Rapid Electrochemical Method for the Evaluation of the Antioxidant Power of Some Lipophilic Food Extracts

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In this paper, a novel electrochemical method to evaluate the antioxidant power of lipophilic compounds present in vegetables, such as carotenoids, chlorophylls, tocopherols, and capsaicin, is reported. The method is based on a flow injection system with an electrochemical detector equipped with a glassy carbon working electrode operating amperometrically at a potential of + 0.5 V (vs Ag/AgCl). The proposed method is selective for lipophilic compounds having antioxidant power. When applied to pure compounds, the order of antioxidant power resulted as follows: lycopene > β-carotene > zeaxanthin > α-carotene > β-cryptoxanthin > lutein > α-tocopherol > capsaicin > chlorophyll a > chlorophyll b > astaxanthin > canthaxanthin. Results obtained on five vegetable and two fruit extracts were compared to those obtained by the 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS) radical cation decolorization assay, one of the most used methods to evaluate the total antioxidant capacity of foods. A good correlation between the two methods was found, except for spinach, because of the different antioxidant powers assigned by the two methods to chlorophylls. In conclusion, results suggest that the proposed electrochemical method can be successfully employed for the direct, rapid, and reliable monitoring of the antioxidant power of lipophilic food extracts.

Keywords: *Antioxidant power; carotenoids; chlorophylls; capsaicin; electrochemical method; amperometric detection*

INTRODUCTION

Free radicals could be important causative agents for several pathological processes including cancer (*1*, *2*), atherosclerosis (*3*), and negative cellular changes associated with aging (*4*). It has been suggested that the consumption of dietary antioxidants plays an important role in protecting against these degenerative events. In particular, an association has been found between intake of high carotenoid-containing fruits and vegetables and protection from certain cancers (*5*). Recent work is also beginning to highlight the role of the phenolic constituents of the diet in contributing to these protective effects. These polar and nonpolar compounds, present in fruits and vegetables, act as reducing agents, hydrogen- or electron-donating agents, or singlet oxygen scavengers (*6*).

Because of the current interest in dietary antioxidants, the total antioxidant power (intended as the cumulative capacity of components to scavenge free radicals) of food extracts (*7*, *8*), spices (*9*, *10*), and beverages such as fruit juices (*11*), wines (*12*), and tea (*13*) has been evaluated by different assays. However, research has been particularly focused on the antioxidant activity of the water-soluble fraction, whereas little information is available on the lipophilic fraction. Among compounds present in the lipophilic food extracts, Miller et al. (*14*) reported the relative antioxidant activities of carotenes and xanthophylls through their abilities to scavenge the 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic) acid (ABTS) radical cation. However, lipophilic food extracts also contain other compounds, such as chlorophylls and essential oil components, that could contribute to food antioxidant activities. For example, it has been demonstrated that capsaicin, a lipophilic phenol, contributes to the antioxidant activity of fresh peppers (*15*, *16*).

In this paper, a novel electrochemical method to evaluate the relative antioxidant power of nonpolar compounds is reported. This method, similar to that recently published for the evaluation of wine and olive oil antioxidant power (*17*, *18*), is based on the chemicophysical properties of the molecules and does not require the use of radical species. Under the established working conditions, the electrochemical method was used for the evaluation of the antioxidant power of some lipophilic pure compounds and vegetable extracts. Finally, the relative antioxidant capacity obtained by the proposed method on vegetable extracts was compared with that obtained by the application of the ABTS radical cation decolorization assay (*19*).

MATERIALS AND METHODS

Chemicals and Reagents. Sodium dodecyl sulfate (SDS), hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azinobis-(3-ethylbenz-thiazoline-6-sulfonic) diammonium salt (ABTS), potassium persulfate (dipotassium peroxodisulfate), and α-tocopherol were obtained from Sigma-Aldrich srl (Sigma-Aldrich srl, Milan, Italy). Crystalline carotenoids used as standards were β-carotene, α-carotene, lycopene, lutein, and astaxanthin obtained from Sigma-Aldrich, and β-cryptoxanthin, and zeaxanthin purchased from Extrasynthese (Extrasynthese, Genay Cedex, France); canthaxanthin was a gift from F. Hoffmann-La Roche (Basel, Switzerland). Chlorophyll a, chlorophyll b, and capsaicin were purchased from Sigma-

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10.1021/jf010731y CCC: $20.00
Published on Web 10/10/2001

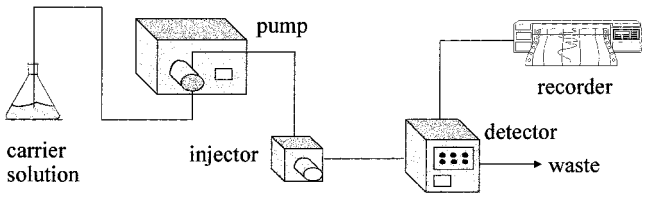

Figure 1. Schematic configuration of the flow system.

Aldrich. The concentrations of stock standard solutions were determined spectrophotometrically using a DU 650 Beckman spectrophotometer (Instruments Inc., Fullerton, CA). All standard solutions were stored in the dark at −20 °C under nitrogen and dissolved in the mobile phase to give individual working standards in the range of 0.1−10 μg mL^{-1} immediately prior to analysis. All solvents were of HPLC grade (Merck, Darmstadt, Germany).

Samples. Five vegetables and two fruits were purchased in a local store. The five vegetables were red, green, and yellow bell pepper, tomato, and spinach; the two fruits were melon and watermelon.

Extraction Procedure. Five grams of the edible portion of fresh food were extracted in duplicate according to the method of Riso and Porrini (*20*). Briefly, the extraction was performed in the dark with about 50 mL of unstabilized tetrahydrofuran (THF). The sample was homogenized by using an Ultra-Turrax homogenizer (Janke & Kunkel, Staufen, Germany) at a moderate speed for few minutes keeping the sample refrigerated by means of an ice bath to avoid overheating and potential carotenoid damage. Substances extracted in THF were partitioned in petroleum ether, and the extracts were transferred into 5-mL glass tubes. Each aliquot was dried under nitrogen flow and stored in the dark at −20 °C.

Electrochemical Method. *Apparatus.* The flow injection (FI) apparatus consisted of a Jasco (Tokyo, Japan) model 880 PU pump and an EG&G Princeton Applied Research (Princeton, NJ) model 400 thin-layer electrochemical detector equipped with a single glassy carbon electrode (surface area 8 mm^2), a reference (Ag/AgCl saturated) electrode, and a platinum counter electrode. The connecting tubes were of PEEK (1.5 mm o.d. × 0.5 mm i.d.). Data were recorded using a Philips (Eindhoven, Netherlands) PM 8252 recorder. The overall configuration of the system is shown in Figure 1.

Procedures. FI experiments were performed in amperometry. Amperometry is based on the oxidization or reduction of an electroactive compound at the working electrode while a constant potential is applied; the measured current in μA is a direct measurement of the electrochemical reaction rate. Analyses were performed at room temperature using a carrier solution composed of methyl tert-butyl ether (MTBE) 50%/methanol 45%/SDS 1.5%/H_2O 3.5%. A flow rate of 0.7 mL min^{-1} and a fixed working potential of + 0.5 V (vs Ag/AgCl) were employed; samples were dissolved in an appropriate volume of the carrier solution. Moreover, to investigate the electrochemical behavior of lipophilic compounds, hydrodynamic voltammetric experiments were carried out. Hydrodynamic voltammograms were obtained by running a series of FI experiments in which the potential was stepped incrementally in the range between 0.2 and 0.8 V (vs Ag/AgCl), and the current for the compounds of interest was measured.

ABTS Radical Cation Decolorization Assay. The antioxidant activities of food extracts were also evaluated by the ABTS radical cation decolorization assay (*19*). This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of Trolox, a water-soluble vitamin E analogue. The ABTS radical cation was prepared by reacting a 7 mM aqueous solution of ABTS with 2.45 mM potassium persulfate (final concentration) and diluted in ethanol to an absorbance of 0.70 (± 0.20) at 734 nm. After addition of 1.0 mL of this diluted solution to aliquots

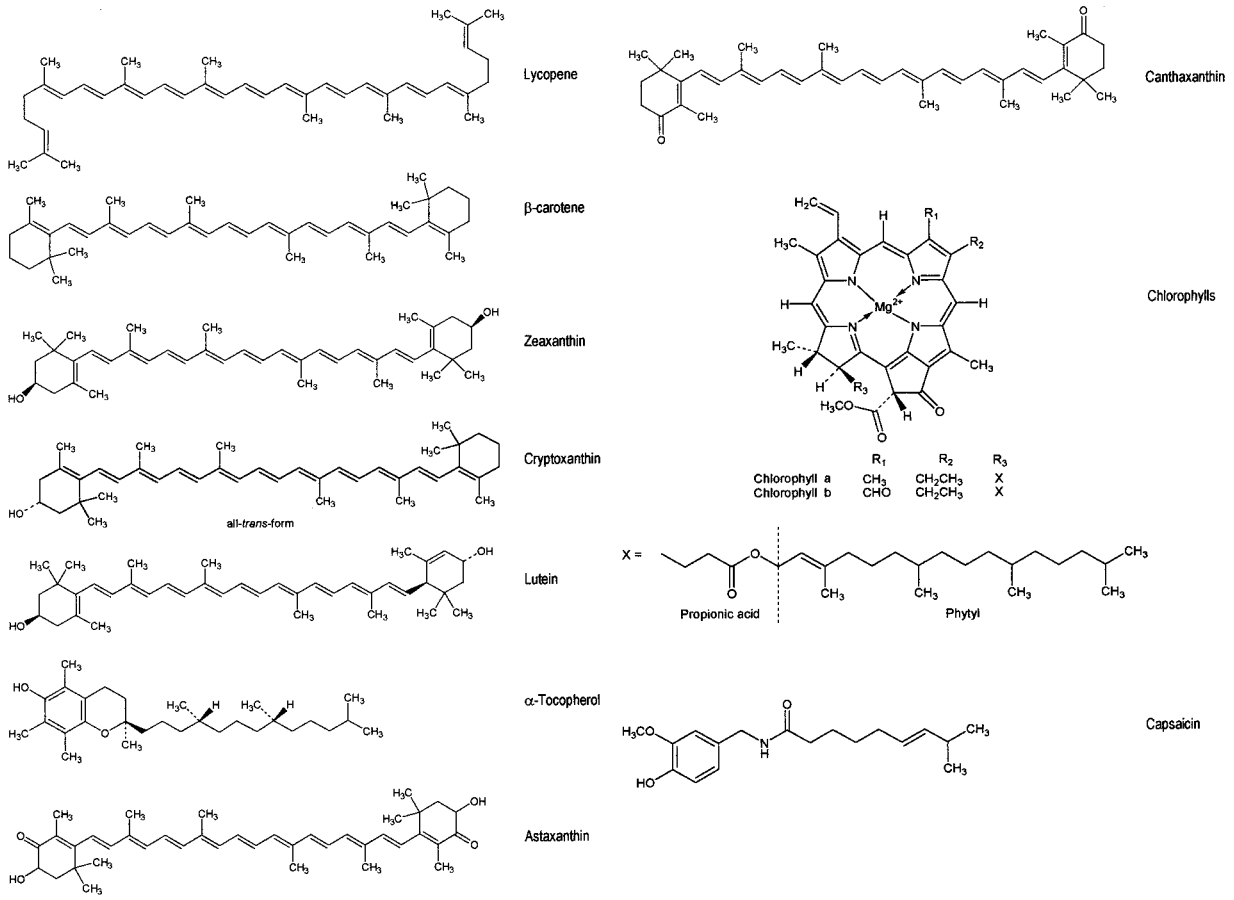

Figure 2. Chemical structures of analyzed lipophilic antioxidant compounds.

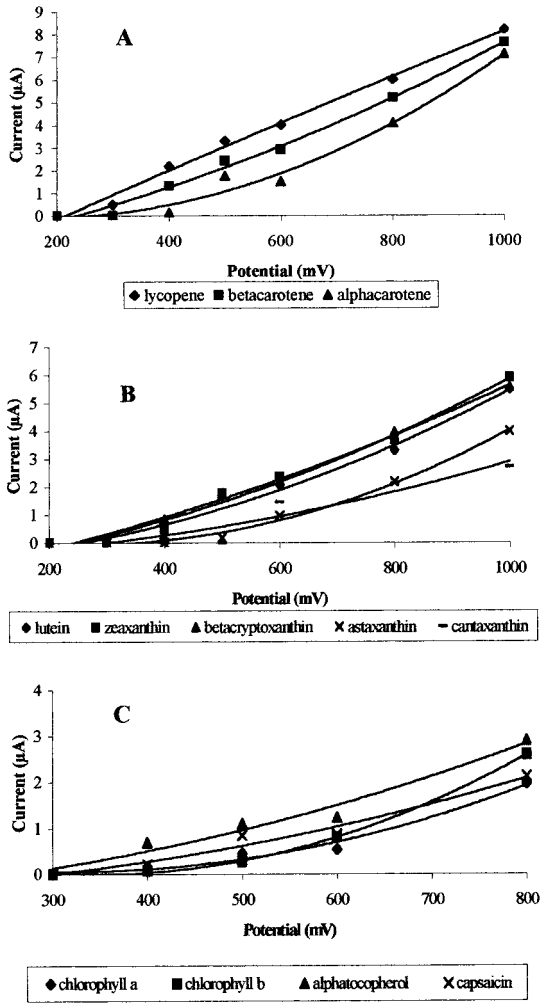

Figure 3. Hydrodynamic voltammetric profiles of (A) carotenes, (B) xantophylls, (C) other lipophilic compounds. Operative conditions: standard concentrations, 9.31×10^{-6} M; flow rate, 0.7 mL min^{-1}; carrier solution, MTBE 50%/methanol 45%/SDS 1.5%/H_2O 3.5%; injection volume, 20 μL.

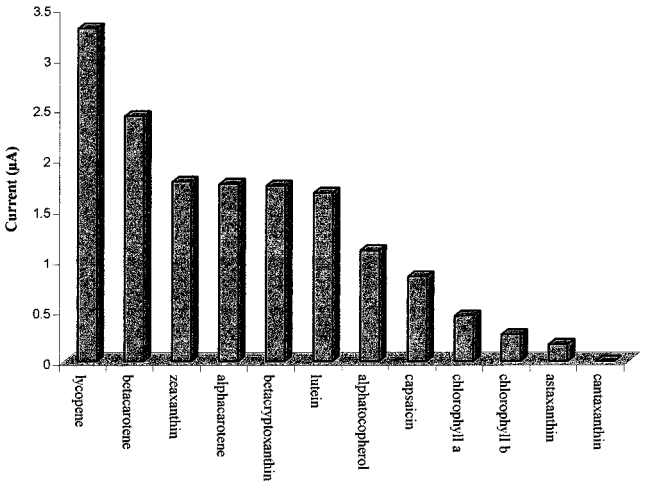

Figure 4. Current responses of lipophilic compounds injected in the FI system with the electrochemical detector operating with a working glassy carbon electrode at the established potential of + 0.5 V (vs Ag/AgCl). Operative conditions: standard concentrations, 9.31×10^{-6} M; flow rate, 0.7 mL min^{-1}; carrier solution, MTBE 50%/methanol 45%/SDS 1.5%/H_2O 3.5%; injection volume, 20 μL.

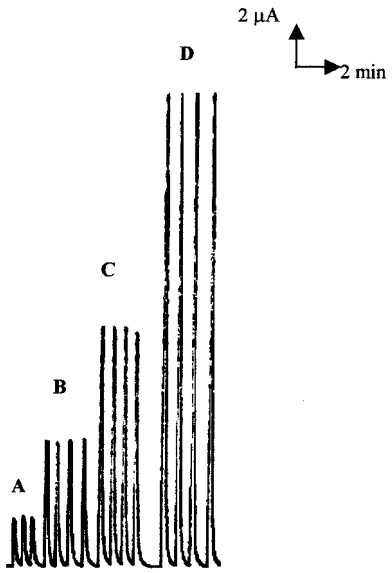

Figure 5. Typical flow injection responses obtained by repetitive injections of β-carotene standard solutions operating at a working potential of + 0.5 V (vs Ag/AgCl). β-carotene concentrations: (A) 1 mg L^{-1}; (B) 2.5 mg L^{-1}; (C) 5 mg L^{-1}; (D) 10 mg L^{-1}. Operative conditions: flow rate, 0.7 mL min^{-1}; carrier solution, MTBE 50%/methanol 45%/SDS 1.5%/H_2O 3.5%; injection volume, 20 μL.

of Trolox or samples, the absorbance reading was taken in a temperature-controlled spectrophotometer cuvette at 30 °C exactly 1 min after initial mixing. Appropriate solvent blanks were run in each assay. Addition of antioxidants to the preformed radical cation reduces it to ABTS determining a decolorization. The extent of decolorization as the percentage inhibition of ABTS radical cation is determined as a function of concentration and calculated relative to the reactivity of Trolox. The total antioxidant activity of food extracts was evaluated directly on extracts dissolved in 1 mL of THF and subsequently diluted at three different dilutions (between 1.6- and 4-fold, depending on their activity) and expressed as millimoles of Trolox per kilogram of fresh food.

HPLC Methods. *Carotenoids and Chlorophylls Determination.* Information about carotenoids and chlorophylls present in all samples evaluated for their antioxidant power were obtained using a HPLC equipment consisting of a 996 Photodiode Array detector (Waters, Milford, USA), a 600E Multisolvent Delivery system (Waters) equipped with a 20 μL loop. The chromatographic conditions were as follows: the column was a YMC-Pack Carotenoids (4.6 × 250 mm i.d.) (YMC Inc., Wilmington, North Carolina, USA) protected by a Waters Nova-Pak C18 guard column and maintained at 20 °C using a Waters column heater. Mobile phase A was a solution on methanol 81%/MTBE 15%/H_2O 4%; mobile phase B was a solution of methanol 6%/MTBE 90%/H_2O 4%. The flow rate was set at 1 mL min^{-1}, and the gradient was run by increasing linearly the percentage of B from 0 to 100% in 90 min, then returning to the initial conditions (100% A; 0% B) in 5 min; the column was reequilibrated for 15 min between runs. Peak responses were measured at 280 nm for capsaicin, 450 nm for carotenoids, 662 nm for chlorophyll a, and 644 nm for chlorophyll b. A photodiode array detector supported by the Millenium[32] chromatography manager computing system (Waters) was used to assess or confirm the spectral identity of carotenoids, chlorophylls, and capsaicin registering the spectra in the range between 200 and 700 nm. Recoveries, checked by using canthaxanthin as internal standard, ranged between 92 and 96%.

Tocopherol Determination. Food extracts were dissolved in 1 mL of *n*-hexane and then diluted at two different dilutions (between 2- and 100-fold). All samples were analyzed in duplicate by normal-phase HPLC coupled with an UV detector set at 295 nm (*21*). The chromatographic conditions were as follows: 20 μL of samples were injected into a LiChrospher Si

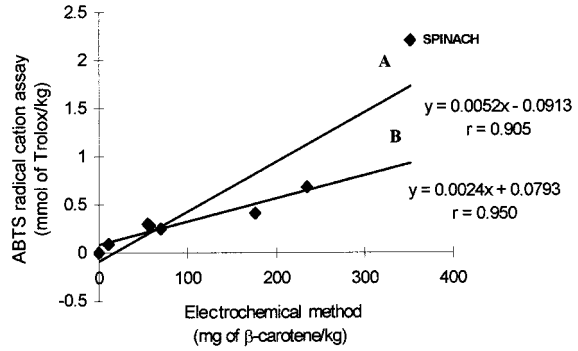

Figure 6. Linear correlation plots of the antioxidant power of vegetable lipophilic extracts evaluated by the electrochemical method *versus* the ABTS radical cation decolorization assay. Line A corresponds to the regression line of all data; line B corresponds to the regression line of all data except the value of spinach.

Table 1. Antioxidant Power of Vegetable Lipophilic Extracts Analyzed Applying the Proposed Electrochemical Method and the ABTS Radical Cation Decolorization Assay (mean ± SD, *n* = 3)

samples	electrochemical method (mg of β-carotene/kg)	ABTS radical cation assay (mmol of Trolox/kg)
red bell pepper	176.56 ± 4.95	0.41 ± 0.015
yellow bell pepper	69.71 ± 2.83	0.25 ± 0.010
green bell pepper	57.19 ± 1.65	0.28 ± 0.007
tomato	235.65 ± 7.07	0.68 ± 0.040
watermelon	55.09 ± 0.95	0.30 ± 0.015
melon	10.63 ± 0.07	0.09 ± 0.004
spinach	351.56 ± 6.14	2.20 ± 0.060

60-5 μm (Merck, Darmstadt, Germany) column using *n*-hexane/ethyl acetate (1000:75, v/v) as the mobile phase at a flow rate of 2 mL min $^{-1}$. Results are expressed as milligrams of α-tocopherol per kilogram of fresh food.

RESULTS AND DISCUSSION

It is known that the antioxidant power of natural compounds is mainly dependent on structural factors (*22*−*24*). Since the electrochemical behavior of these compounds depends on the same structural features, it can provide a chemical basis to describe their ability to act as electron donors and thus their antioxidant capacity.

In Figure 2 the chemical structures of lipophilic compounds assayed are reported.

The hydrodynamic voltammetric profiles of these compounds are shown in Figure 3. These profiles show the oxidative behavior of compounds in relation to the established potential; as it can be seen, no compounds can be detected at potential equal or lower than + 0.2 V (vs Ag/AgCl). Lycopene is the only compound revealed at + 0.3 V, whereas astaxanthin and cantaxanthin are oxidizable only at potentials greater than + 0.5 V. Since the oxidization potential of a compound provides an estimate of the energy required to donate an electron, the lower the oxidization potential the easier the compound will donate an electron and the higher will be its expected antioxidant activity. For this reason, the potential of + 0.5 V (vs Ag/AgCl) appears to be selective to discriminate only the compounds having high reducing capacity, hence with effective antioxidant power. The responses of lipophilic compounds at the established potential of + 0.5 V are displayed in Figure 4. Lycopene has more antioxidant power than the other analyzed compounds, and β-carotene is more effective than its isomer α-carotene. Molecules such as β-cryptoxanthin, zeaxanthin, and lutein show similar reducing capacity, and the presence of hydroxyl groups on the terminal rings seems to lower their reducing capacity with respect to carotenes. In addition, the incorporation of carbonyl groups in the rings has a negative effect on the electrochemical response at + 0.5 V; this is evident for canthaxanthin and astaxanthin whose antioxidant power at + 0.5 V is 0.0 and 0.17 μA, respectively.

Among chlorophylls, chlorophyll a shows an antioxidant activity stronger than chlorophyll b, in accord with Endo et al. (*25*). This is not surprising, since the latter contains a more oxidized (formyl) residue, instead of a methyl group present in chlorophyll a.

Finally, regarding capsaicin, we can ascribe its antioxidant power to the 4-hydroxy-3-methoxy phenyl residue, as in the case of both vanillic and ferulic acids (*16*).

The electrochemical ranking of lipophilic compounds found in our working conditions is in good agreement with the antioxidant capacity reported in the literature (carotenes > hydroxycarotenoids > ketocarotenoids) (*14*, *23*, *26*). This agreement allows us to consider the direct injection of samples in the FI system with electrochemical detector operating at a potential of + 0.5 V (vs Ag/AgCl) as a simple method for determining the antioxidant power of lipophilic vegetable extracts.

Figure 5 displays typical flow injection peaks obtained for increasing concentrations of β-carotene used as standard. As it can be seen, the detector responds rapidly to the dynamic changes in β-carotene concentration, allowing about 60 determinations h^{-1}. These

Table 2. Content of Carotenoids, Chlorophylls, Capsaicin, and α-Tocopherol of the Vegetable Lipophilic Extracts (mean ± SD, *n* = 3)

	carotenoids (mg/kg)						chlorophylls (mg/kg)			
	lutein	9-*cis*-β-carotene	15-*cis*-β-carotene	*trans*-β-carotene	α-carotene	lycopene	chlorophyll a	chlorophyll b	capsaicin (mg/kg)	α-tocopherol (mg/kg)
red bell pepper		1.6 ± 0.2		9.1 ± 0.4					440.6 ± 15.3	19.8 ± 0.4
yellow bell pepper	31.4 ± 0.9		2.7 ± 0.04	3.6 ± 0.1	1.1 ± 0.01				117.2 ± 11.2	23.6 ± 3.6
green bell pepper	6.7 ± 0.1		0.6 ± 0.2	2.3 ± 0.05			3.4 ± 0.01	21.5 ± 0.1	130.6 ± 12.0	6.0 ± 0.5
tomato			2.6 ± 0.03	7.0 ± 0.05		133.8 ± 21.3				8.7 ± 0.4
watermelon				1.9 ± 0.02		46.7 ± 1.2				1.2 ± 0.1
melon				10.9 ± 0.6						nd[a]
spinach	105.6 ± 34.7	6.3 ± 0.9		56.2 ± 2.3			927.21 ± 48.8	463.6 ± 33.6		19.8 ± 0.4

[a] nd: not detectable.

measurements are part of a calibration experiment over the 1−10 mg L^{-1} range of β-carotene concentration. The resulting calibration plot is highly linear (slope 0.4725 μA L mg^{-1}; intercept 0.070 μA). The relative standard deviation at the concentration level of 5 mg L^{-1} is 3.5% ($n = 12$) and the estimated detection limit, calculated using the linear regression technique from Miller and Miller (*27*), is 0.2 mg L^{-1}.

Vegetables and fruits with different lipophilic composition were analyzed by the proposed procedure and the results were expressed as milligrams of β-carotene equivalents per kilogram of fresh food. In Table 1, the antioxidant power of lipophilic extracts, determined electrochemically, is reported and compared to that obtained by the ABTS radical cation decolorization assay (*19*). Among food analyzed, spinach exhibits the highest antioxidant power, followed by tomato, whose high antioxidant power is likely due to its high lycopene content (*28*). Conversely, red bell pepper exhibits a high antioxidant capacity, despite its low carotenoid content (*29*). These results demonstrate that carotenoids are not the only contributors to the antioxidant activity of lipophilic food extracts. As shown in Figure 6, there is a good correlation between the two methods (regression line A), even thought it is evident that the spinach sample is an outlayer. In fact, the correlation coefficient improves when this value is dropped out (regression line B). This could be explained by the fact that the ABTS radical cation decolorization assay and the electrochemical method evaluate differently the antioxidant capacity of chlorophylls. In fact, experimental data (not shown) demonstrate that the ABTS radical cation decolorization assay estimates chlorophyll a as an antioxidant as β-carotene, whereas chlorophyll a results in five times less antioxidant than β-carotene when evaluated by the electrochemical method (Figure 4).

To better understand the observed differences in the antioxidant power, lipophilic extracts were analyzed by HPLC and the results are reported in Table 2. The HPLC data suggest that the main contributor to the antioxidant power of melon is β-carotene. On the other hand, other vegetables that exhibit a relevant antioxidant activity, such as spinach and red, yellow, and green peppers, contain very little carotenoids, but they are important source of chlorophylls and capsaicin. Even if all these compounds are electrochemically less antioxidant than carotenes, when present in high concentrations their contribution to antioxidant power became determinant. Finally, in all food extracts analyzed, the smallα-tocopherol content, combined with the low extractive capacity of THF on this compound, results in negligible contribution to the antioxidant power as compared to other lipophilic compounds.

Our results suggest that the proposed electrochemical method can be successfully employed for the direct, rapid, and reliable monitoring of antioxidant power in lipophilic food extracts. Particularly interesting is the rapidity of analysis (60 determination h^{-1}) that makes the flow system an attractive alternative over other reported methods. Furthermore, the proposed method is based only on the chemicophysical properties of the molecules and does not require the use of reactive species to evaluate the antioxidant power of food. Moreover, HPLC data on the lipophilic food extract composition demonstrate that carotenoids are not the only contributors to antioxidant activity of lipophilic extracts.

ABBREVIATIONS USED

SDS, sodium dodecyl sulfate; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; ABTS, 2,2′-azinobis(3-ethylbenz-thiazoline-6-sulfonic) acid; FI, flow injection; THF, unstabilized tetrahydrofuran; MTBE, methyl tert-butyl ether.

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Received for review June 4, 2001. Revised manuscript received August 14, 2001. Accepted August 16, 2001.

JF010731Y