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ARTICLE

Effect of Pressurized Liquids on Extraction of Antioxidants from *Chlorella vulgaris*

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Chlorella vulgaris is a green microalga that contains various antioxidants, such as carotenoids and chlorophylls. In this study, antioxidants from *C. vulgaris* were extracted using pressurized liquid extraction (PLE), which has been recently used for bioactive compound extraction. The antioxidant capacity of individual compounds in chlorella was determined by online HPLC $ABTS^{\bullet+}$ analysis. According to the antioxidant analysis of total extracts, the extraction yield, radical scavenging activity, and phenolic compounds using PLE were relatively high compared to those obtained using maceration or ultrasound-assisted extraction. On the basis of online HPLC $ABTS^{\bullet+}$ analysis, the 15 major antioxidants from chlorella extracts were identified as hydrophilic compounds, lutein and its isomers, chlorophylls, and chlorophyll derivatives. Using PLE at high temperature (85−160 °C) significantly increased antioxidant extraction from chlorella, improving the formation of hydrophilic compounds and yielding more antioxidative chlorophyll derivatives. Online HPLC $ABTS^{\bullet+}$ analysis was a useful tool for the separation of main antioxidants from PLE extracts and allowed the simultaneous measurement of their antioxidant capacity, which clearly showed that PLE is an excellent method for extracting antioxidants from *C. vulgaris*.

KEYWORDS: Pressurized liquid extraction; antioxidant; *Chlorella vulgaris*; online HPLC $ABTS^{\bullet+}$ analysis

INTRODUCTION

The unicellular alga *Chlorella vulgaris* is a well-known health food worldwide, especially in Asia. It is composed of many bioactive substances with medical properties, such as protein, vitamins, chlorophyll, and carotenoids (*1*). Various experimental studies have demonstrated the biological properties of chlorella. Chlorella can decrease oxidative stress and stress-induced ulcers in mice (*2*−*6*). In rabbits fed a high-cholesterol diet for 10 weeks, *C. vulgaris* showed antilipidemic and antiatherosclerotic activity (*7*). Such results have been attributed to the effects of specific components in chlorella, including dietary fiber and a wide range of antioxidants, such as carotenoids and chlorophylls. The extraction techniques for these antioxidants are important for the growing nutraceutical industry.

Maceration (MAC), sonication, and Soxhlet methods have been previously examined for carotenoid and chlorophyll extraction from several microalgae (*8*, *9*). These traditional extraction techniques have some drawbacks, such as the use of large quantities of toxic organic solvents, long extraction times, low selectivity and/or low extraction yields, and the exposure of the extracts to excessive heat, light, and oxygen. As an alternative extraction method, pressurized liquid extraction (PLE) has been recently used in the extraction of biologically active constituents (*10*−*13*). This method utilizes conventional solvents at elevated temperatures and pressures and is well-established for environmentally clean extraction. Because PLE is automated, uses less solvent in a shorter period of time, and maintains the sample in an oxygen- and light-free environment, PLE has the potential to be a powerful tool in the industry.

Recently, online HPLC methods for analyzing radical-scavenging activity have been developed (*14*−*17*), which require a stable model free radical system. One such system is the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), in which radical-scavenging activity is assessed by comparing it to that of the synthetic vitamin E derivative 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Online assessment of antioxidant activity allows complex mixtures to be separated by HPLC and the antioxidant contribution of individual ingredients to be evaluated.

The aim of this study was to evaluate the antioxidant activity of a pressurized liquid extract of chlorella. Experiments were performed to study the total phenolic content and total antioxidant activity of chlorella extracts. In addition, an HPLC system linked to an $ABTS^{\bullet+}$-based online antioxidant detection system was used to assess the contribution of individual elements of chlorella extracts to the overall antioxidant capacity.

MATERIALS AND METHODS

Samples and Chemicals. The biomasses of *C. vulgaris* were obtained from Daesang Corp. (Kunsan, Korea). Chlorella cells were collected, freeze-dried in a vacuum freezer-dryer (Ilshin Lab, Gyeonggi, Korea), and

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stored under vacuum in darkness until extraction. HPLC-grade *tert*-butyl methyl ether, ethanol, methanol, and water were purchased from Fisher Scientific (Springfield, NJ). Folin−Ciocalteu reagent, 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, sodium carbonate, gallic acid, Trolox, β-carotene, and chlorophylls *a* and *b* were from Sigma-Aldrich (St. Louis, MO). Standard lutein (98.2%) was from Chromadex, Inc. (Santa Ana, CA). The chlorophyll derivatives pheophorbide *a* and pheophytin *a* were purchased from Wako Chemicals (Osaka, Japan). All other chemicals were of analytical grade.

Extraction Procedures. The antioxidant activities of chlorella extracts extracted via PLE were compared with those extracted by traditional methods [i.e., maceration (MAC) or ultrasound-assisted extraction (UAE)]. For MAC, a 0.5 g sample of freeze-dried chlorella powder was macerated in 90% ethanol at room temperature for 6 h. For UAE, a 0.5 g sample of chlorella powder was sonicated in an ultrasonic cleaning bath (model RK 158s, Sonorex, Bandelin, Germany) with 50 mL of 90% ethanol for 2 h. Extracts resulting from MAC or UAE were filtered through Whatman no. 1 filter paper into a 50 or 100 mL volumetric flask. Extracts were brought to the correct volume with the appropriate solvent and refiltered through a 0.2 μm membrane filter for HPLC analysis.

PLE was performed with 0.5 g of chlorella powder using a Dionex ASE 200 accelerated solvent extractor (Sunnyvale, CA) equipped with 33 mL stainless steel extraction cells and 60 mL collection vials. The extraction procedure was as follows: (i) the sample was loaded in the cell; (ii) the cell was filled with solvent to a pressure of 1500 psi; (iii) heat was applied for the initial heat-up time; (iv) static extraction was performed with all system valves closed; (v) the cell was rinsed with 60% of the cell volume with extraction solvent; and (vi) the system was depressurized. The experiment was conducted at 35, 60, 85, 110, 135, and 160 °C, respectively. Each extract was collected into a glass collection vial and then transferred to a 50 mL volumetric flask that was brought up to its final volume with methanol. Extracts were filtered prior to injection into the HPLC system. Extraction yield was calculated as the ratio of the dry weight of extract to the dry weight of chlorella used for the extraction.

Total Phenolics Determination. The total phenolics content of chlorella extracts was quantified using the Folin−Ciocalteu method (*18*). To prepare the calibration curve, 10, 20, 40, 60, and 80 mg/L dilutions of gallic acid standard solutions were prepared by dilution with HPLC water. Fifteen microliters of the extract solutions was mixed with 170 μL of HPLC water in the 96-well plate. Next, 12 μL of Folin−Ciocalteu reagent and 30 μL of sodium carbonate (200 g/L) were added. The mixtures were incubated for 1 h at room temperature in the dark. After this reaction period, 73 μL of HPLC water was added with the multichannel pipet. The absorbance of blue solutions was read at 734 nm, the calibration curve was constructed, and the content of total phenolic compounds in chlorella extracts was calculated as gallic acid equivalents (GAE).

Trolox Equivalent Antioxidant Capacity (TEAC). The TEAC method is based on $ABTS^{\bullet+}$ performance. We mixed 6.62 mg of potassium persulfate in 10 mL of HPLC water with 38.4 mg of ABTS in 10 mL of HPLC water. After 24 h at room temperature in the dark, the $ABTS^{\bullet+}$ is at an optimum concentration in the reaction mixture (*19*). The $ABTS^{\bullet+}$ solution was then diluted with absolute ethanol to obtain an initial absorbance of 0.70 ± 0.05 at 734 nm. To prepare the calibration curve from fresh 2.0 mM Trolox standard solutions, 0.0125, 0.050, 0.100, 0.150, and 0.200 mM dilutions were prepared with ethanol. Twenty microliters of Trolox standard solution and ethanol as a blank were mixed with 200 μL of fresh $ABTS^{\bullet+}$ solution. The reduction in $ABTS^{\bullet+}$ emission was measured at 734 nm and plotted against the Trolox concentration. Linear regression ($R^2 = 0.999$) was used to calculate the antioxidant capacity of the samples.

Online HPLC $ABTS^{\bullet+}$ Analysis. The antioxidant activity of chlorella extracts was determined using the $ABTS^{\bullet+}$ assay, based on methods used by Kim et al. (*15*) with some modifications. A 2 mM $ABTS^{\bullet+}$ stock solution containing 3.5 mM potassium persulfate was prepared and incubated at room temperature in the dark overnight to allow for stabilization of the radical. The $ABTS^{\bullet+}$ reagent was prepared by diluting the stock 30-fold in water. The chlorella extract was injected and separated using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a vacuum degasser, binary pumps, autosampler, diode array detector (DAD), UV−vis detector, and an additional reagent pump.

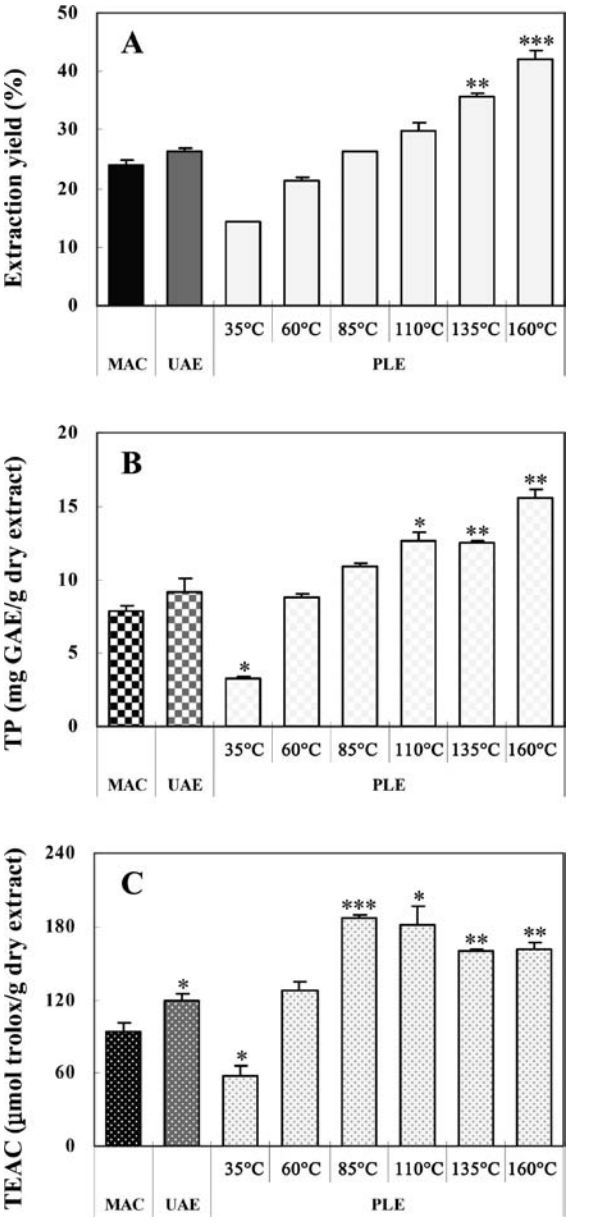

Figure 1. Effects of several extraction methods, such as MAC, UAE, and PLE, on antioxidant extraction from *C. vulgaris*: (**A**) extraction yields (%) of chlorella extracts; (**B**) total phenolics (mg of gallic acid equiv/g of dry chlorella extract); (**C**) Trolox equivalent antioxidant capacities (μmol of Trolox/g of dry chlorella extract) using each extraction method. ***, $P < 0.001$, **, $P < 0.005$, and *, $P < 0.01$, compared to MAC (as a traditional method) using Student's *t* test with $n = 3$.

A YMC carotenoid column (3 μm particle size, 250 mm × 4.6 mm, Waters, Milford, MA) was used to analyze the carotenoids and chlorophylls.

The most suitable mobile phase system was composed of methanol (100%) (A) and *tert*-butyl methyl ether (B) under the following conditions: isocratic at 0% B for 7 min followed by a linear gradient from 0 to 5% B in 1 min, isocratic at 5% B for 25 min, a linear increase to 35% B in 1 min, and isocratic at 35% B for 14 min, followed by a linear decrease to 0% B in 4 min. The column was equilibrated for 15 min at the starting conditions before each injection. A flow rate of 1.0 mL/min at 30 °C with an injection volume of 20 μL was used. The DAD was operated in the 200−800 nm range, and the chromatographic profile was recorded at 265 nm for phenolic compounds, at 445 nm for carotenoids, and at 660 nm for chlorophylls. The HPLC eluent from the DAD arrived at a "T" piece, where the $ABTS^{\bullet+}$ was added. The $ABTS^{\bullet+}$ flow rate was 0.5 mL/min delivered by an additional Agilent 1200 pump. After mixing through a 1 mL loop maintained at 30 °C, the absorbance was measured at 734 nm by

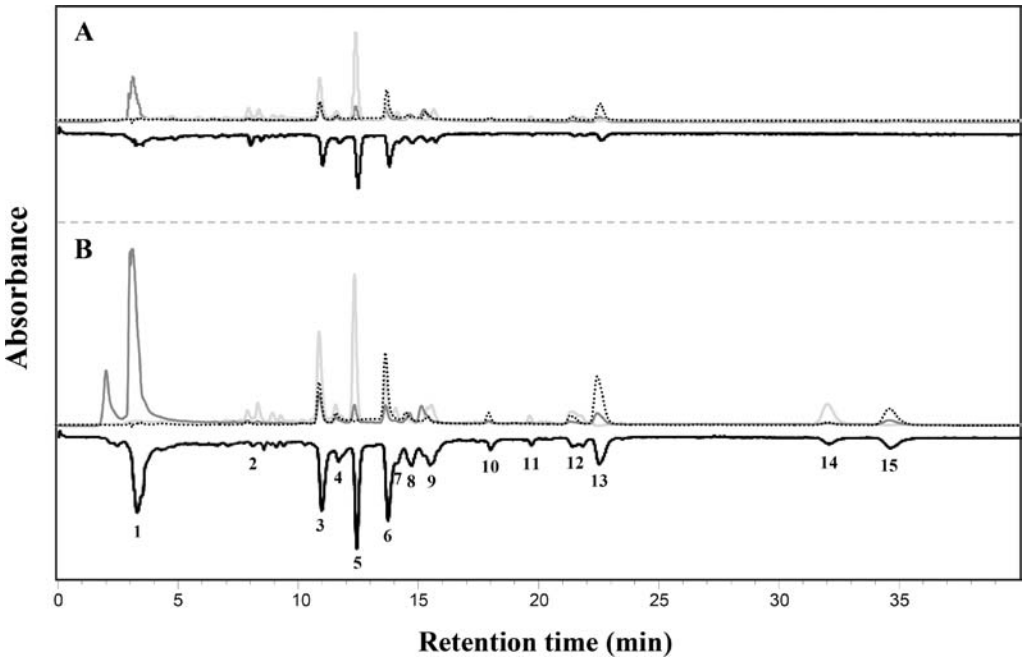

Figure 2. Online HPLC ABTS$^+$ profiles of antioxidants in chlorella extracts using MAC (**A**) and PLE 160 °C (**B**). Aliquots of chlorella extracts (20 μL) were analyzed by gradient reverse phase HPLC with a PDA detector at 265 nm for phenolic compounds (solid dark gray line), at 445 nm for carotenoids (solid light gray line), and at 660 nm for chlorophylls (dashed line), prior to reaction with ABTS radical cation and analysis of antioxidant potential at 734 nm (solid black line, negative trace). **Table 1** shows the identities of the numbered peaks.

a UV−vis detector. Finally, data were analyzed using ChemStation software (Agilent Technologies). The antioxidant potential was quantified by reference to a Trolox standard calibration curve.

Identification and Quantification. The carotenoids, chlorophylls, and their derivatives were identified by comparing retention times and absorption spectra of unknown peaks with reference standards and cochromatography with added standards. In addition, quadruple mass spectrometry (MS) with atmospheric pressure chemical ionization (APCI) (Varian, Palo Alto, CA) in positive mode was used for detection. The Varian MS workstation software (version 6.3) was used for data acquisition and processing. The HPLC conditions were the same as described above for the online detection of radical-scavenging activity. Mass spectra were acquired over the m/z 500−1000 scan range using a 0.1 unit step size with a drying gas flow of 4 L/min, a nebulizing gas flow of 50 psi, a vaporizer temperature of 550 °C, and a dry gas temperature of 350 °C.

RESULTS AND DISCUSSION

Antioxidant Capacity of the Total Extracts of Chlorella. Maceration, UAE, and PLE were tested to evaluate the influence of the extraction method on the antioxidants extracted from chlorella. **Figure 1** shows the extraction yield (**A**), total phenolics content (**B**), and TEAC (**C**) of each extract.

PLE had the highest extraction yield (~40%) at 160 °C, whereas the extraction yields of MAC and UAE were comparatively lower (25−30%) (**Figure 1A**), perhaps due to the improvement in extraction efficiency by the high temperature of PLE. The total phenolics content of PLE 160 °C extracts was significantly higher ($P < 0.005$) than that of MAC extracts (**Figure 1B**), and the phenolics contents of PLE extracts increased in a temperature-dependent manner. The maximum total phenolics content of PLE 160 °C extracts was ~15 mg of GAE/g of dry extract. These results indicate that the high extraction efficiency of PLE affected the extraction of total phenolics. The antioxidant capacity of PLE (at 60−160 °C) extracts measured by TEAC was higher (120−180 μmol of Trolox/g of dry extract) than that of MAC extracts (90 μmol of Trolox/g of dry extract) (**Figure 1C**). Although severe heat processing (110−160°) slightly decreased the antioxidant capacity of extracts, the total antioxidant capacity of the extraction solutions was enhanced, owing to the high extraction efficiency of heat treatment.

Recently, it was reported that the antioxidant activity of chlorella extract obtained by hydrothermal extraction (120−200 °C) is higher than that obtained by hot water extraction (*20*). The increased extraction temperature resulted in a higher antioxidant activity, indicating that the antioxidant compounds of chlorella extracts have higher resistance to temperature. These previous findings help explain the high antioxidant capacity of heat-processed extracts in our study. High heat treatment with PLE improved the extraction of antioxidants, such as heat-stable phenolic compounds, carotenoids, and chlorophylls, into the extraction solvents.

Identification of Antioxidants in PLE Extracts. Figure 2 shows the HPLC chromatograms of 15 major antioxidants using MAC (**A**) and PLE 160 °C (**B**). The peaks were identified on the basis of their retention behaviors and visible absorption spectra compared to available authentic standards and further confirmed by LC-MS (**Table 1**).

Peak 1 was tentatively labeled as a complex of hydrophilic compounds; peaks 5, 7, 9, and 11 were carotenoids (lutein, *cis*-lutein, *cis*-lutein, and β-carotene, respectively); and peaks 2, 3, 4, 6, 8, 10, 12, 13, 14, and 15 were chlorophyll derivatives (pheophorbide *a*, chlorophyll *b*, chlorophyll *b*′, chlorophyll *a*, chlorophyll *a*′, pyrochlorophyll *b*, pheophytin *b*, pheophytin *a*, pyropheophytin *b*, and pyropheophytin *a*, respectively). Peak 1 was the primary antioxidant peak in PLE 160 °C, whereas the content of peak 1 was relatively small in MAC (**Figure 2B**).

Interestingly, antioxidant chlorophyll derivatives such as pyropheophytins *a* and *b* were significantly found in PLE 135 and 160 °C extracts. We concluded that these chlorophyll derivatives were formed through the severe thermal processing associated with this process, which generates chemical structure deformations (*21*, *22*). According to recent studies, chlorophyll derivatives have potent antimutagenic activity and can inhibit tumor cell growth (*23*). Thus, the increased production of

chlorophyll derivatives by PLE may enhance the biological benefit of PLE extracts.

Online HPLC ABTS$^{\bullet+}$ Radical Scavenging Assay of Chlorella Extracts. The antioxidant potential of individual ingredients in chlorella extracts was assessed by the online HPLC ABTS$^{\bullet+}$ method (**Table 2**). Following HPLC separation, HPLC eluate was mixed with a stabilized solution of the ABTS$^{\bullet+}$ radical and directed to a UV−vis detector monitoring absorbance at 734 nm. The radical solution has a deep blue color, and any quenching of the ABTS radical cation results in a loss of color, indicated by a negative peak on the absorption profile monitored at 734 nm. As shown in **Table 2**, carotenoids and chlorophylls, particularly lutein, chlorophylls *a* and *b*, and pheophytin *a*, were the most abundant antioxidants in the chlorella extracts. These four compounds contributed ~50% of the total antioxidant potential in most extracts, with their isomers and derivatives comprising the rest. In particular, the greatest antioxidant contribution to PLE 160 °C came from hydrophilic compounds, which accounted for 23% of the HPLC-derived antioxidant potential. This significant contribution to antioxidant activity was due to the increase in hydrophilic compound content through high heat processing (~160 °C). PLE 85 °C was the most effective extraction method for improving the antioxidant capacity, and harsh heat treatments (110−160°) slightly decreased the antioxidant capacities of the extracts. This result is compatible with the general TEAC data using the total extract (**Figure 1C**). A significant correlation (R^2 = 0.9654) was found between the total antioxidant capacity using conventional TEAC method and the sum of the antioxidant capacities of each HPLC-separated peak (**Figure 3**). This result indicates that the online ABTS assay system is an efficient and validated method for antioxidant separation and chlorella extract analysis.

The TEACs of chlorophyll *a*, chlorophyll *b*, pheophytin *a*, and pyropheophytin *a* were previously reported as 0.73, 0.23, 0.02, and 0.16, respectively (*24*). Thermal processing and/or acidification results in pheophytinization, due to the conversion of natural chloropylls to Mg^{2+}-free pheophytins. Severe heat treatment particularly accelerates pyropheophytin formation (*21*, *22*). In PLE 110 °C, PLE 135 °C, and PLE 160 °C, the decrease in the antioxidant potential per gram of dry extract was explained by the deformation of chlorophylls to both pyrochlorophylls and pheophytins, the antioxidant capacities of which are comparatively lower than those of chlorophylls.

Table 1. Peak Identification of 15 Antioxidants Isolated from Chlorella Extracts

peak	t_R (min)	APCI-MS $[M + H]^+$	λ_{max}[a] (nm)			tentative identification
1	3.27		265			hydrophilic compounds
2	8.19	575.5	407	508	666	pheophorbide *a*
3	10.94	907.6	466	600	650	chlorophyll *b*
4	11.63	907.6	466	602	652	chlorophyll *b′*
5	12.39	551.4	444	472		lutein
6	13.69	893.6	432	618	664	chlorophyll *a*
7	14.04	551.4	438	466		*cis*-lutein
8	14.68	893.6	432	620	666	chlorophyll *a′*
9	15.56	551.4	442	468		*cis*-lutein
10	17.96	849.6	466	600	648	pyrochlorophyll *b*
11	19.64	537.3	446	472		*β*-carotene
12	21.36	885.6	436	598	652	pheophytin *b*
13	22.46	871.6	408	610	666	pheophytin *a*
14	32.02	827.6	436	600	654	pyropheophytin *b*
15	34.56	813.6	410	610	666	pyropheophytin *a*

[a] A gradient mobile phase of *tert*-butyl methyl ether and methanol was used.

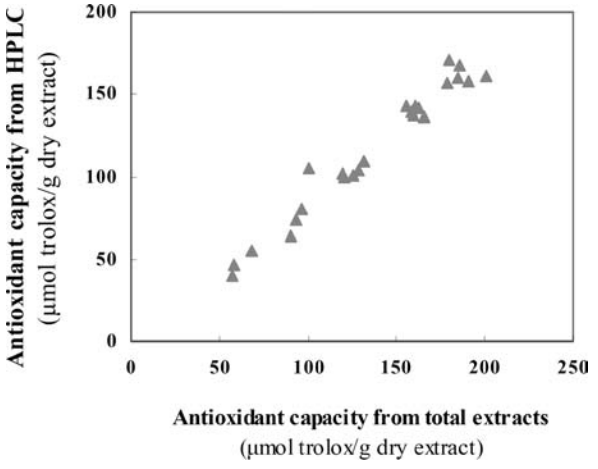

Figure 3. Correlation between the antioxidant capacity calculated from the conventional TEAC method and the HPLC antioxidant capacity derived from the addition of the antioxidant activity for each peak separated by HPLC.

Table 2. Antioxidant Potential of Individual Compounds from Chlorella Extracts Using Online HPLC ABTS$^{\bullet+}$ Analysis

	TEAC (μmol of Trolox/g of dry extract)							
			PLE					
major antioxidants	MAC	UAE	35 °C	60 °C	85 °C	110 °C	135 °C	160 °C
hydrophilic compounds	4.466 ± 1.310	5.087 ± 0.451	6.402 ± 0.841	10.051 ± 1.128	17.393 ± 1.047	17.956 ± 0.712	16.390 ± 0.931	33.180 ± 3.145
pheophorbide *a*	3.383 ± 0.245	4.258 ± 0.651	2.556 ± 0.741	3.273 ± 0.241	2.844 ± 0.123	0.711 ± 0.052	1.017 ± 0.125	0.445 ± 0.100
chlorophyll *b*	10.738 ± 2.014	16.816 ± 4.125	9.457 ± 1.113	15.786 ± 1.685	29.441 ± 2.452	26.393 ± 2.985	17.346 ± 3.214	17.624 ± 1.247
chlorophyll *b′*	1.267 ± 0.450	1.097 ± 0.110	1.315 ± 0.241	2.873 ± 0.387	5.663 ± 0.912	4.830 ± 0.678	3.256 ± 0.541	3.251 ± 0.241
lutein	28.096 ± 2.541	27.249 ± 3.124	7.885 ± 2.142	27.212 ± 2.145	31.436 ± 0.457	29.987 ± 3.548	24.586 ± 1.214	20.005 ± 2.214
chlorophyll *a*	13.288 ± 1.954	21.690 ± 0.847	11.637 ± 3.012	21.547 ± 1.245	35.824 ± 2.859	30.293 ± 2.124	25.042 ± 1.783	23.953 ± 1.456
cis-lutein	1.200 ± 0.210	2.445 ± 0.089	1.136 ± 0.124	3.186 ± 0.214	3.671 ± 0.541	3.654 ± 0.312	3.208 ± 0.471	2.935 ± 0.215
chlorophyll *a′*	0.844 ± 0.312	4.075 ± 0.125	0.997 ± 0.121	4.564 ± 0.966	7.736 ± 1.214	6.442 ± 0.854	5.518 ± 1.658	5.265 ± 1.119
cis-lutein	1.351 ± 0.214	6.299 ± 0.359	1.220 ± 0.098	7.566 ± 0.980	7.013 ± 1.148	8.097 ± 1.222	10.888 ± 1.090	9.339 ± 0.569
pyrochlorophyll *b*	1.002 ± 0.055	1.200 ± 0.152	0.153 ± 0.001	0.720 ± 0.241	1.432 ± 0.158	1.950 ± 0.214	2.111 ± 0.209	1.986 ± 0.231
β-carotene	0.652 ± 0.005	1.216 ± 0.214	0.175 ± 0.045	1.031 ± 0.158	1.549 ± 0.144	1.443 ± 0.222	1.242 ± 0.104	1.157 ± 0.312
pheophytin *b*	0.557 ± 0.085	0.629 ± 0.154	0.170 ± 0.003	1.590 ± 0.352	7.920 ± 0.521	8.959 ± 0.952	5.865 ± 0.315	4.117 ± 0.577
pheophytin *a*	6.419 ± 1.215	9.611 ± 1.199	2.639 ± 0.321	4.582 ± 0.248	15.688 ± 1.258	17.469 ± 2.252	12.934 ± 1.300	10.219 ± 2.228
pyropheophytin *b*	0.008 ± 0.000	0.007 ± 0.000	0.172 ± 0.012	0.085 ± 0.019	0.054 ± 0.082	0.067 ± 0.008	2.916 ± 0.210	2.708 ± 0.369
pyropheophytin *a*	0.080 ± 0.000	0.001 ± 0.000	0.071 ± 0.001	0.088 ± 0.009	0.096 ± 0.005	0.076 ± 0.010	4.910 ± 0.352	6.181 ± 0.521
totals	73.351 ± 10.610	101.680 ± 11.600	45.986 ± 8.816	104.154 ± 10.018	167.759 ± 12.922	158.326 ± 16.145	137.230 ± 13.517	142.366 ± 14.544

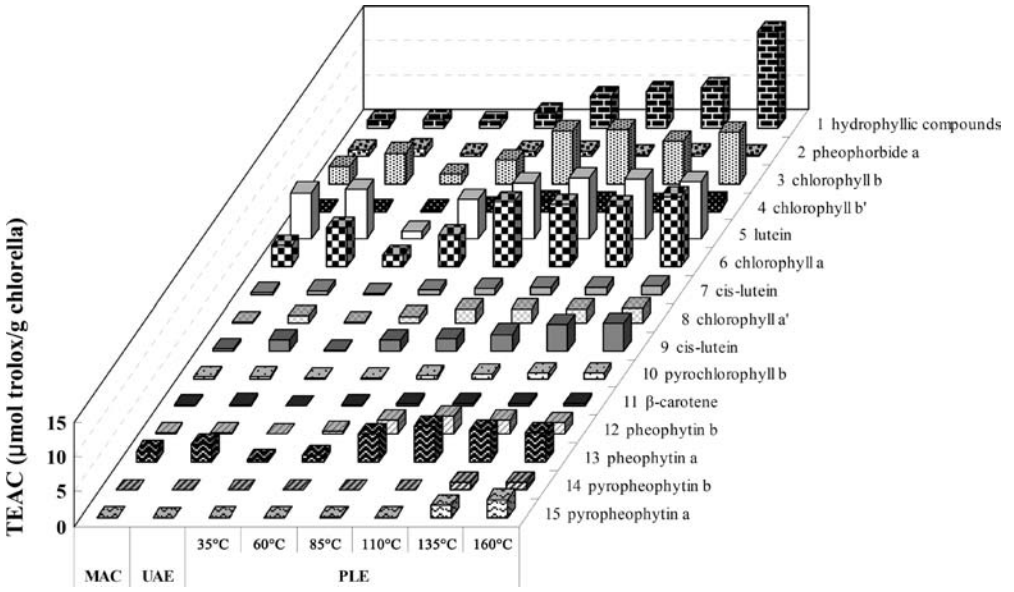

Figure 4. Overall antioxidant potential of chlorella extracts, considering the extraction yields and antioxidant capacities of individual ingredients. TEAC was expressed as the antioxidant capacity per the same amount of chlorella sample.

Because PLE increased the extraction yield of chlorella temperature-dependently, the overall antioxidant capacity calculated from the same amount of chlorella sample (not per dry extract) was greatest in PLE 160 °C (**Figure 4**). This result indicates that hydrophilic compounds, lutein, chlorophylls *a* and *b*, and pheophytin *a* were major contributors to the antioxidant capacity of PLE in chlorella.

In conclusion, we demonstrated the effect of PLE on antioxidant extraction from chlorella. The use of HPLC with an online $ABTS^{\bullet+}$ system enabled us to separate the antioxidants such as hydrophilic compounds, carotenoids, and chlorophylls from chlorella extracts and to simultaneously quantify the antioxidant potential of the individual components, demonstrating the excellence of PLE in antioxidant extraction from *C. vulgaris*.

ABBREVIATIONS USED

PLE, pressurized liquid extraction; MAC, maceration; UAE, ultrasound-assisted extraction; GAE, gallic acid equivalents; TEAC, Trolox equivalent antioxidant capacity; DAD, diode array detector; APCI, atmospheric pressure chemical ionization.

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