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# Determination of Total Antioxidant Capacities of Algal Pigments in Seaweed by the Combination of High-Performance Liquid Chromatography (HPLC) with A Cupric Reducing Antioxidant Capacity (CUPRAC) Assay

Sibel Yalçın[a], Merve Uzun[b], Özge Karakaş[b], Kevser Sözgen Başkan[a], Emine Şükran Okudan[c], and Mustafa Reşat Apak[a]

[a]Chemistry Department, Faculty of Engineering, Istanbul University-Cerrahpasa, Avcilar, Istanbul, Turkey; [b]Institute of Graduate Students, Chemistry Department, Istanbul University-Cerrahpasa, Avcilar, Istanbul, Turkey; [c]Faculty of Aquatic Sciences and Fisheries, Akdeniz University, Antalya, Turkey

**ABSTRACT**

This study mainly aimed to analyze the essential antioxidant algal pigments present in marine algae to quantify the total antioxidant capacity (TAC) of these pigment constituents with CUPRAC (cupric ion reducing antioxidant capacity) and ABTS spectrophotometric methods, and to correlate the TAC values combined with high-performance liquid chromatography (HPLC) results. *S. vulgare*, *C. barbata* brown and *F. petiolata* green algae species demonstrated the highest antioxidant activity with the values of $12.11 \pm 0.01$; $18.56 \pm 0.01$; and $13.98 \pm 0.02$ (mmol trolox (TR) $g^{-1}$ dw) obtained by the CUPRAC method. The individual antioxidant constituents of acetonic seaweed extracts were detected and quantified by HPLC on a C30 column using a modified mobile phase of gradient elution comprised of methanol-acetonitrile (50:50, v/v) with 0.1% (v/v) triethylamine (TEA) and acetone and ultraviolet detection for caroteneoids at 450 nm and for chlorophylls at 650 nm. The theoretical TAC values of seaweed samples were calculated by multiplying individual concentrations of pigments determined by HPLC with the spectrophotometric trolox equivalent or $\beta$-carotene equivalent antioxidant capacity (TEAC or CEAC) coefficient of each identified pigment and summing up this values. Among the studied seaweeds, those with the highest carotenoid content determined by HPLC were *S. vulgare* (51.77 μg/g), *H. incurva* (32.89 μg/g) and *F. petiolata* (200.12 μg/g), respectively. On the other hand, those with the highest total chlorophyll content were *F. petiolata* (1694.67 μg/g), *S. vulgare* (127.07 μg/g), and *C. barbata* (100.78 μg/g), respectively. As expected, fucoxanthin was present as the main carotenoid accumulated in the brown algae species presented in the study.

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## Introduction

Throughout the history of mankind, plants and marine origin plants have been widely used to treat diseases in addition to being used as a food source. Seaweeds are

CONTACT S. Yalçın sibelyal@istanbul.edu.tr Chemistry Department, Faculty of Engineering, Istanbul University-Cerrahpasa, Avcilar, 34320, Istanbul, Turkey.

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invaluable renewable marine bioresources, and each species is different from another; that is, each species has its own unique composition. Due to the biodiversity of algal active constituents, seaweeds are known to be potentially rich sources of valuable nutrients and almost the only choice in many different industrial fields: hydrocolloid extraction, fertilizers, extracts for cosmetics and pharmaceuticals, biofuels, bioplastics, and wastewater treatment (Andrade et al. 2013; Silva, Bahcevandziev, and Pereira 2019; Sanjeewa et al. 2018; Yalçın and Özyürek 2018).

The major biologically active compounds found in marine algae are commonly affirmed to be polysaccharides, proteins, polyunsaturated fatty acids, pigments, polyphenols, minerals, plant growth regulators, and others (Magnusson et al. 2015; Pangestuti and Kim 2011). Marine algae pigments can be classified into three major groups: chlorophylls, carotenoids, and phycobiliproteins. The color of marine algae typically refers to phycobilins for red, chlorophyll for green, and fucoxanthin for brown algae. Some of the most important components amongst the many other vital bioactive constituents present in seaweeds are carotenoids, including $\beta$-carotene ($\beta$-Car), lutein (Lt), antheraxanthin (Anthx), violaxanthin (Viox), neoxanthin (Neox), zeaxanthin (Zx), fucoxanthin (Fx), and siphonaxathin (Spx) (Kadam, Tiwari, and ÓDonnell 2013). In recent decades, the large-scale production of carotenoids from algal sources has gained much importance regarding scientific research and industrial applications in nutraceutical (Shannon and Abu-Ghannam 2019) and cosmeceutical fields (Jahan et al. 2017). The isolated form of fucoxanthin, which is an essential pigment mainly specific to brown algae, has been widely reported to be a rich potential antioxidant and has revealed inhibitory effects on various cancer cells such as human leukemia, breast and colon (Hosokawa et al. 1999; Konishi et al. 2006).

Yan et al. (1999) screened the radical scavenging antioxidant activity of edible brown seaweed and calculated the individual antioxidant contribution of isolated fucoxanthin. The protective effect of fucoxanthin isolated from a species of brown marine algae on ultraviolet radiation-induced cell damage was also investigated by Heo and Jeon 2009. Siphonaxathin (Spx) is one of the other precious carotenoids specific to green algae. It was extracted from *Codium fragile* green algae and exhibited a high inhibitory effect on human leukemia (HL-60) cells in comparison with other carotenoids (Ganesan et al. 2011).

Chlorophylls and derivatives, as well as carotenoids, have been reported to show strong antioxidant activity (Cho et al. 2011). Thus, pyropheophitin-*a* isolated from *E. bicyclis* marine algae, one of the chlorophyll *a*-related compounds, was shown to exhibit higher antioxidative activity than $\alpha$-tocopherol (Cahyana, Shuto, and Kinoshita 1992). The suppressive effects of the antioxidant marine pigment constituents on the release of the associated inflammatory mediators are most likely related to their antioxidant activities. Antioxidants act as scavengers of reactive oxygen species and are capable of repairing oxidative damage caused by free radicals and reactive species such as superoxide-, hydroxyl-, and hydrogen peroxide. Chlorophylls and pheophytins (magnesium-free chlorophyll derivatives) have been stated to exhibit antimutagenic and antioxidant activity to block radical chain reactions arising from autooxidation of edible vegetable oils (stored in the dark) by means of an effective proton donor mechanism (Ferruzzi et al. 2002; Osuna-Ruíz et al. 2019).

Therefore, algal pigments can play a key role in the prevention and treatment of chronic degenerative diseases. Many recent studies have focused only on the

determination of the total antioxidant capacity of macroalgal extracts rather than the individual antioxidant capacity of the ingredients present in algae (Chew et al. 2008). The total antioxidant capacity offers the antioxidant contribution of all other bioactive molecules that can be extracted together with the target compounds. As a result, the total antioxidant capacity may be associated with increased bioactivity with synergistic effects between different molecules/groups co-extracted in addition to the contribution of each bioactive component (Başkan et al. 2013). The main advantage of the methods calculating the antioxidant activity of a single compound and its contribution to the total activity of a complex mixture is that the activity of a single compound is comparable to those of other constituents in the complex matrix.

Reversed phase - high performance liquid chromatography (RP-HPLC) is commonly used to determine carotenoids (Inbaraj, Chien, and Chen 2006; Simonovska et al. 2013) and chlorophylls (Kao, Chen, and Chen 2011; Loh et al. 2012), and both simultaneously (Gökmen, Bahçeci, and Acar 2005; Puspitasari-Nienaber, Ferruzzi, and Schwartz 2002) in seaweed samples.

The most commonly applied methods and reagents to measure the antioxidant activity of plants are ABTS/TEAC (2,2′-azinobis [3-ethyl benzo thiazoline-6-sulfonate])/(trolox equivalent antioxidant capacity) (Arnao, Cano, and Acosta 2001) (Re et al. 1999), DPPH (2,2-diphenyl-1-picrylhydrazyl) (Brand-Williams, Cuvelier, and Berset 1995), and CUPRAC (cupric reducing antioxidant capacity) assays. The CUPRAC assay developed by Apak et. al. (2004) is simple, diversely applicable to both hydrophilic and lipophilic antioxidants, and its reagents are stable and easily available at low cost. The CUPRAC method was classified as one of the electron transfer-based methods, and its superiorities to other antioxidant assays were summarized by Prior, Wu, and Schaich (2005). They also reported that some substrates including reducing sugars and citric acid with no antioxidant properties can be oxidized with other antioxidant assays but not with the CUPRAC reagent advantageously having lower redox potential. The CUPRAC method was also utilized by Fung, Hamid, and Lu (2013) to measure the antioxidant activity of brown algae *Undaria pinnatifida* from New Zealand. Gorinstein et al. (2006) reported that, unlike most other electron-transfer based assays, CUPRAC operates at a realistic pH close to the physiological pH .

The main aim of this research is to identify the antioxidant pigments of the acetonic extracts of different seaweed samples by a combined HPLC-CUPRAC assay.

In order to separate, identify and quantify the individual carotenoid and chlorophyll pigments in the selected seaweeds, the most suitable mobile phase composition for HPLC analysis was applied using a synthetic mixture of antioxidant algal pigments; calibration curves were generated for each pigment, and the pigments present in the seaweed extracts were quantified by virtue of these curves. The HPLC-specified concentration of each pigment was multiplied with its TEAC (trolox equivalent antioxidant capacity, defined as the mM trolox (TR) equivalent concentration of 1 mM solution of the tested antioxidant) coefficient, and these values were summed to obtain the theoretical TAC value by means of the additivity of absorbances of constituents in a mixture. In conclusion, the HPLC results provided a comparison of the contribution of each pigment in the seaweed species to overall antioxidant capacity with the equivalent antioxidant capacity of both trolox and $\beta$-Car.

## Materials and methods

### Seaweed collection

The seaweeds in the full-grown period were collected from Çanakkale located on the western coast and Antalya on the southern coast of Turkey. The names of the collected seaweed samples and their abbreviations are as follows: *Sargassum vulgare*: *S. vulgare; Cystoseira barbata*: *C. barbata; Cystoseira montagnei J. Agardh* 1842: *C. montagnei, Halopithys incurva*: *H. incurva, Gracilaria bursa-pastoris*: *G. b-pastoris; Palisada perforata*: *P. perforata, Ellisolandia elongata*: *E. elongata, Caulerpa scalpelliformis f. denticulata*: *C.s.f. denticulata; Penicillus capitatus*: *P. capitatus; Flabellia petiolata*: *F. petiolata.*

The availability of the algae and their potential cultivation were presented in the two papers by Yalçın et al. (2019; 2020). The macroscopic epiphytes and other impurities on the collected seaweeds were removed by rinsing and brushing. Next the seaweeds were immersed in containers with liquid nitrogen. After transfer to the laboratory, they were lyophilized, ground and stored in polypropylene bottles in the dark at room temperature until needed. Identification of the materials was done withn Olympus SZX16 stereo zoom and BX51 model binocular light microscopes.

### Chemicals and standards

$\beta$-Carotene ($\beta$-Car), $\alpha$-carotene ($\alpha$-Car), lutein (Lt), zeaxanthin (Zx), fucoxanthin (Fx), violaxanthin (Viox), astaxanthin (Astx), triethylamine (TEA), potassium persulfate, neocuproine (Nc) (2,9-dimethyl-1,10-phenanthroline), ammonium acetate ($NH_4Ac$), (Sigma-Aldrich), ABTS (2,2′-azinobis[3-ethylbenzothiazoline-6-sulfonate]) chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) (Sigma), $CuCl_2{\cdot}2H_2O$ (copper (II) chloride dihydrate), hydrogen peroxide (Merck), HPLC grade solvents (acetone, methanol (MeOH), ethanol (EtOH), acetonitrile (ACN)) (Riedel-de Haën) were supplied from the indicated sources.

### Instrumentation

A Metrohm Herisau E512 pH-meter was used for pH measurements (Herisau, Switzerland). The extraction operations were made using a Bransonic 221 ultrasonic bath, a HB4 basic KIKA-WERKE water bath, and an Elektro-mag vortex mixer (U.S., Shelton). Seaweed samples were lyophilized using a Telstar Cryodos freeze-dryer (Terrassa, Spain). Spectrophotometric measurements were made with a Cary 1E spectrophotometer (Varian Instruments) (Sydney, Australia), and chromatographic separation and identification of pigment constituents were performed using a Waters HPLC system (Milford, MA, U.S.A.) equipped with a 1525 binary pump, a column oven with thermostat, a 2998 PDA (photodiode array) detector (Chelmsford, MA, U.S.A.). Pure distilled water was used throughout, as obtained from Millipore Simpak1 Synergy 185 ultra-pure water system.

### Preparation of samples

The extraction procedure for carotenoids and chlorophylls was applied as follows: (i) 2 g of the lyophilized sample powder of seaweeds was weighed in flasks and (ii) extracted

for 10 min with 5 mL of acetone in an ultrasonic bath at room temperature protected from light; (iii) the upper phase was decanted, and (ii) and (iii) were repeated a total of five times. The extracts were combined and diluted to 25 mL with acetone and centrifuged at 4000 rpm for 5 min; the supernatants were passed through GF/PET (glass fiber/polyethylene terephthalate) 1.0/0.45 $\mu$m microfilters.

According to a previous study performed in our laboratory (Başkan et al. 2013), acetone was chosen as the most suitable solvent for pigment extraction due to the advantage of being miscible with water. Hence, CUPRAC and ABTS methods were applied without difficulty. Furthermore, Warkoyo and Saati (2011) reported that the pigment extraction of three algae species (red, green and brown) with acetone was more effective in comparison with other solvents (ethanol, hexane, and petroleum benzene).

### Reagents and solutions

$\beta$-Car, $\alpha$-Car, Lt, Zx, Fx, Viox, Neox, Astx, Chl *a*, and Chl *b* solutions were prepared as stock solutions in HPLC grade acetone and were used freshly; if necessary, they were stored at $-20\,^{\circ}C$ for less than one week and checked by absorbance at maximum absorption wavelength before use (Başkan et al. 2013). For the CUPRAC test of TAC, the following solutions were prepared: $CuCl_2$ solution, $1.0 \times 10^{-2}$ M, prepared by dissolving $CuCl_2{\cdot}2H_2O$ in water; ammonium acetate ($NH_4Ac$) buffer at pH 7.0, 1.0 M, prepared from $NH_4Ac$ in water; neocuproine (Nc) solution, $7.5 \times 10^{-3}$ M, prepared daily by dissolving Nc in 96% ethanol (Apak et al. 2004). For the ABTS test of TAC determination, the chromogenic radical reagent ABTS at 7.0 mM concentration was prepared by dissolving this compound in water and adding $K_2S_2O_8$ to this solution such that the final persulfate concentration in the mixture was 2.45 mM. The resulting ABTS radical cation solution allowed to stand at room temperature in darkness for 12 to 16 h, and then used for ABTS/TEAC assays. The reagent solution was diluted with acetone at a volume ratio of 1:10 prior to use.

## Spectrophotometric methods

### CUPRAC method

A mixture comprised of 1 mL of $1.0 \times 10^{-2}$ M copper(II) chloride, 1 mL of 1 M $NH_4Ac$ buffer at pH 7.0, and 1 mL of $7.5 \times 10^{-3}$ M Nc solution was prepared, x mL of sample solution and $(1.0 - x)$ mL acetone were added, and mixed well (total volume: 4.0 mL). This final mixture in a stoppered test tube was allowed to stand at room temperature for 30 min. At the end of this period, the absorbance at 450 nm was measured against a reagent blank. This method was applied to the pigment standards and seaweed extracts.

### ABTS method

The ABTS/persulfate method reported by Re et al. (1999) was employed. Briefly, 1 mL acetone was added to 3 mL of the radical cation ($ABTS^{\bullet+}$) solution produced by the reaction between ABTS and potassium persulfate, and the absorbances were recorded at 734 nm at the end of the sixth minute (i.e., this optimal time was experimentally

determined for $\beta$-Car and other pigments). The procedure was repeated for the antioxidant pigment by adding 3 mL of the radical cation solution to x mL of sample solution and $(1.0 - x)$ mL of acetone, and recording the absorbance readings at the fifth min. The absorbance of the reagent blank ($A_0$) diminished in the presence of antioxidant pigment, the absorbance decrease ($\Delta A$) being proportional to antioxidant pigment concentration. Linear regression equations were obtained using both TR and $\beta$-Car as $\Delta A$ *versus* molar concentration.

### Chromatographic analysis

The analyses were performed using a Waters YMC C30 (250 mm × 4.6 mm, 5 $\mu$m particle size) chromatographic column. Detection was carried out within the wavelength range from 200 to 800 nm with a photodiode array (PDA) detector. The elution rate was 1.5 mL $min^{-1}$ and the column temperature was 35 °C. For pigment compounds, two different solutions of the mobile phase, i.e., methanol/acetonitrile (50:50, v/v) with 0.1% (v/v) triethyl amine (A) and acetone (B), were used in gradient elution. The following working mode was adopted for gradient elution (Başkan et al. 2013): 15 min 100% A (slope 0); 10 min from 100% to 30% A (slope, 4); 15 min from 30% to 0% A (slope, 4) (using Empower 2 Software, Waters). Using the above working mode, the calibration curves and linear equations of peak area *versus* concentration were determined for the antioxidant pigments.

With the aid of these calibration curves, plant extracts in acetone, and synthetic mixtures of pigments were analyzed. Qualitative and quantitative analyses of sample solutions were conducted with at least three injections whose volume was 20 µL. The chromatographic column was washed for 10 min with acetone prior to each injection and equilibrated for 10 min with a solvent mixture containing 0.1% (v/v) triethyl amine.

### Theoretical TAC calculation

Theoretical TAC values as trolox equivalents (TE) were obtained by multiplying individual concentrations of pigments determined by HPLC with their spectrophotometric TEAC coefficients and summing the values:

$$\text{Theoretical TAC} = \sum_{i=1}^{n} C_i \ (\text{TEAC})_i \tag{1}$$

where $c_i$ is the concentration of antioxidant component $i$ found with the help of HPLC, and $(TEAC)_i$ is the trolox equivalent antioxidant capacity (TEAC) coefficient of component i with respect to a given spectrophotometric method (either CUPRAC or ABTS). The theoretical TAC (thus calculated) and experimental TAC (directly measured with a given spectrophotometric method) values were compared as $\mu$mol trolox ($TEAC_{CUPRAC} = (\varepsilon_{Pigment}/\varepsilon_{Trolox})$) and $\mu$mol $\beta$-Car ($\beta$-$CEAC_{CUPRAC} = (\varepsilon_{Pigment}/\varepsilon_{\beta\text{-carotene}})$) equivalent per g dw (dry weight) seaweed samples. Both TEAC and $\beta$-CEAC coefficients were unitless, as they represented antioxidant power relative to a reference compound (i.e., TR and $\beta$-Car, respectively). Since the calculated (theoretical) capacities involve the combined use of chromatographic and spectrophotometric methods, HPLC – CUPRAC term was

used to express the added products of HPLC-assayed individual antioxidant concentrations and their TEAC (or $\beta$-CEAC) coefficients found by CUPRAC.

### Statistical analysis

Spectrophotometric measurements were performed in triplicate for each sample and standard. The results of statistical analyses were obtained using Excel (Microsoft Office 2016) for determining the means and the standard error of mean. Results were presented as mean values ± standard deviation (SD). HPLC measurements were obtained as the average of three values for each standard and the mixture of standard. In order to verify the statistical correlation for antioxidant capacity values of seaweed extracts, the Spearman's rank correlation coefficient (or Spearman's rho) was employed.

## Results and discussion

### Spectrophotometric results of the analyzed carotenoid and chlorophyll standards

The total antioxidant capacity values originated from carotenoid and chlorophyll pigment standards expected to be present in the seaweed samples were determined in trolox equivalents as the standard reference antioxidant compound, and thus, the linear calibration equations of TR were $y = 1.634 \times 10^4 c + 0.0011$ ($R^2 = 0.9992$) for CUPRAC method and as $y = 1.473 \times 10^4 c - 0.0605$ ($R^2 = 0.9998$) for ABTS/persulfate spectrophotometric reference method. The parametric symbols used in the calibration equations were y for absorbance, c for antioxidant molar concentration, and $R^2$ for the determination coefficient. The absorbance values measured for carotenoid and chlorophyll pigments were recorded against their tested concentrations (absorbance measurements were realized in quartz cuvettes having 1 cm light path) and linear calibration equations were drawn between concentration and absorbance values. The molar absorption coefficients ($\varepsilon$) and linear working ranges conforming to Beer's law are given in Supplemental Table S1. The TAC values were expressed as TR and $\beta$-Car equivalents.

To determine these values, the molar absorptivity coefficient of the tested pigment was divided by that of TR and $\beta$-Car under the same conditions ($TEAC_{CUPRAC} = \varepsilon_{Pigment}/\varepsilon_{TR}$) and ($CEAC_{CUPRAC} = \varepsilon_{Pigment}/\varepsilon_{\beta\text{-Carotene}}$); $\beta$-Car was selected to evaluate the total antioxidant capacity of seaweed samples since it is one the most common carotenoids and often considered the general representative of pigments. In a study reported by Kobayashi et al. 2007, the redox potential of Chl *d* was examined and this value was compared to the redox potentials of Chl *a* and *b*. In this study, when acetonitrile was used as solvent, $E_{red}$ = (-1.12 V) - (-1.46 V) for Chl *a* and $E_{red}$ = (-1.02 V) - (- 1.41 V) for Chl *b* (against standard hydrogen electrode) were determined. On the other hand, the reduction potential of the $[Cu(Nc)_2]^{2+}$ complex used in the CUPRAC method is known to be 0.6 V (Apak et al. 2016; Özyürek et al. 2011). As a result, chlorophylls are oxidized rapidly and efficiently with $[Cu(Nc)_2]^{2+}$ and the quantity of colored product (i.e., $[Cu(Nc)_2]^{+}$ chelate) emerging at the end of the redox reaction is equivalent to that of reacted Cu(II)-Nc. Since chlorophyll is completely oxidized, there is no unreacted chlorophyll in the medium. In fact, the r values (0.998) determined from the chlorophylls (*a* and *b*) calibration graphs confirm this.

### HPLC results of the analyzed carotenoid and chlorophyll standards

The chromatogram of the mixture of ten pigment standards analyzed by RP-HPLC is presented in Figure S1. The linear calibration graphs were created between concentration and chromatographic peak area and the calculated equations and $R^2$ values are given in Table S2. The maximum absorption wavelengths ($\lambda_{max}$) were detected by the absorbance spectrum of pigment standards and these values were also compared with literature findings. These experimental (found $\lambda_{max}$) and theoretical (reported $\lambda_{max}$) value and retention times were presented in Table S2. The individual contribution of each carotenoid and chlorophyll pigment to the TAC of algae samples was determined with the help of calibration curves in HPLC.

Taking advantage of the additivity property of TAC in a complex sample, the theoretical total antioxidant capacities of seaweed samples were calculated by multiplying the concentration with the TEAC coefficient of each identified antioxidant (pigment), and adding the products. The sum of the individual antioxidant capacities of compounds is very close to the total antioxidant capacity (TAC) of the combination of these compounds (Im et al. 2014). The CUPRAC spectrophotometric method obeys Beer's law in regard to the additivity of absorbances due to individual antioxidant constituents, because a single chromophore, cuprous-neocuproine, is formed upon reduction of the CUPRAC reagent with antioxidants. Consequently, the CUPRAC-TAC values of antioxidants in complex mixtures are perfectly additive (Apak et al. 2016).

In the calibration equations given in Table S2, y represents peak area, c pigment molar concentration, and $R^2$ linear correlation coefficient. The chromatograms were achieved with the optimized separation scheme at 450 nm and 650 nm for carotenoid and chlorophyll pigments, respectively. The results are displayed in Figure S1. All of the pigment standards were separated within 30 min. The other pigments that can be found in seaweeds, namely Spx, Anthx, chlorophyll *c* (Chl *c*), pheophytin *a* (Php *a*), were identified depending on the literature source (Heriyanto et al. 2017; Kuczynska, Jemiola-Rzeminska, and Strzalka 2015, Sanz et al. 2015, Wright et al. 1991).

### Antioxidant capacities of the seaweed extracts

Seaweed extracts were spectrophotometrically analyzed with CUPRAC and ABTS methods to determine TAC values. As given in Table 1, the TAC values (in TR and $\beta$-Car equivalents, as TE and $\beta$-CE, respectively) were found with both spectrophotometric CUPRAC and ABTS methods and combined HPLC-spectrophotometric method (HPLC-CUPRAC) for seaweed samples. Inspecting the results presented in Table 1, although $\beta$-Car equivalent TAC values for the same algae were high, they were much less than the TE capacity values. This was due to the fact that the absorption coefficient obtained for $\beta$-Car was approximately 3.2 times greater than that of TR (Table S1). Since $\beta$-Car is the general representative of carotenoids, it was selected to be the reference constituent.

The theoretical TAC values obtained by HPLC were compared with the TAC values determined spectrophotometrically. Thus, it became possible to evaluate the HPLC-TAC values corresponding to the percentage of the TAC of constituents in seaweeds. The percentage ratios of 100 × HPLC-TAC (TE)/TAC (TE) and 100 × HPLC-TAC ($\beta$-CE)/

**Table 1.** Theoretical and experimental TAC (total antioxidant capacity) values of seaweed samples in the units of mmol trolox $g^{-1}$ dw algae (TE) and $\mu$mol $\beta$-Caroten $g^{-1}$ dw algae ($\beta$-CE).

| | CUPRAC | | HPLC-CUPRAC* | | ABTS |
|---|---|---|---|---|---|
| Algae sample | TAC (TE) | TAC ($\beta$- CE) | TAC(TE) | TAC($\beta$- CE) | TAC(TE) |
| Brown (Phaeophyceae) | | | | | |
| *S. vulgare* | 12.11 ± 0.01 | 3.56 ± 0.009 | 1.33 ± 0.06 | 0.41 ± 0.021 | 4.71 ± 0.01 |
| *C. barbata* | 18.56 ± 0.01 | 5.59 ± 0.010 | 0.80 ± 0.03 | 0.25 ± 0.011 | 9.22 ± 0.01 |
| *C. montagnei* | 0.30 ± 0.04 | 0.10 ± 0.004 | 0.16 ± 0.01 | 0.050 ± 0.002 | 0.20 ± 0.01 |
| Red (Rhodophyceae) | | | | | |
| *H. incurva* | 3.22 ± 0.04 | 0.96 ± 0.036 | 0.80 ± 0.04 | 0.24 ± 0.010 | 1.64 ± 0.01 |
| *G. bursa-pastoris* | 1.0 ± 0.004 | 0.32 ± 0.004 | 0.20 ± 0.01 | 0.06 ± 0.001 | 1.0 ± 0.01 |
| *P.perforata* | 0.40 ± 0.01 | 0.10 ± 0.010 | 0.04 ± 0.001 | 0.01 ± 0.002 | 0.20 ± 0.01 |
| *E.elongata* | 1.18 ± 0.003 | 0.32 ± 0.003 | 0.68 ± 0.02 | 0.21 ± 0.012 | 1.08 ± 0.03 |
| Green (Chlorophyceae) | | | | | |
| *C.s.f. denticulata* | 3.61 ± 0.01 | 1.09 ± 0.006 | 0.61 ± 0.01 | 0.19 ± 0.010 | 1.0 ± 0.01 |
| *P.capitatus* | 0.48 ± 0.01 | 0.13 ± 0.010 | 0.05 ± 0.002 | 0.012 ± 0.001 | 0.28 ± 0.01 |
| *F.petiolata* | 13.98 ± 0.02 | 4.36 ± 0.03 | 12.86 ± 0.12 | 4.27 ± 0.081 | 2.4 ± 0.01 |

*HPLC-CUPRAC values represent the theoretically found TAC using the additivity principles.

TAC ($\beta$-CE) were found to be approximately 11.0% and 11.5%, 4.3% and 4.4%, and 53.0%, and 50.0 for *S. vulgare*, *C. barbata*, and *C. montagnei brown* algae species, respectively. The ratios for the *H. incurva, G. bursa-pastoris, P. perforata, and E. elongata* algae species from the red algae group were 25.0% and 25.0%, 29.0% and 19.0%, 10.0% and 10.0%, 57.6% and 65.6% respectively. The same ratios were 17.4% and 17.0%, 92.0% and 97.9% and 10.4%, 9.2% for *C. scalpelliformis f. denticulata, F. petiolata* and *P. capitatus* green algae species, respectively.

For all analyzed algae species, the calculated HPLC-CUPRAC TAC values corresponded to the percentage ratios ranging from approximately 4.3% to 97.9% of experimental TAC values. This means that experimental and theoretical antioxidant capacity values are generally quite different from another. These somewhat incompatible results, apart from the compounds determined by standards and literature on the studied algae species, may stem from distinct pigment components in the chromatogram whose content and quantity cannot be assayed. Another highly possible reason is that the phenolic components, which are partially extracted with acetone but do not form a peak (i.e., do not exhibit absorption) in the range from 450 to 650 nm, probably increase the TAC value.

Scientists from New Zealand using the CUPRAC method to determine the antioxidant capacity of *U. pinnatifida* seaweed also reported that the antioxidant effect of seaweeds, particularly the brown species, was mainly derived from phenolic compounds called phlorotannins (Fung, Hamid, and Lu 2013). In this work, apart from algal pigments, the acetonic seaweed extract may possibly contain a variety of constituents, including sulfated polysaccharides, alkaloids, and phenolics that may participate in total antioxidant activity. Recent studies have linked the antioxidant activity of brown marine macroalgae to chiefly sulfated polysaccharides and phenolics as well as various compounds including pigments; hence both have the ability to quench free radicals. For instance, sulfated polysaccharides were isolated with acetonic solvent from the *Cystoseira* species by Kosanić, Ranković, and Stanojković 2015; Sellimi et al. 2014 and *S. vulgare* brown algae by Dore et al. 2013. This means that synergistic effects may take place between these constituents, resulting in an increase in total antioxidant activity of algal extracts containing the antioxidant active components.

Synergism or antagonism occurs when the antioxidative protection/scavenging of two antioxidants is greater or smaller than the sum of their individual effects, respectively (Apak et al. 2016). The 'synergistic antioxidant interaction' means that when two or more antioxidants are present together, the combined effect of the antioxidants in the mixture is greater than the sum of the effects of individual antioxidants found separately (Tsao 2015). This does not depend on the mechanism of the assay, which may be based on electron-transfer or radical scavenging action, as long as the mixture response would be larger than the additive response of individual antioxidants. As the composition and the quantity of constituents present in algae may change greatly depending on many factors such as seasonal changes, location, growth period, and species of algae, variable results are actually expected.

Among the seaweeds from the same color group, there may be great differences in terms of content and quantity, but even for the same algae, significant differences may possibly be determined. In this study, the seaweed samples were particularly collected in the abundant periods in which they were considered to be more productive. As the results of HPLC-TAC (TE)/TAC (TE) and HPLC-TAC ($\beta$-CE)/TAC ($\beta$-CE) ratios were very compatible, in addition to TR, $\beta$-Car was found suitable and necessary as a reference to assess the antioxidant potential of seaweeds. In the spectrophotometric total antioxidant capacity determination, the commonly used ABTS/TEAC method having partially a similar mechanism to that of the CUPRAC/TEAC method was applied as a reference method due to the use of TR, a water-soluble synthetic derivative of vitamin E as standard reference material.

The differences between the CUPRAC and ABTS results in Table 1 occur because the mechanisms of the two methods are different (i.e., ABTS is a mixed-mode assay comprising both electron transfer and hydrogen atom transfer mechanisms). The Spearman's rho values for CUPRAC and ABTS were 0.933; $p(2\text{ tailed}) = 8 \times 10^{-5}$. The data indicated a strong correlation between the chosen methods. On the other hand, a study using different methods by Müller, Fröhlich, and Böhm (2011) compared the antioxidant activities of carotenoids. The methods used include FRAP, ABTS and DPPH, and the results are compared as α-tocopherol equivalents mol α-TE/mol).

In this study, neither the carotenes nor the xanthophylls showed any DPPH• scavenging activity. 20 carotenoid compounds were examined, and there were differences depending on the method and the structure of the compound. According to the findings of the FRAP method, the ranking in terms of antioxidant activities was Lt > Zx > Anthx > Viox > Neox > Astx, while in the ABTS method the order was α-Car > $\beta$-Car > Lt = Anthx > Zx > Viox > Neox. FRAP and ABTS assays are based on the same mechanism.

Additionally, the redox potential of (0.70 V) is comparable to that of the redox couple (0.68 V). Therefore, it was expected that the compounds should react similarly in the analysis of αTEAC (ABTS) and FRAP and that there would be a good correlation between their results. However, the reaction conditions between these assays differ, especially the pH-value (FRAP: 3.6; αTEAC: 7.4) and steric claims of the oxidizing molecules and ferric di-TPTZ and ABTS. Consequently, the authors calculated only a slight correlation between FRAP and αTEAC results for carotenoids (R = 0.444) (Müller, Fröhlich, and Böhm 2011). The FRAP method is generally indicated for hydrophilic compounds.

In addition, as stated in the literature, FRAP results can vary greatly depending on the time scale of the analysis, because the Fe(II)-TPTZ color may not sufficiently

develop within the protocol period for slowly reacting antioxidants (Müller, Fröhlich, and Böhm 2011; Rufino et al. 2010). This situation may explain the low correlation between applied methods. Başkan et al. (2013) reported that the TEAC coefficients determined by CUPRAC method for chlorophyll a and b are 7.2 and 6.2, while the values determined by ABTS method are 1.2 and 0.3. The half-wave oxidation potentials of $\pi$-monocation radical and dication of chlorophyll were reported by Borg et al. (1970) to be $E_{1/2} = 0.52$ V for $Chl/Chl^{\bullet+}$ and $E_{1/2} = 0.77$ V for $Chl^{\bullet+}/Chl^{2+}$ redox couples measured by cyclic voltammetry (CV). By comparison of the standard potential of the CUPRAC reagent (i.e., 0.60 V), it can be envisaged that $[Cu(Nc)_2]^{2+}$ can oxidize Chl to $\pi$-monocation radical: $Chl^{\bullet+}$. Also, when typical heterocyclic compounds substituted with various functional groups found in Maillard reaction products were examined by Yanagimoto et al. (2002) for antioxidant activity, it was found that pyrroles exhibited the greatest antioxidant activity among all heterocyclic compounds tested. Porphyrin seems to be an essential chemical structure for the antioxidant activity of chlorophyll; chlorophyll did not decompose the hydroperoxides, but reduced free radicals such as 1,1-diphenyl-2-picrylhydrazyl (Endo, Usuki, and Kaneda 1985).

## HPLC analyses of seaweed samples

All of the pigments present in the selected algae samples were identified and quantified by RP-HPLC. The chromatograms of the samples of brown, red and green algae extracts showing the pigment compositions are presented in Figure 1. Since the color of the algae is variable with regard to the composition and amount of the pigments they contain, the chromatogram of each algae was evaluated by corresponding color classification. The total quantities of the pigment compounds in the seaweed samples were calculated as μg pigment/g dry weight (dw). Accordingly, the pigment constituents were reported as the percentage of the total.

In general, most seaweed species analyzed in the present study displayed a rather simple composition of carotenoids and primarily consisted of one or two major xanthophylls (Figure 1 and Table 2). As seen from Figure 1A, *C. barbata* brown marine algae was determined to contain dominantly brown species-specific Fx and Anthx in addition to a small quantity of Zx. Another brown algae *S. vulgare* had Fx and *β*-Car as the main components and Anthx, Viox and Zx as the accessorial carotenoids. *C. montagnei* brown algae contained a low concentration of totally in comparison with the two brown algae that primarily was composed of Fx and Anthx in addition to a lesser Lt and Zx.

Lt, *β*-Car, and Anthx constituents were found in high concentrations (3.86 μg/g algae-dw, 27.12 g/g algae-dw, 3.0 g/g algae-dw) in the the red marine algae species, *H. curva* and *E. elongata*. *Corallina elongata*, which is currently recognized as a synonym of *Ellisolandia elongata* (Hind and Saunders 2013), was also reported previously to accumulate antheraxanthin as the main carotenoid (Esteban et al. 2009). Based on the PDA ($\lambda_{max}$ of 444 nm) spectra presented in the literature sources (Schubert, García-Mendoza, and Pacheco-Ruiz 2006; Esteban et al. 2009), the presence of antheraxanthin component in the seaweeds was identified in this study. It was also determined that *H. incurva* contains *α*-Car and Lt, and *E. elongata* contains Neox in small concentrations (Daigo et al. 2008; Hegazi et al. 1998).

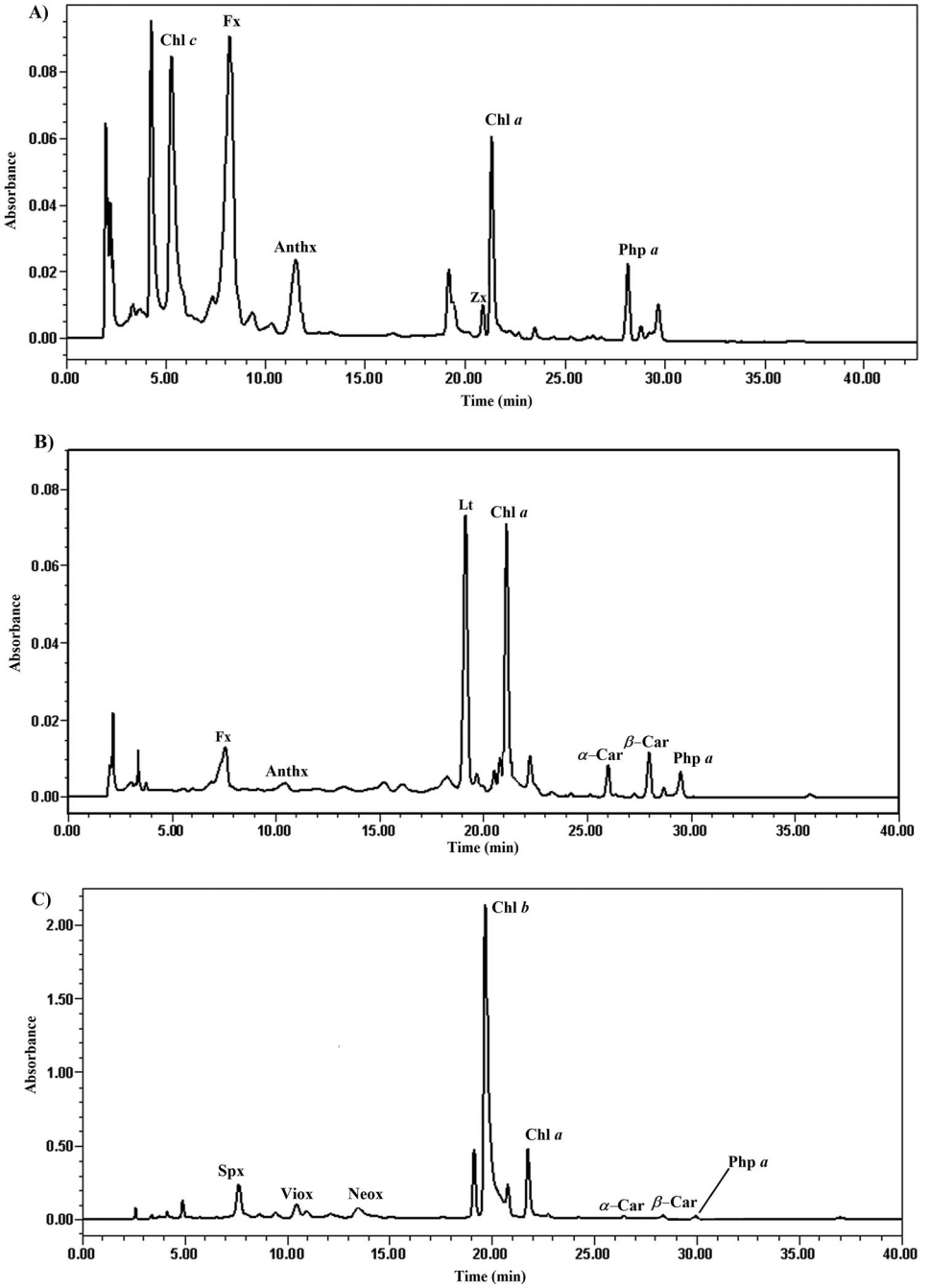



**Figure 1.** HPLC chromatograms and identified peaks of (A) *C. barbata*, (B) *H. incurva*, and (C) *F. petiolata* algae extracts (carotenoids and chlorophyll *c* at 450 nm, chlorophyll *a*, *b* and pheophytin *a* at 650 nm were determined).

One of the most important benefits of investigating algal pigments is to identify different pigment profiles that are characterized by the dominance of one or more species among others as indicated in the patterns of *H. Incurva,* and *E. elongata*.

The distribution of chlorophylls and particularly carotenoids having diverse molecular structures have been reported to be used as a functional index for the classification of algae. Although the mechanism of certain biosynthetic pathways is not yet exactly revealed, each pigment present in alga species is also supposed to help algal classification, which means pigment constituents can serve as chemotaxonomic markers (Schubert, García-Mendoza, and Pacheco-Ruiz 2006). For example, green marine algae *C. fragile*, an edible species, is characterized by an abundance of Spx. Spx, one of the rare pigments extracted particularly from green algae *C. fragile*, exhibited an inhibition effect on human leukemia HL-60 cells (Ganesan et al. 2011).

In this study, *F. petiolate* green algae was found to contain Spx (81.08 µg/g algae-dw equal to 40.52% of total carotenoid content) as the major carotenoid (Table 2). Raniello et al. (2006) and Yokohama (1983) notified an increase in Spx with depth, and thus demonstrated the role of this pigment in the acclimation to the low light of deep environments and in the harvesting of green light concerning siphonein (a derivative of Spx) and chl *b*.

As shown in Figure 1 and Table 2, Neox, Viox and $\beta$-Car were other major carotenoids, and a small quantity of $\alpha$-Car was also accumulated in *F. petiolata* species. Both Anthx and Spx pigments were calculated as Fx pigment equivalent having almost the same maximum absorption wavelength ($\sim$ 450 nm) with the Anthx and Spx pigments. Thus, the concentration of the Anthx and Spx was obtained from the calibration curve generated for Fx (Sanz et al. 2015, Wright et al. 1991).

Brown and green species of marine macroalgae all possess the xanthophyll cycle referred to as the Viox cycle, which involves a reversible sequential conversion of Zx *via* Anthx into Viox with the catalytic effects of zeaxanthin epoxidase and violaxanthin epoxidase enzymes (Viox $\leftrightarrow$ Antx $\leftrightarrow$ Zx) (Esteban et al. 2009; Takaichi 2011). This light-dependent regulation mechanism of the xanthophyll cycle is characterized by the prevention of oxidative damage produced not only by excessive or low light but also by other reactive oxygen species caused by environmental effects such as salinity, drought or heat stress. In low light, the xanthophyll cycle pigments have the capability of harvesting available light and passing the energy to the chlorophyll molecules. In the presence of high light, the reverse reaction occurs, which means that these carotenoids absorb excessive energy from chlorophyll and dissipate it thermally hence preventing chlorophyll from photodamage. However, the presence of this cycle in red species has not yet been precisely shown.

Since red algae generally do not have a xanthophyll biosynthesis cycle after zeaxanthin, Zx and Lt (Daigo et al. 2008) often result in the accumulation as major carotenoids, as seen in *G. bursa-pastoris, P. perforata* and *H. Incurva* red seaweed samples, respectively (Table 2). Schubert, García-Mendoza, and Pacheco-Ruiz (2006) identified Anthx to be the main carotenoid in addition to Zx and Lt in the *E. elongate* red algae. On the other hand, in our previous studies on some putative hormones in seaweeds, we determined that *Halopithys incurva*, *Gracilaria bursa-pastoris*, and *Ellisolandia elongata* red algae possess abscisic acid (ABA) plant growth regulator (Yalçın et al. 2019; 2020).

Since the quantitative detection of ABA was successful in the mentioned red seaweeds, the findings strongly support the presence of an ABA biosynthetic pathway which is produced from zeaxanthin and ends with the production of ABA, similar to

**Table 2.** Carotenoid and chlorophyll contents (μg/g dw algae) in the seaweed samples.

| | Pigment amount, μg/g algae | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Carotenoids | | | | | | | | | | Chlorophylls | | | | |
| Algae species | Fx | Spx | Anthx | Viox | Neox | Lt | Zx | α-Car | β-Car | Total | Chl *c* | Chl *b* | Chl *a* | Php *a* | Total |
| *S. vulgare* | 32.24 ± 0.26 | – | 0.71 ± 0.01 | 0.76 ± 0.01 | – | – | 0.25 ± 0.01 | – | 17.81 ± 0.35 | 51.77 | – | – | 94.94 ± 0.45 | 32.13 ± 0.21 | 127.07 |
| *C. barbata* | 16.03 ± 0.43 | – | 0.80 ± 0.02 | – | – | – | 0.45 ± 0.25 | – | – | 16.88 | 15.40 ± 0.21 | – | 48.41 ± 0.46 | 36.97 ± 0.31 | 100.78 |
| *C. montagnei* | 0.89 ± 0.40 | – | 0.40 ± 0.09 | – | – | 0.17 ± 0.02 | 0.11 ± 0.01 | – | – | 1.57 | – | – | 0.17 + 0.004 | 18.85 ± 0.23 | 19.02 |
| *H. incurva* | 2.97 ± 0.05 | – | 0.44 ± 0.10 | – | – | 3.86 ± 0.06 | – | 0.39 ± 0.01 | 27.10 ± 0.11 | 32.89 | – | – | 68.28 ± 0.23 | 1.69 ± 0.01 | 69.97 |
| *G.b-pastoris* | – | – | – | – | – | – | 0.23 ± 0.01 | – | – | 0.23 | – | – | – | 1.52 ± 0.02 | 1.52 |
| *P.perforata* | – | – | – | – | – | – | 0.27 ± 0.02 | – | – | 0.27 | – | – | 2.48 ± 0.05 | 1.66 ± 0.01 | 4.14 |
| *E.elongata* | 0.79 ± 0.05 | – | 3.00 ± 0.14 | – | 0.12 ± 0.01 | 0.51 ± 0.03 | – | – | – | 4.43 | – | 8.07 ± 0.3 | 71.89 ± 0.35 | – | 79.96 |
| *C.denticulata* | – | – | – | – | – | – | – | 0.13 ± 0.01 | – | 0.13 | – | 33.09 ± 0.1 | 35.67 ± 0.2 | 8.75 ± 0.1 | 77.51 |
| *P.capitatus* | – | 0.67 ± 0.01 | – | – | – | – | – | – | – | 0.67 | – | 0.84 ± 0.3 | 0.81 ± 0.03 | – | 1.65 |
| *F.petiolata* | – | 81.08 ± 0.9 | – | 56.19 ± 0.85 | 11.94 ± 0.1 | – | – | 0.50 ± 0.05 | 50.41 ± 0.72 | 200.12 | – | 816.15 ± 9.0 | 754.09 ± 8.2 | 124.43 ± 2.3 | 1694.67 |

that in terrestrial plants (Mikami et al. 2016). Apart from carotenoid pigments, the chlorophyll composition of the investigated algae species primarily consisted of Chl *a* and Php *a*, but their concentration was much higher than that of the carotenoids. Since Php *a* is a derivative of Chl *a*; and the maximum absorption wavelength of both components is at 665 to 666 nm, the quantitative result of Php *a* was obtained from the calibration curve generated for the Chl *a* pigment and calculated as the equivalent of Chl *a*. In addition, the Chl *c* pigment determined in brown algae *C. barbata* extract was identical with the literature results that present the retention times and spectrum recorded by the diode array detector (Heriyanto et al. 2017; Kuczynska, Jemiola-Rzeminska, and Strzalka 2015; Li, Gong, and Hsiung 2002).

Unlike the other chlorophyll components analyzed at 650 nm ($\lambda_{max}$ of 650 nm (Chl *b*), 665 nm (Chl *a*)), the wavelength at which the maximum absorption occurred was appproximately 445 nm and easily analyzed at 450 nm with carotenoids. As a result, the Chl *c* component can be identified since there is no overlap problem between the peak of the Chl *c* component and the peaks of other chlorophyll components (Figure 1) (Haryatfrehni et al. 2015). The quantitative result of Chl *c* pigment was determined as the equivalent of Fx, since Chl *c* had almost the same maximum absorption wavelength.

As presented in Table 2, all of the three green algae species and red algae *E. elongata* included in the study also contained Chl *b*. Among algae species analyzed in this study, the *F. petiolata* green algae was shown to contain a remarkable variety of chlorophylls and the highest total concentration. Thus, the total concentration of chlorophylls in *F. petiolata* algae was determined by HPLC to be 1694.67 µg/g- dw. As shown in Table 2, the total content consisted of approximately 48.16% Chl *b*, 44.50% Chl *a*, and 7.34% Php *a*. constituents.

*F. petiolata* was previously considered endemic to the Mediterranean but is generally distributed mainly in tropical and subtropical regions, and often associated with other algae species and particularly endangered seagrass *Posidonia oceanica* and hosted with a wide variety of fungi in the sea. In comparision with many other green algae, the crude extract of *F. petiolata* species has been detected to exhibit strong antibacterial, antiviral, antimitotic, antifungal and cytotoxic activities (Ballesteros, Martin, and Uriz 1992). Finally, the extent of *F. petiolata* algae as a source of bioactive compounds has not been fully determined, and this study is the first comprehensive report since Casazza and Mazzella (2002) about the potential antioxidant capacity and pigment content of this species.

Although Fx is known as the main carotenoid particularly specific to brown algae, it has been reported that this pigment may also be present at a lower quantity within some red species. Our findings in this study have been corroborated and correlated well with the works of Takashi (2011) and Schubert, García-Mendoza, and Pacheco-Ruiz (2006). Hence, we detected Fx 1.1 µg/g- algae in *H. incurva* equal to 3.34% of total carotenoid content and 0.80 µg/g- algae in *E. elongata* red seaweeds equal to 18% of total carotenoid content on a dry weight basis.

## Conclusion

The antioxidant capacity of seaweed was evaluated in ten species from the coast of Çanakkale and Antalya in the Aegean and Meditereanean Seas of Turkey. The results

suggest that the spectrophotometric CUPRAC assay, useful for testing both hydrophilic and lipophilic antioxidants, may be used to determine the antioxidant capacity of algal carotenoids and chlorophylls. Theoretical antioxidant capacities of acetonic seaweed extracts were calculated by multiplying the concentration of each pigment determined by HPLC with its TEAC coefficient and summing the products. The relative contribution of the identified pigments to the experimental TAC value was determined by taking the ratio of the theoretical TAC value to that determined by the spectrophotometric CUPRAC method.

The combined HPLC-CUPRAC method enabled the practical comparison of antioxidant constituents of algae extracts by HPLC and their calculated TAC values in TR and $\beta$-Car equivalents. This comparison was possibly more meaningful than the total pigment contents made on a mass basis. CUPRAC assay showed that some crucial pigments particularly found in algae species such as Chl *a,* Chl *b,* Fx, Lt, Zx were efficient antioxidants, and Chl *a* had the highest TEAC coefficient in the following sequence: Chl *a* > Chl *b* > α-Car > Lt > $\beta$-Car > Fx > Zx.

The seaweeds exhibiting the highest antioxidant capacity were *S. vulgare* and *C. barbata* from brown species, *H. incurva* from red species, and *C. s. f. denticulata* and *F. petiolata* from green species. The detection and quantification of precious specific algal pigments such as Fx, Chl *c*, Anthx, and Spx explained the antioxidant effect assessed in selected seaweed species which can be effectively utilized as a source of potential antioxidants. Seaweeds have been consumed since ancient times as human food, more commonly in Asian countries. However, in the last few decades, seaweed and seaweed-based products have found a progressively increasing demand as alternative food sources for the overpopulated world. Some of the seaweed analyzed in this study, *S. vulgare*, *C. barbata*, *G. bursa-pastoris*, *P. perforata*, *E. elongata*, and *C. scalpelliformis f. denticulata* were classified as edible seaweeds by international experts (Pereira 2016). Edible algae are either eaten directly or used as ingredients in both medicinal and food preparations (as gelling or emulsifying agents). Thus, this study may find potential use in promoting the use of marine algae in commercial marine food and pharmaceutical products and developing large-scale aquaculture.

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## ORCID

Kevser Sözgen Başkan http://orcid.org/0000-0002-3486-0231

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