JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Rapid Screening and Guided Extraction of Antioxidants from Microalgae Using Voltammetric Methods

Koen Goiris,*[,†,‡,§] Peter De Vreese,[#] Luc De Cooman,[†,‡] and Koenraad Muylaert[§]

[†]KAHO Sint-Lieven, Laboratory of Enzyme, Fermentation, and Brewing Technology, Gebroeders De Smetstraat 1, 9000 Ghent, Belgium

[‡]Leuven Food Science and Nutrition Research Centre (LFoRCe), Department M2S, K. U. Leuven, Kasteelpark Arenberg 20, B-3001 Leuven, Belgium

[§]Research unit Aquatic Biology, K.U. Leuven Kulak, Etienne Sabbelaan 53, 8500 Kortrijk, Belgium

[#]KAHO Sint-Lieven, Laboratory for Chemical Process Technology, Department of Industrial Engineering Division Chemistry, Gebroeders De Smetstraat 1, 9000 Ghent, Belgium

ABSTRACT: Currently, microalgae draw much attention as a promising source of natural antioxidants to replace synthetic antioxidants for food applications. In this paper, the use of voltammetric techniques as a fast alternative for chemical assays to determine the antioxidant power of microalgal biomass is discussed. It was found that antioxidant activities determined by square wave voltammetry correlate well with the results from other established antioxidant assays, such as Trolox equivalent antioxidant capacity (R^2 = 0.737), ferric reducing antioxidant potential (R^2 = 0.729), and AAPH-induced oxidation of linoleic acid (R^2 = 0.566). Besides yielding quantitative data on the antioxidant activity, square wave voltammetry provides additional information on the antioxidant profile of microalgal biomass as the peak potentials of antioxidant components are determined. Consequently, square wave voltammetry can be used as a tool for optimizing the extraction processes to recover antioxidant components from microalgae.

KEYWORDS: *microalgae, antioxidants, square wave voltammetry*

INTRODUCTION

Antioxidants have many applications in the food and chemical industries. In the chemical industry, they are used for stabilizing fuels and lubricants[1] and for preventing oxidative degradation of plastics and rubbers. In the food industry, antioxidants are important additives for enhancing the shelf life of foodstuffs, mainly by retarding lipid oxidation.[2] Whereas industry usually relies on synthetic antioxidants, there is a potential to replace synthetic antioxidants with natural antioxidants. Particularly in food applications, due to the potential carcinogenic effect of synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), there is an increasing interest in antioxidants of natural origin.[3] Many natural antioxidants are also claimed to have beneficial health effects and are increasingly being used as nutraceuticals.[4]

Most natural antioxidants are currently derived from plant sources. Nevertheless, it is believed that microalgae might be an alternative source of natural antioxidants.[5−9] Due to their phototrophic nature, microalgae are subjected to intense oxidative stress generated by reactive oxygen species (ROS) produced during photosynthesis. To prevent cellular damage by these ROS, microalgae possess antioxidant defense mechanisms[10] such as antioxidant enzymes (e.g., superoxide dismutase or catalase) and low molecular weight antioxidants (LMWA). These LMWA include carotenoids, glutathione, vitamins (ascorbate and tocopherols), and phenolics. Particularly, carotenoids have been intensively studied in microalgae, and microalgae are already used as a commercial source of carotenoid antioxidants (astaxanthin from *Haematococcus* and β-carotene from *Dunaliella*[11,12]). Phenolic compounds are another important class of LMWA in plants but have received little attention in microalgae. Several recent studies, however, have shown that phenolics contribute significantly to the antioxidant activity in microalgae.[9,13−15] In previous work,[9] the antioxidant capacity of microalgae was compared with those of medicinal plants and common edible fruits and vegetables. It was concluded that microalgae are indeed a potential source for the production of antioxidant formulations.

Antioxidants comprise a highly heterogeneous group of chemical compounds, and many methods have been developed to evaluate the antioxidant potential of biomass extracts.[16,17] Most assays for determination of antioxidant activity are based on chemical reactions of antioxidants with a probe, which has a different color depending on its redox state, followed by spectrophotometric measurement. Frequently used methods include the total radical-trapping antioxidant potential assay (TRAP), oxygen radical absorbance capacity (ORAC), and the ferric-reducing antioxidant power assay (FRAP). A disadvantage of many chemical methods is that they are based on color formation, which can be problematic when applied to algal extracts due to high concentrations of colored chlorophylls and carotenoids. Furthermore, as the underlying redox reactions have a specific redox potential, these chemical assays measure

Received: May 29, 2012
Revised: July 10, 2012
Accepted: July 10, 2012
Published: July 10, 2012

only a fraction of the total antioxidant potential in the extract. Moreover, many tests are limited for use with either lipophilic or hydrophilic extracts.

Voltammetric methods have been proposed as an alternative to chemical antioxidant assays. The evaluation of antioxidant capacity by voltammetric methods offers many benefits.[16] Voltammetric measurements do not need laborious sample preparation, can be performed rapidly with relatively simple equipment, can be used for analysis of both lipophilic and hydrophilic extracts, and can be carried out on intensely colored (e.g., due to presence of chlorophyll) or even turbid extracts. All of these characteristics make voltammetric measurements suitable for screening large numbers of heterogeneous samples. The peak potentials detected in the voltammogram can be used as an indicator of the redox potential of major antioxidants present in the sample.[18] Although voltammograms cannot provide conclusive evidence on the nature of the antioxidant components present in the sample, they do provide information about the reducing power of the antioxidants present. Voltammetry further provides quantitative information on the total antioxidant capacity of the sample given by the total area under the curve (AUC). This is especially important when crude samples are measured, in which usually several compounds with various oxidation potentials contribute to the AUC.[19,20]

To our knowledge, voltammetric methods have not yet been used to screen microalgae for antioxidant activity. Two recent studies have used voltammetric methods to determine the antioxidant potential of macroalgae.[21,22] However, in contrast to microalgae, macroalgae are rich in phlorotannins, which may contribute significantly to antioxidant activity. The aim of the present study was to investigate the potential of voltammetric techniques for the determination of the antioxidant capacity of microalgae and to find out whether voltammetric measurements could provide insight into the antioxidant composition of microalgae. Therefore, the antioxidant activities of extracts from eight species of microalgae from different phylogenetic classes were evaluated. Antioxidant activity was measured in different biomass fractions (i.e., nonpolar, medium polar, and polar fractions) by using square wave voltammetry. The resulting data are compared with antioxidant activities determined by well-established chemical antioxidant assays, that is, Trolox equivalent antioxidant capacity (TEAC), FRAP, and AAPH-induced oxidation of linoleic acid (AIOLA). Furthermore, the antioxidant data are related to the phenolic and carotenoid contents of the samples.

MATERIALS AND METHODS

Algal Biomass Samples. Six samples of freeze-dried biomass from pure cultures were provided by Necton S.A. (Olhão, Portugal): *Nannochloropsis oculata* (Eustigmatophyceae), *Isochrysis* sp. (Prymnesiophyceae), *Tetraselmis* sp. (Chlorophyta), *Botryococcus braunii* (Chlorophyta), *Porphyridium cruentum* (Rhodophyta), and *Neochloris oleoabundans* (Chlorophyta) .

Samples of freeze-dried biomass were further obtained from pure cultures of two species produced in-house under controlled conditions in pilot-scale photobioreactors, *Scenedesmus obliquus* CCAP 276/3A (Chlorophyta) and *Phaeodactylum tricornutum* UGent Pt86 (Bacillariophyceae). The algal biomass of these species was produced in-house using 130 L plexiglass tubular airlift reactors. Algae were cultured in Wright's Cryptophyte (WC) medium[23] prepared either with deionized water for *Scenedesmus* or with synthetic seawater (30 g L^{-1} Homarsel, Zoutman, Belgium) for *Phaeodactylum*. The medium was filter-sterilized before addition to the photobioreactors (0.2 μm PTFE filters, Sartopore 2 150 cm^2, Sartorius Stedim, Vilvoorde, Belgium). The reactors were illuminated continuously (125 μmol photons m^{-2} s^{-1}, Philips Cool White fluorescent tubes) and mixed with filter-sterilized air (flow rate = 25 L min^{-1}). The culture was maintained at pH 8.5 by automated addition of CO_2 to the stream of air to compensate for CO_2 consumption by the microalgae. The biomass was harvested at the end of the logarithmic phase by centrifugation, and the wet biomass was immediately freeze-dried and stored frozen at −20 °C.

Preparation of Extracts. Extracts from the biomass samples were prepared using two solid−liquid extraction procedures. Extractions were performed in the dark at room temperature under inert nitrogen atmosphere to avoid losses of antioxidants during extraction.

The first extraction procedure was applied to obtain "crude total antioxidant extracts" by extracting both nonpolar and polar compounds using an ethanol/water mixture. To this end, 200 mg of freeze-dried biomass was ground using a pestle and mortar and extracted with 2 mL of an ethanol/water (3:1 v/v) mixture for 30 min. After centrifugation (4500*g*, 10 min), the pellet was resuspended in 2 mL of the ethanol/water mixture and extracted for a second time. Both extracts were pooled and stored under nitrogen atmosphere at −20 °C prior to analysis.

The second extraction procedure was applied to fractionate polar from nonpolar compounds through sequential extraction in hexane, ethyl acetate, and hot water.[5] In this way, the distribution of the antioxidant capacity over fractions with various polarities could be studied. To this end, 400 mg of freeze-dried biomass was ground in a mortar and extracted with 4 mL of hexane for 30 min. After centrifugation (4500*g*, 10 min), the pellet was resuspended in hexane and extracted for a second time, and both extracts were combined. The biomass pellet was subsequently extracted with ethyl acetate using the same procedure and finally with hot deionized water (80 °C). The hexane and ethyl acetate extracts were dried under a stream of nitrogen and redissolved in 4 mL of pure ethanol. All extracts were stored under nitrogen atmosphere at −20 °C prior to analysis.

Phenolics and Carotenoid Content. Phenolic content was estimated via the Folin−Ciocalteu method according to the slightly modified procedure of Hajimahmoodi et al.[15] For this, 200 μL of extract was mixed with 1.5 mL of Folin−Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min. Next, 1.5 mL of sodium bicarbonate solution (60 g L^{-1}) was added to the mixture. After incubation for 90 min at room temperature, the absorbance was measured at 750 nm. Total phenolics were calibrated against gallic acid standard solutions (25−150 mg L^{-1} in methanol/water (1:1 v/v)) and are expressed as milligrams of gallic acid equivalent (GAE) per gram of dry biomass.

Carotenoid content was estimated spectrophotometrically according to the method of Lichtenthaler and Buschman.[24] Aliquots of the extracts were diluted 15−300 times with 90% (v/v) methanol in water. Absorbances were measured at 470, 652, and 665 nm, and carotenoid content was calculated using the Lichtenthaler equation.

Antioxidant Capacity. The antioxidant capacity was measured by three assays commonly used in the food industry. In each assay, Trolox was used as a reference.

The TEAC assay was carried out according to the method of Li et al.[5] In the TEAC assay, antioxidant action by hydrogen atom transfer (HAT) as well as single-electron transfer (SET) is measured.[17] The $ABTS^{\bullet+}$ radical cation was generated by preparing a mixture of 7 mM ABTS and 2.45 mM potassium persulfate in Milli-Q water. This mixture was allowed to stand in the dark for 16 h at room temperature and was then used within 2 days. The $ABTS^{\bullet+}$ solution was diluted with deionized water to give an absorbance of 0.700 ± 0.050 at 734 nm. Next, 50 μL of microalgal extract or Trolox solution was mixed with 1.9 mL of diluted $ABTS^{\bullet+}$ solution. After 10 min of incubation at room temperature, the absorbance was measured at 734 nm.

The FRAP assay was carried out according to the method of Hajimahmoodi et al.[15] The FRAP assay measures the antioxidant action via the SET mechanism[17] but cannot detect compounds that act only by radical quenching. The FRAP reagent was freshly prepared by mixing equal volumes of a 10 mM TPTZ (2,4,6-tripyridyl-*S*-triazine)

solution in 40 mM HCl and an aqueous 20 mM $FeCl_3$ solution and diluting this mixture five times in a 0.3 M acetate buffer (pH 3.6), followed by warming to 37 °C. Next, 100 μL of microalgal extract or Trolox solution was mixed with 3 mL of FRAP reagent, and the absorbance of the reaction mixture was measured at 593 nm after incubation for 10 min at 37 °C.

The AIOLA assay was described by Liégois et al.[25] and measures the ability of an antioxidant to prevent oxidation of linoleic acid induced by alkylperoxyl radicals, generated from the water-soluble azo compound 2,2′-azobis(2-amidinopropane) dihydrochloride. For this test, a linoleic acid emulsion was prepared by slowly adding 0.25 mL of linoleic acid under continuous stirring to 5 mL of 0.05 M borate buffer (pH 9) containing 0.25 mL of Tween 20. The resulting dispersion was clarified by adding 1 mL of 1 M sodium hydroxide. Next, the volume was adjusted to 50 mL with borate buffer, and the emulsion was stored at 4 °C in the dark under nitrogen until required for further analysis. Before use, the substrate was always checked for autoxidation by measuring the absorbance at 234 nm, and solutions exhibiting >3% autoxidation were discarded. For the assay, 30 μL of substrate and 10 μL of antioxidant solution were added to a quartz cuvette containing 2.81 mL of 0.05 M phosphate buffer (pH 7.4) at 40 °C. The oxidation reaction was initiated at 37 °C by adding 150 μL of 40 mM AAPH solution freshly prepared in 0.05 M phosphate buffer (pH 7.4). For the blank, 10 μL of phosphate buffer was added instead of antioxidant solution. The radical reactions were monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. Absorbances were measured at 37 °C using a Thermo L spectrophotometer, equipped with a seven-position automatic sample changer, connected to an external water bath. All measurements were run against an AAPH reference (mixture of 150 μL of AAPH solution and 2.85 mL of phosphate buffer) because AAPH has a relatively high absorbance below 260 nm, which changes as it decomposes. Through plotting the absorbance at 234 nm versus the reaction time, the inhibition time was calculated as the point of intersection between the tangents to the inhibition and propagation phase curves.

Voltammetry. The potentiostat used for both cyclic and square wave voltammetry was an Autolab PGSTAT12 (Metrohm Autolab B.V., Utrecht, The Netherlands). The system was controlled and data were collected using NOVA 1.7.8 software (Metrohm Autolab B.V.). A three-electrode setup was used for all measurements. As a working electrode, a glassy carbon electrode (3 mm diameter) was used. Between measurements, the working electrode was polished using a polishing cloth (Basi Inc., West Lafayette, IN, USA) with 0.05 μm alumina suspension (Buehler, Dusseldorf, Germany). After polishing, the electrode was immersed in a 5% HNO_3 solution, rinsed subsequently with ethanol and water, and dried. As a counter electrode, a dimensionally stable anode was used, consisting of a titanium mesh, coated with a RuO_2/IrO_2 alloy (Magneto special anodes B.V., Schiedam, The Netherlands). Ag|AgCl in 3 M KCl was used as a reference electrode. Tetrabutylammonium perchlorate was added to the organic extracts to increase the conductivity of the samples. For all measurements, the working electrode was conditioned for 60 s at a potential of 0.00 V. For cyclic voltammetry (CV), a scan rate of 25 mV/s (staircase CV with 2.5 mV steps) was set. The conditions for square wave voltammetry (SWV) were as follows: frequency, 25 Hz; amplitude, 20 mV; step size, 5 mV. For the measurement of the reference material (β-carotene, fucoxanthin, (+)-catechin, gallic acid, and ascorbic acid; all from Sigma-Aldrich, Bornem, Belgium) pure compounds were dissolved ethanol/water (3:1, v/v), except for β-carotene, which was dissolved in a mixture of dichloromethane and ethanol (1:1, v/v), and voltammetric measurements were performed under the same conditions as for the microalgal extracts. For quantification, a calibration was performed using a dilution series of the synthetic vitamin E derivative Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 0.1−1.0 mM in ethanol/water 3:1, v/v). The AUC was used as a quantitative measure of antioxidant capacity.

Statistical Analysis. Linear regression analysis was used to evaluate whether total antioxidant activity estimated from the AUC in SWV was correlated with antioxidant activity estimated using the TEAC, FRAP, and AIOLA assays. All data were normalized using a $\log(x + 1)$ transformation.[26] R-software (version 2.13.0) was used for regression analysis.

RESULTS AND DISCUSSION

Cyclic Voltammetry versus Square Wave Voltammetry. Both CV and SWV are common techniques to study electrochemical processes. Cyclic voltammetry has a linear potential scheme, so the potential is scanned at a fixed rate between two potentials. The measured current as a function of the applied potential reflects the electrochemical behavior of any electroactive species present in the solution. Square wave voltammetry, however, is a differential technique whereby a square wave potential scheme is applied to the working electrode. Subtracting the measured forward and backward currents at each point results in an improved resolution, especially for reversible couples. However, the complicated potential scheme makes it mathematically much more difficult to interpret the resulting total differential current versus potential plot. To determine which technique is the most appropriate for measuring the antioxidant properties of microalgal extracts, a comparison was made between the cyclic voltammogram and the square wave voltammogram for an ethanol/water extract of *Isochrysis* (Figure 1). With CV, only

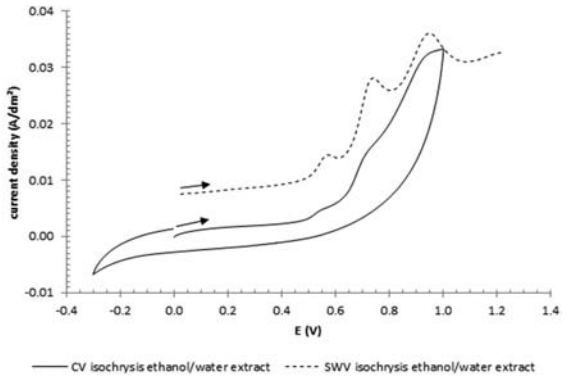

Figure 1. Comparison of the voltammetric profiles of an ethanol/water extract of *Isochrysis* sp. obtained with cyclic voltammetry (glassy carbon electrode vs RuO_2/IrO_2 alloy counter electrode and Ag|AgCl reference electrode, scan rate = 25 mV/s, staircase CV = 2.5 mV steps) and square wave voltammetry (glassy carbon electrode vs RuO_2/IrO_2 alloy counter electrode and Ag|AgCl reference electrode, frequency = 25 Hz, amplitude = 20 mV, step size = 5 mV).

two minor peaks were detected (peak potentials of 0.544 and 0.710 V), whereas with SWV three well-defined peaks were detected (peak potentials of 0.544, 0.710, and 0.920 V). Similar observations were made with extracts from other microalgae (data not shown). Because SWV yields more and better defined peaks, this method was selected over CV for further determination of the antioxidant profile of microalgal extracts. This finding is in line with work from others, indicating that SWV is more appropriate than CV for quantitative measurements as more distinct peaks are observed.[22,27]

Linearity and Repeatability of Square Wave Voltammetry. After SWV was selected as the technique of choice, the linearity and repeatability of the method were verified. For that purpose, the AUC was integrated, the value of which represents an estimate of the total antioxidant activity of the extracts. The repeatability of the method was evaluated for two algal samples,

that is, *Neochloris* (EtOH/water extract; RSD = 3.89%, n = 4) and *Isochrysis* (EtOH/water extract; RSD = 4.17%, n = 3). The relative standard deviations of AUC with the algal extracts were comparable to that of a Trolox standard solution of 0.25 mM (RSD = 3.12%, n = 4). Furthermore, the repeatability of our experiments on microalgal extracts is in line with previous studies on plant extracts.[28] To express total antioxidant capacity in Trolox equivalents, a calibration was carried out in which AUC was plotted against Trolox concentration. The calibration curve displayed good linearity (R^2 = 0.9983, n = 5) over a concentration range of 0.1−1 mM Trolox with a repeatability of 3.97%. Limits of detection and quantification were calculated from the calibration line and were 0.015 and 0.051 mM Trolox, respectively. The sensitivity of the method was calculated as the slope of the calibration curve and was 0.0026 mM^{-1} (RSD slope = 1.25%). This calibration curve was further used to calculate the Trolox equivalent antioxidant activity for all of the extracts, on the basis of the experimentally determined AUC.

Analyses of Reference Compounds. The antioxidant fraction of microalgae comprises a complex mixture of different classes of LMWA,[29] some of which mainly act as radical scavengers (e.g., carotenoids), whereas others also act as antioxidants through electron transfer (e.g., polyphenols). Voltammetric methods determine antioxidant activity by measuring the electron-donating capacity of antioxidants and may therefore not be capable of detecting all LMWA in microalgal extracts. Hence, the electrochemical response of different antioxidant classes present in microalgae was tested by applying SWV to reference components (phenolics, carotenoids, ascorbic acid). From the voltammograms in Figure 2, it is clear that different classes of antioxidants present in microalgae can be detected under our experimental SWV conditions. Peak potentials differed between the compounds and could be related to their known antioxidant activities. The peak potentials obtained for the carotenoids β-carotene and fucoxanthin (0.499 versus 0.715 V) correspond well with literature data[27] and indicate that β-carotene is a more efficient electron donor than fucoxanthin and hence has a higher ability to act as an antioxidant by electron transfer. Also, the phenolic compounds gallic acid and catechin show a differential behavior in their electrochemical properties. Whereas gallic acid shows two major peaks in the voltammogram at 0.609 and 0.856 V and a minor peak at 0.300 V, two major peaks at 0.665 and 0.921 V and a minor peak at 0.281 V can be distinguished in the voltammogram of catechin. The first peaks in the voltammograms of the phenolic components can be attributed to the formation of the semiquinone radicals, whereas the second peak corresponds with the following oxidation to the quinone form.[30] The low peak potentials of the phenolic reference components are an illustration of the well-known strong antioxidant properties of phenolics. The voltammogram of ascorbic acid shows a single peak at 0.436 V, which confirms the good antioxidant properties of this water-soluble vitamin.

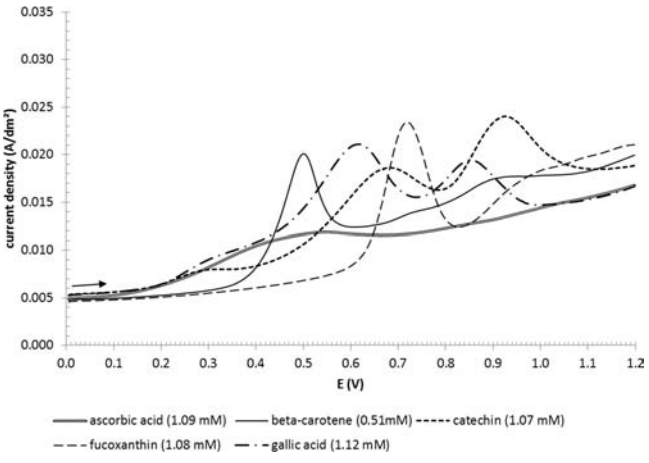

Figure 2. Square wave voltammograms of reference components of various antioxidant classes including polyphenols, carotenoids, and antioxidant vitamins (voltammetric conditions; glassy carbon electrode vs RuO_2/IrO_2 alloy counter electrode and AglAgCl reference electrode, frequency = 25 Hz, amplitude = 20 mV, step size = 5 mV).

Selection of Appropriate Extraction Protocols. As microalgae contain both polar and nonpolar antioxidants, a suitable extraction protocol is required to extract these compounds of various polarities. This could be achieved by multiple, successive extractions using solvents of different polarities or by a single extraction using a solvent mixture of intermediate polarity. Consequently, besides preparation of extracts using an ethanol/water (3:1, v/v) mixture of intermediate polarity, the antioxidants present in microalgal biomass were fractionated by successively extracting microalgal biomass with hexane, ethyl acetate, and hot water, respectively. In this way, antioxidant components are enriched into nonpolar, medium polar, and polar microalgal fractions. Figure 3 shows an overview of the square wave voltammograms of the different fractions in comparison with the square wave voltammograms of the corresponding ethanol/water extracts (voltammograms were corrected for the amount of biomass per volume of extract). In the sequential extraction, different peak potentials are often observed in the different extracts, indicating that different LMWA were extracted using different solvents. Generally, the antioxidant activity as estimated from the AUC was higher for the ethyl acetate fraction than for the hexane and hot water fractions. However, in *Nannochloropsis* and especially in *Tetraselmis*, the highest antioxidant activity was measured in the hot water fraction, suggesting that these species are rich in water-soluble LMWA. For the ethanol/water extracts the AUC was substantially higher than the AUC of the individual fractions from the sequential extraction. In most cases, the AUC of the ethanol/water extract even exceeded the combined AUCs of the three fractions from the sequential extraction. Furthermore, more peaks were detected in the voltammograms of the ethanol/water extracts than in the voltammograms of the individual fractions obtained by sequential extraction. This indicates that the proposed ethanol/water mixture is a good compromise to extract the antioxidants of various chemical natures and polarities. In general, the major peaks that were detected in the voltammograms of the fractions were also found in the voltammograms from the ethanol/water extracts. For *Nannochloropsis*, however, several peaks with a potential below 0.750 V were detected in the ethanol/water extract, but not in the three fractions obtained by sequential extraction. This suggests that the ethanol/water mixture extracted specific compounds that were not extracted using the sequential procedure. On the basis of the above results, it can be concluded that a single-step extraction using ethanol/water is capable of extracting the majority of LMWA present in the microalgal biomass and is more efficient than a sequential extraction procedure using solvents of different polarities. A sequential extraction, however, may yield information on the

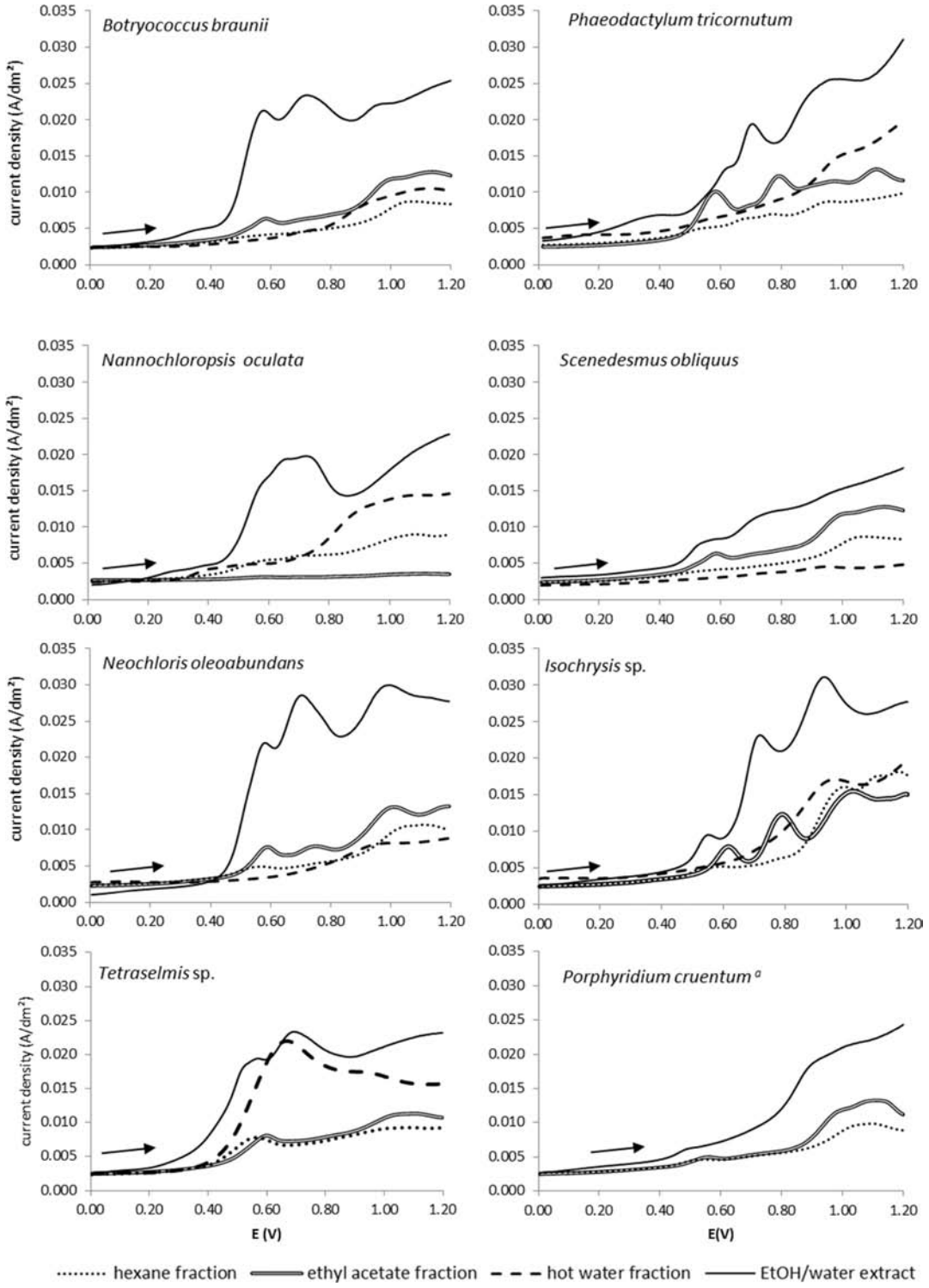

Figure 3. Square wave voltammograms of ethanol/water (3:1), hexane, ethyl acetate, and hot water extracts from eight microalgal species (voltammetric conditions; glassy carbon electrode vs RuO_2/IrO_2 alloy counter electrode and Ag|AgCl reference electrode, frequency = 25 Hz, amplitude = 20 mV, step size = 5 mV). [a] No hot water extract could be obtained from *Porphyridium cruentum* due to strong gelling properties of its polysaccharide fraction.

polarity of the LMWA present in microalgae, which is important in terms of possible applications.

Correlation of Square Wave Voltammetry with Other Antioxidant Assays. Table 1 contains the data on the antioxidant measurements of microalgal extracts using the four different assays, that is, SWV, TEAC, FRAP, and AIOLA, all expressed as micromoles of Trolox equivalent per gram of dry biomass. Regression analysis was applied to verify whether antioxidant activity, as estimated from the AUC in SWV, can be used to predict antioxidant activity measured using chemical antioxidant assays. Significant correlations were found between SWV and TEAC (Figure 4A; $R^2 = 0.737$, $p < 0.001$), between SWV and FRAP (Figure 4B; $R^2 = 0.729$, $p < 0.001$), and between SWV and AIOLA (Figure 4C; $R^2 = 0.566$, $p < 0.001$).

Table 1. Antioxidant Activities (Expressed as Micromole Trolox Equivalents per g Dry Biomass) in Extracts of Various Polarities from Eight Microalgal Species As Measured by Four Antioxidant Assays (SWV, TEAC, FRAP, and AIOLA)

	μmol TE/g biomass															
	EtOH/water extract				hexane fraction				ethyl acetate fraction				hot water fraction			
	SWV	TEAC	FRAP	AIOLA	SWV	TEAC	FRAP	AIOLA	SWV	TEAC	FRAP	AIOLA	SWV	TEAC	FRAP	AIOLA
Phaeodactylum	12.00	9.10	37.19	na[a]	1.16	4.55	1.78	na	7.55	2.83	5.41	n.a.	2.75	1.98	1.37	na
Porphyridium	6.42	5.14	10.89	1.82	2.10	1.26	1.42	1.37	3.95	1.81	2.42	2.78	na	na	na	na
Tetraselmis	35.00	69.40	46.58	56.38	3.63	3.55	4.75	3.18	3.98	4.15	6.64	8.96	42.17	17.17	39.91	39.21
Nannochloropsis	21.67	20.16	40.68	6.47	1.80	2.52	2.79	2.83	0.66	3.82	7.43	7.59	9.09	8.24	8.86	4.26
Isochrysis	26.67	22.50	53.73	12.90	4.44	0.76	6.50	2.36	5.24	3.51	9.29	5.72	6.99	12.80	8.77	6.93
Neochloris	40.00	64.30	40.72	16.56	2.43	2.73	0.78	1.84	3.72	3.73	1.63	2.61	3.85	7.67	1.37	1.08
Botryococcus	22.50	53.90	21.83	10.42	1.53	1.65	2.18	1.28	2.74	3.99	5.85	8.08	5.78	7.40	2.57	4.16
Scenedesmus	4.25	5.87	19.68	na	0.77	0.32	0.19	na	1.40	3.39	2.74	n.a.	2.31	1.21	0.68	na

[a]na, not available.

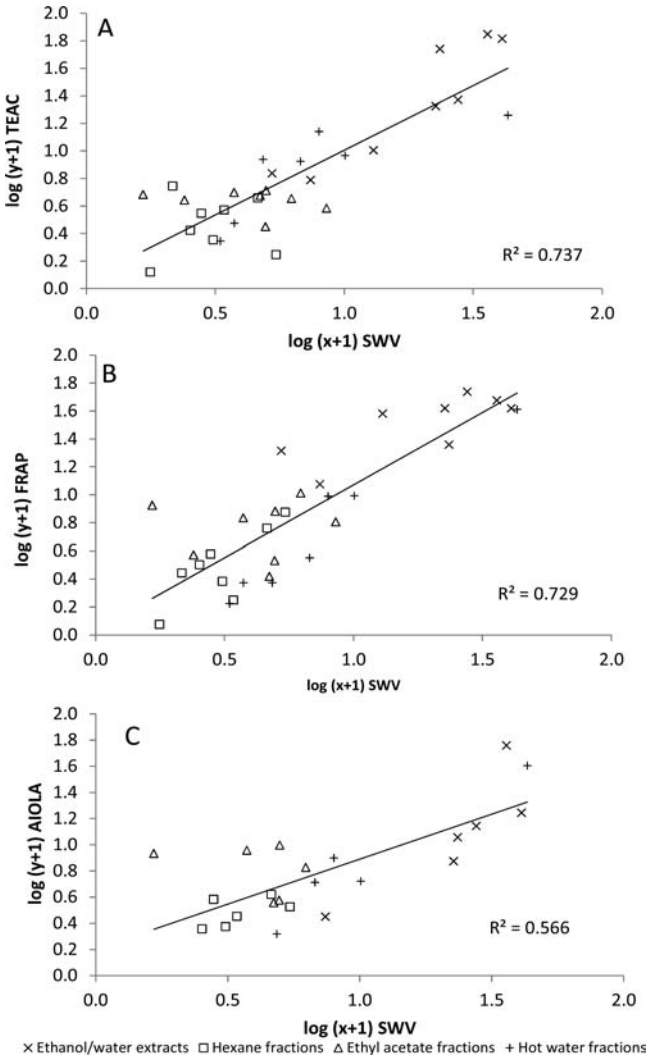

Figure 4. Correlation between measurements with square wave voltammetry and other established antioxidant assays, expressed as μmol Trolox equivalents per g dry biomass.

The significant R^2 values indicate that results from SWV and chemical antioxidant assays are closely related. The total AUC of SWV is therefore a good alternative for chemical-based antioxidant assays. This was also observed by Pohanka et al.[31] when comparing data from FRAP and SWV on antioxidant levels in blood plasma.

The good correlation between SWV and both TEAC and FRAP assays can be explained by comparing the peak oxidation potentials that are measured by SWV with the redox potentials of the reactive compounds in the TEAC and FRAP assays. Most of the area that is quantified from the voltammograms has a potential below 0.700 V (Figure 3). The TEAC assay quantifies antioxidant activity by reduction of $ABTS^{\bullet+}$, which has a redox potential of 0.680 V.[17] The FRAP assay quantifies antioxidant compounds that have redox potentials below 0.700 V (i.e., the redox potential of the Fe^{3+}−TPTZ complex). Whereas both TEAC and FRAP detect antioxidants that act through single-electron transfer, TEAC can also detect compounds that act as radical quenchers by hydrogen atom transfer.[17] The AIOLA assay, on the other hand, measures the ability of an antioxidant to prevent lipid oxidation and thus provides an indication of the potential of antioxidants for applications in foodstuffs. The good correlation between the SWV measurements and the results from the AIOLA assay indicate that the antioxidant components that are detected using SWV also reduce the oxidation of linoleic acid in our model system. Hence, SWV could be used as a fast screening technique in the search for new antioxidants to prevent lipid oxidation in foodstuffs. This is in agreement with the findings of Ceballos and Fernandez,[32] who used SWV for the determination of the well-known synthetic antioxidants BHT and BHA that are used to protect edible oils from oxidation.

Antioxidant activity in the extracts, as estimated from the AUC, was also compared with phenolic content and carotenoid content to estimate the contribution of these two classes of LMWA to total antioxidant activity. A significant correlation was found between SWV (AUC) and phenolic content (R^2 = 0.547; $p < 0.001$), suggesting that polyphenols make an important contribution to antioxidant activity as measured by SWV. A much weaker correlation was also found between SWV and carotenoid content (R^2 = 0.121; p = 0.031). In previous work, it was also stated that both phenolics and carotenoids contribute to the antioxidant activity of microalgae.[9,15] The weaker correlation for carotenoids might be due to the fact that carotenoids act mainly as antioxidants by hydrogen atom transfer, which cannot be detected by SWV, whereas polyphenols are more active as electron donors and are therefore more easily detected by voltammetric methods.

Comparison of Microalgal Species. Not only the AUC but also the peak potential is a good indicator of the antioxidant capacity of a biomass extract. On the basis of the AUC and the peak potentials observed in the voltammograms of Figure 3, an evaluation can be made of the potential of the eight microalgal

species as a source of antioxidants. The voltammograms of the ethanol/water extracts of *Scenedesmus obliquus* and *Porphyridium cruentum* had very low total AUC values and did not show pronounced peaks, indicating that these species are not promising sources of antioxidants. The voltammograms of the ethanol/water extracts of *Phaeodactylum* and *Isochrysis* had a relatively high AUC, but most of it was found at relatively high peak potentials, which suggests that the majority of the antioxidants they contain are relatively inefficient electron donors. For *Neochloris oleoabundans*, *Tetraselmis* sp., *Botryococcus braunii*, and *Nannochloropsis oculata* the AUC was high and the voltammograms contained peaks with potentials below 0.700 V, indicating that these microalgae contain powerful antioxidants. Differences in antioxidant activity between the species may be inherent properties of the species but may also be due to the conditions under which the species were cultured. The content of antioxidants in microalgae is highly variable and often increases with increasing light intensity.[33] The fact that *Scenedesmus* and *Phaeodactylum* were cultured using relatively weak artificial light, whereas others species were cultured outdoors in full sunlight, may explain why these two species had a relatively low antioxidant content.

In conclusion, the results indicate that square wave voltammetry represents a fast and relatively low-cost technique for measuring the antioxidant activity of microalgae, given the good correlation with established antioxidant assays. The voltammograms can be used for quantification of antioxidant activity, based on the area under the curve. Moreover, square wave voltammetry provides information on the antioxidant profile of the extracts, based on the oxidation potentials of the peaks in the voltammograms. Because both quantitative information and qualitative information are obtained, square wave voltammetry is better suited as a tool for optimizing the extraction of antioxidants from microalgae than the simple photometric tests that were used in this study.

AUTHOR INFORMATION

Corresponding Author

*E-mail: koen.goiris@kahosl.be. Phone: +32 (0)9 265 8613. Fax: +32 (09) 265 8724.

Funding

This research was financially supported by Research Foundation − Flanders (FWO), Project GA12911N.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Dr. Edward Matthijs of the Laboratory for Chemical Process Technology, KAHO Sint-Lieven, for the use of the equipment for voltammetric measurements.

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