris* | 16.94 ± 7.26 | 32.91 ± 4.47 | 38.9 ± 0.1 | 19.97 ± 1.27 | 10.44 ± 1.11 | 34.90 ± 3.87 | 17.64 ± 1.24 |
| ***Algae*** | | | | | | | |
| *Sargassum vulgare* | 26.43 ± 2.46 | 70.86 ± 4.40 | 3.7 ± 0.1 | 2.20 ± 0.07 | 0.91 ± 0.09 | 2.95 ± 0.22 | 4.93 ± 0.05 |
| *Sargassum muticum* | 10.73 ± 0.52 | 58.67 ± 8.65 | 14.0 ± 0.0 | 18.58 ± 1.78 | 4.62 ± 0.39 | 7.44 ± 0.42 | 4.03 ± 0.23 |
| *Porphyra spp.* | 12.86 ± 1.09 | 35.30 ± 5.12 | 31.9 ± 0.0 | 25.51 ± 1.42 | 8.67 ± 1.03 | 25.70 ± 3.25 | 39.16 ± 1.49 |
| *Cystoseira abies-marina* | 6.81 ± 3.07 | 48.09 ± 4.51 | 7.5 ± 0.0 | 5.73 ± 0.55 | 1.83 ± 0.05 | 9.06 ± 0.92 | 14.18 ± 1.41 |
| *Undaria pinnatifida* | 3.79 ± 0.61 | 67.11 ± 6.00 | 12.9 ± 0.2 | 21.37 ± 1.36 | 3.18 ± 0.63 | 5.21 ± 0.21 | 6.52 ± 0.28 |
| *Halopitys incurvus* | 33.04 ± 3.43[a] | 41.78 ± 8.15[a] | 24.3 ± 0.3 | 29.37 ± 1.12 | 14.97 ± 0.66 | 6.71 ± 0.28 | 11.93 ± 0.76 |
| ***Plants*** | | | | | | | |
| Rosemary | 156.93 ± 4.51 | 192.84 ± 25.04 | 4.6 ± 0.1 | 0.36 ± 0.03 | 0.46 ± 0.03 | 47.29 ± 1.51 | 35.88 ± 1.83 |
| Thyme | 187.89 ± 14.04[b] | 188.48 ± 1.84[b] | 6.5 ± 0.2 | 0.50 ± 0.03 | 0.44 ± 0.05 | 41.50 ± 1.58 | 32.14 ± 4.12 |
| Verbena | 36.15 ± 4.09 | 81.14 ± 2.30 | 12.6 ± 0.2 | 1.55 ± 0.07 | 0.17 ± 0.02 | 42.62 ± 3.35[c] | 37.89 ± 2.67[c] |

**Table 2.** Fluorescence and browning of the SWE extracts produced at 100 and 200ºC. Pairs of extracts with values not statistically different ($p>0.05$) are marked with superscript letters. All measurements were done at least by triplicate.

| Sample | Flurescence | | Browning | | | |
|---|---|---|---|---|---|---|
| | $\lambda_{exc}$ 360/40; $\lambda_{em}$ 460/40 | | Absorbance at 360 nm | | Absorbance at 420 nm | |
| | 100 ºC | 200 ºC | 100 ºC | 200 ºC | 100 ºC | 200 ºC |
| ***Microalgae*** | | | | | | |
| *Chlorella vulgaris* | 326.667 ± 23.594 | 5867.833 ± 474.085 | 1.020 ± 0.038[a] | 1.056 ± 0.026[a] | 0.978 ± 0.036 | 0.602 ± 0.041 |
| ***Algae*** | | | | | | |
| *Sargassum vulgare* | 227.167 ± 26.453 | 1662.333 ± 7.095 | 0.298 ± 0.024 | 1.056 ± 0.014 | 0.519 ± 0.098[c] | 0.534 ± 0.007[c] |
| *Sargassum muticum* | 433.333 ± 9.201 | 3544.333 ± 289.010 | 0.236 ± 0.016 | 1.169 ± 0.006 | 0.104 ± 0.007 | 0.608 ± 0.001 |
| *Porphyra spp.* | 671.167 ± 35.290 | 3964.500 ± 454.442 | 0.271 ± 0.006 | 1.006 ± 0.031 | 0.125 ± 0.004 | 0.630 ± 0.019 |
| *Cystoseira abies-marina* | 215.667 ± 24.695 | 2981.333 ± 130.849 | 0.120 ± 0.007 | 0.932 ± 0.009 | 0.056 ± 0.004 | 0.468 ± 0.004 |
| *Undaria pinnatifida* | 211.167 ± 6.969 | 6585.667 ± 526.935 | 0.211 ± 0.004 | 1.170 ± 0.114 | 0.028 ± 0.001 | 0.537 ± 0.039 |
| *Halopitys incurvus* | 1459.333 ± 55.985 | 4501.000 ± 130.565 | 0.138 ± 0.010 | 0.545 ± 0.009 | 0.138 ± 0.010 | 0.307 ± 0.011 |
| ***Plants*** | | | | | | |
| Rosemary | 568.667 ± 20.027 | 2351.667 ± 192.062 | 2.781 ± 0.081 | 2.101 ± 0.070 | 0.307 ± 0.011 | 0.223 ± 0.004 |
| Thyme | 517.667 ± 25.477 | 2407.333 ± 328.124 | 2.439 ± 0.059 | 1.953 ± 0.107 | 0.182 ± 0.005 | 0.355 ± 0.023 |
| Verbena | 998.833 ± 81.710 | 5134. 500 ± 70.219 | 1.630 ± 0.024[b] | 1.649 ± 0.047[b] | 0.190 ± 0.003 | 0.466 ± 0.025 |

**Table 3.** TEAC, $ORAC_{FL}$ and superoxide radical antioxidant capacity values obtained for the studied natural extracts after SWE at 100 and 200 ºC. Pairs of extracts with values not statistically different ($p>0.05$) are marked with superscript letters. TE, trolox equivalents. All measurements were done at least by triplicate.

| Sample | TEAC (mmol TE/g d.m.) | | $ORAC_{FL}$ (µmol TE/g) | | Superoxide radical scavenging capacity $IC_{50}$ (mg/mL) | |
|---|---|---|---|---|---|---|
| | 100 ºC | 200 ºC | 100 ºC | 200 ºC | 100 ºC | 200 ºC |
| ***Microalgae*** | | | | | | |
| *Chlorella vulgaris* | 0.146 ± 0.004 | 0.789 ± 0.068 | 242.652 ± 32.017 | 1007.663 ± 125.296 | 10.752 ± 0.773 | 8.260 ± 0.303 |
| ***Algae*** | | | | | | |
| *Sargassum vulgare* | 0.852 ± 0.025 | 1.561 ± 0.077 | 439.534 ± 18.085 | 1742.670 ± 161.237 | 11.442 ± 1.054 | 4.391 ± 0.285 |
| *Sargassum muticum* | 0.302 ± 0.005 | 1.045 ± 0.099 | 284.172 ± 23.235 | 1449.344 ± 72.647 | > 50 | 3.564 ± 0.371 |
| *Porphyra spp.* | 0.193 ± 0.015 | 1.834 ± 0.164 | 329.191 ± 22.450 | 1349.683 ± 114.519 | > 50 | 13.565 ± 0.481 |
| *Cystoseira abies-marina* | 0.156 ± 0.014 | 1.006 ± 0.093 | 275.906 ± 34.658 | 1313.570 ± 139.662 | > 50 | 7.312 ± 0.296 |
| *Undaria pinnatifida* | 0.047 ± 0.006 | 1.512 ± 0.191 | 45.356 ± 4.829 | 1522.692 ± 150.928 | > 50 | 5.371 ± 0.284 |
| *Halopitys incurvus* | 0.355 ± 0.051 | 1.042 ± 0.017 | 629.802 ± 68.752 | 1498.756 ± 221.673 | 7.901 ± 0.164 | 5.851 ± 0.077 |
| ***Plants*** | | | | | | |
| Rosemary | 2.811 ± 0.091[a] | 2.805 ± 0.055[a] | 4891.529 ± 333.847[c] | 4614.561± 665.174[c] | 0.665 ± 0.113 | 0.464 ± 0.013 |
| Thyme | 2.316 ± 0.055[b] | 2.484 ± 0.213[b] | 3697.638 ± 307.177 | 4707.348 ± 276.959 | 0.540 ± 0.175[d] | 0.489 ± 0.004[d] |
| Verbena | 0.959 ± 0.120 | 2.156 ± 0.145 | 1757.403 ± 220.097 | 4214.173 ± 101.928 | 2.212 ± 0.080 | 0.636 ± 0.031 |

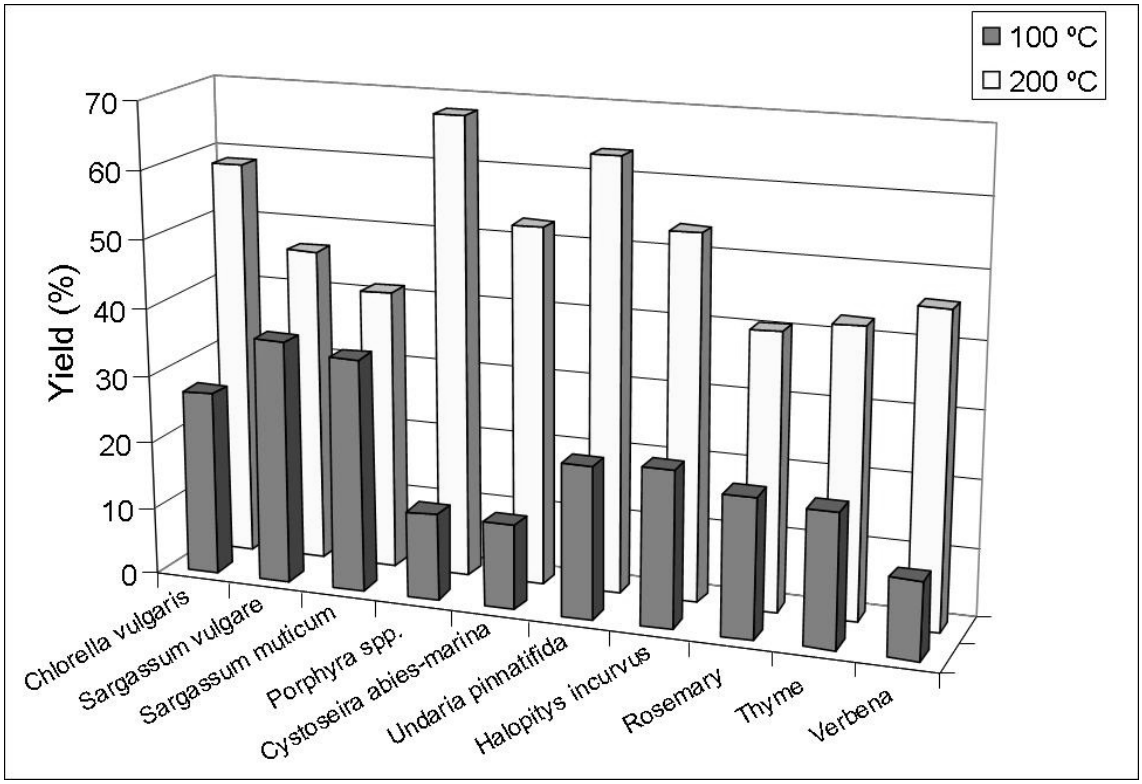
100 ºC
200 ºC
Yield (%)
70
60
50
40
30
20
10
0
Chlorella vulgaris
Sargassum vulgare
Sargassum muticum
Porphyra spp.
Cystoseira abies-marina
Undaria pinnatifida
Halopitys incurvus
Rosemary
Thyme
Verbena

**Figure 1.**

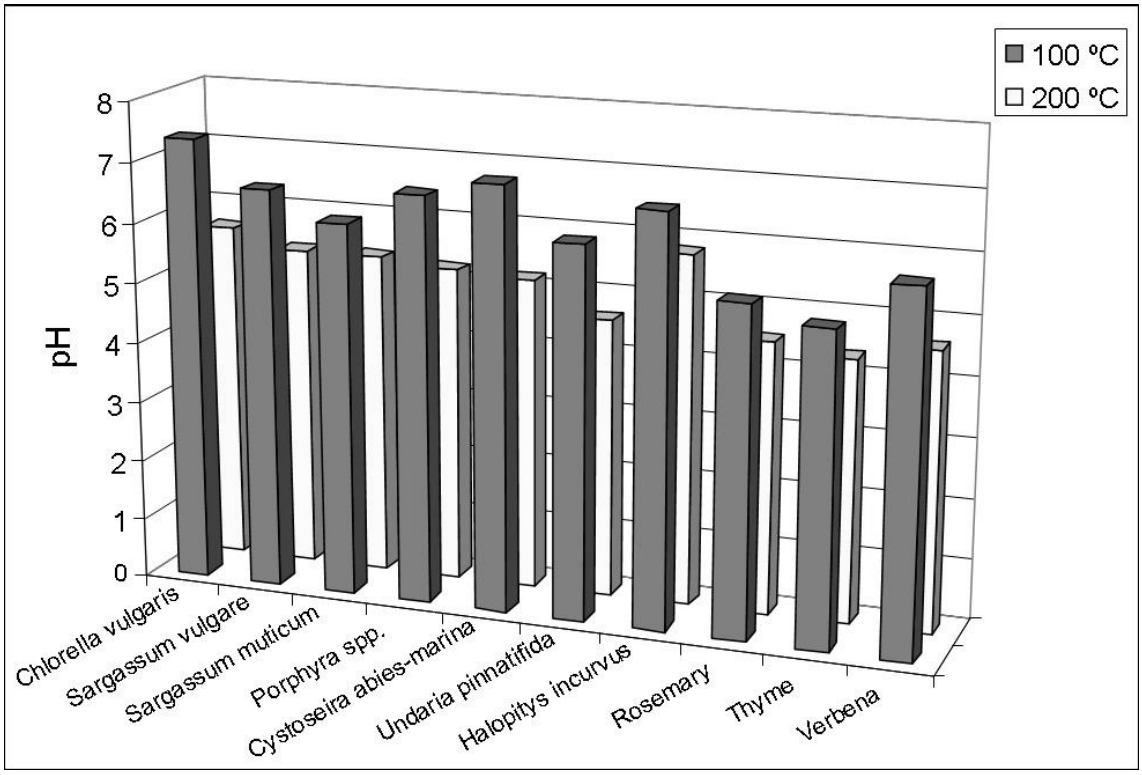
100 ºC
200 ºC
pH
8
7
6
5
4
3
2
1
0
Chlorella vulgaris
Sargassum vulgare
Sargassum muticum
Porphyra spp.
Cystoseira abies-marina
Undaria pinnatifida
Halopitys incurvus
Rosemary
Thyme
Verbena

**Figure 2.**

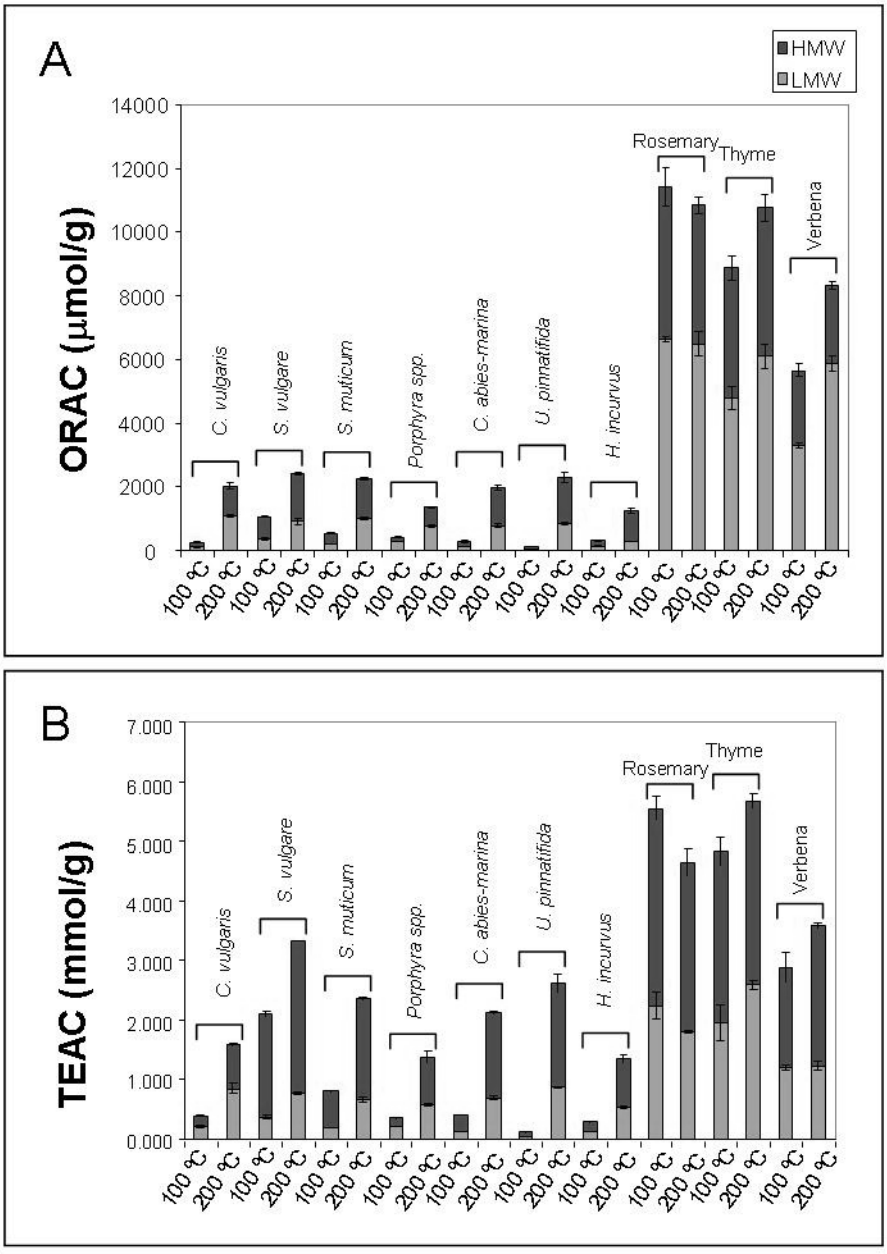
A
HMW
LMW
ORAC (µmol/g)
14000
12000
10000
8000
6000
4000
2000
0
C. vulgaris
S. vulgare
S. muticum
Porphyra spp.
C. abies-marina
U. pinnatifida
H. incurvus
Rosemary
Thyme
Verbena
100 ºC
200 ºC
B
TEAC (mmol/g)
7.000
6.000
5.000
4.000
3.000
2.000
1.000
0.000

**Figure 3.**