JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

High-Throughput Methods To Assess Lipophilic and Hydrophilic Antioxidant Capacity of Food Extracts in Vitro

D. JIMENEZ-ALVAREZ,[†] F. GIUFFRIDA,*[,†] F. VANROBAEYS,[†] P. A. GOLAY,[†] C. COTTING,[†] A. LARDEAU,[†] AND BRENDAN J. KEELY[§]

Nestlé Research Centre, Lausanne, Switzerland, and Department of Chemistry, University of York, York, England

Assays comprising three probes for different mechanisms of antioxidant activity in food products have been modified to allow better comparison of the contributions of the different mechanisms to antioxidant capacity (AOC). Incorporation of a common format for oxygen radical absorbance capacity (ORAC), ferric reducing antioxidant power (FRAP), and iron(II) chelating activity (ICA) assays using 96-well microplates provides a comprehensive and high-throughput assessment of the antioxidant capacity of food extracts. The methods have been optimized for aqueous extracts and validated in terms of limit of quantification (LoQ), linearity, and precision (repeatability and intermediate reproducibility). In addition, FRAP and ORAC assays have been validated to assess AOC for lipophilic extracts. The relative standard deviation of repeatability of the methods ranges from 1.2 to 6.9%, which is generally considered to be acceptable for analytical measurement of AOC by in vitro methods. Radical scavenging capacity, reducing capacity, and iron chelating properties of olive mill wastewaters (OMWW), oregano, and parsley were assessed using the validated methods. OMWW showed the highest radical scavenging and reducing capacities, determined by ORAC and FRAP assays, respectively, followed by oregano and parsley. The ability to chelate Fe^{2+} was, in decreasing order of activity ($p > 0.05$) parsley $\cong$ oregano $>$ OMWW. Total phenol content, determined by the Folin−Ciocalteu method, correlated to the radical scavenging and reducing capacities of the samples but not to their chelating properties. Results showed that the optimized high-throughput methods provided a comprehensive and precise determination of the AOC of lipophilic and hydrophilic food extracts in vitro.

KEYWORDS: Antioxidant capacity; phenols; ORAC; FRAP; chelating activity; Folin−Ciocalteu; olive mill wastewater; oregano; parsley

INTRODUCTION

Antioxidants have been used for a long time in foods to prevent lipid oxidation. The effectiveness of antioxidants depends on chemical, physical, and environmental factors such as pH and ionic strength (*1*). These factors vary in different food matrices, and thus the antioxidant effectiveness must be determined in each individual food. The procedure to evaluate antioxidant effectiveness in food generally requires storage of the food for a period of time and regular evaluation of lipid oxidation compounds over the storage period. This approach is time-consuming and often expensive. Accordingly, rapid in vitro methods have been developed to estimate the chemical antioxidant capacity (AOC). In vitro methods are one-dimensional tests that estimate the chemical reactivity of antioxidants with respect to a free radical or oxidant source. In vitro assays provide relevant information about the antioxidant mechanisms of action (*2*). Unfortunately, in vitro methods do not take into account important factors affecting antioxidant effectiveness in food systems such as the physical location of the antioxidant (*3*). Protocols including a battery of in vitro assays are needed because a single one-dimensional method cannot reflect the complexity of the antioxidant action in food (*2, 4*).

Many in vitro assays to assess the radical scavenging capacity of antioxidants have been developed. Methods differ in the free radical and oxidant sources [e.g., $O_2^{\bullet -}$, $^1O_2$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^-$, HOCl, $RO(O)^{\bullet}$, $LO(O)^{\bullet}$], pH, reagents, and the analytical techniques used to evaluate the AOC (*5*). Meaningful comparison of the results obtained by different methods is practically impossible due to the variability in experimental conditions (*5*).

* Address correspondence to this author at the Nestlé Research Centre, Vers-Chez-les-Blanc, 1000 Lausanne, Switzerland (e-mail francesca.giuffrida@rdls.nestle.com; telephone +41 21 785 8085; fax +41 21 785 8553).

[†] Nestlé Research Centre.

[§] University of York.

10.1021/jf703723s CCC: $40.75 © 2008 American Chemical Society
Published on Web 04/24/2008

The First International Congress on Antioxidant Methods (*6*) discussed the current status of the methods being used and attempted to drive standardization of the methods, which could allow researchers to compare results more readily. Prior et al. (*7*) proposed standardization on three methods: Folin−Ciocalteau assay (FC), oxygen radical absorbance capacity (ORAC), and Trolox equivalent antioxidant capacity (TEAC). The FC method is based on a single electron transfer (SET) reaction and has normally been used to estimate the phenol content of foods. The assay is based on the oxidation of phenol compounds at high pH by a molybdotungstophosphoric heteroplyanion reagent, yielding a colored product with λ_{max} at 765 nm. The FC assay is simple, fast, and robust and does not require specialized equipment. A drawback of the FC assay is that reducing agents such as ascorbic acid can interfere in the analysis and thus overestimate the content of phenol compounds. Recently, several modifications of the FC method have been published that avoid interferences of reducing compounds (*8, 9*). The ORAC assay measures antioxidant inhibition of peroxyl radical, relevant in lipid oxidation in food, and thus reflects classical radical chain-breaking antioxidant activity by a hydrogen transfer mechanism (HAT) (*4, 7, 10*). In the ORAC assay, artificially generated peroxyl radicals react with fluorescein. As the reaction progresses fluorescein is consumed and the fluorescence decreases. In the presence of an antioxidant a competitive reaction takes place between the fluorescent probe and the antioxidant, retarding the consumption of fluorescein. Thus, AOC can be calculated by the difference in the area under the curve (AUC) between the blank and the sample. Moreover, the ORAC assay estimates the AOC of both hydrophilic and lipophilic compounds (*11*). The estimation of the lipophilic antioxidants is important because they account significantly for the total antioxidant capacity of some foods (*12, 13*). The TEAC assay is based on a SET reaction and estimates the ability of an antioxidant to reduce the artificially generated $ABTS^{\bullet+}$ radical cation. Similar to TEAC, the ferric reducing antioxidant power (FRAP) assay is based on a SET reaction and measures the ability of the antioxidant to reduce a ferric 2,4,6-tripyridyl-*s*-triazine salt (Fe^{3+}−TPTZ) to the blue-colored ferrous complex (Fe^{2+}−TPTZ) at low pH. Both TEAC and FRAP provide similar results because the redox potentials of the Fe^{3+}−TPTZ salt and $ABTS^{\bullet+}$ radicals are similar (0.70 and 0.68 V, respectively) (*4*). Iron, used as substrate in the FRAP assay, is more relevant in lipid oxidation processes than the artificially generated $ABTS^{\bullet+}$ radicals.

The presence of metals such as iron and copper has been shown to catalyze lipid oxidation in food (*14–17*). Thus, assays to estimate the metal chelating properties of antioxidants have been developed (*18, 19*). The iron chelating activity (ICA) assay (*20*) estimates the ability of antioxidants to chelate Fe^{2+}. In the ICA method, Fe^{2+} is quantified by spectrophotometry using ferrozine (FZ) to form a colored complex with iron (Fe^{2+}−FZ) and thus the amount of iron chelated by a food extract is calculated by difference in absorbance between a blank (Fe^{2+} and FZ) and a sample (Fe^{2+}, food extract and FZ).

The objective of this study was to develop a standard procedure for the measurement of the AOC of food extracts using assays that target different antioxidant mechanisms, that is, radical scavenging capacity, reducing capacity, and metal chelating properties. Three methods were chosen, ORAC, FRAP, and ICA, and validated in terms of linearity and precision. In addition, total phenol content was measured by the FC method. The hydrophilic and lipophilic antioxidant capacity of food extracts was assessed by the ORAC and by an optimized and validated lipophilic FRAP assay (L-FRAP), and the ICA assay was adapted to microplate reader format to allow high-throughput analyses at low pH (3.6). Finally, the AOC of dried oregano, dried parsley, and olive mill wastewater (OMWW) was assessed using the developed procedures.

MATERIALS AND METHODS

Chemicals. (±)-6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid, fluorescein, 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ), iron(III) chloride, ethylenediaminetetraacetic acid (EDTA), and anhydrous iron(II) chloride (beads) were obtained from Sigma-Aldrich (Buchs, Switzerland). Folin−Ciocalteu reagent was purchased from Merck (Dietikon, Switzerland). 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was purchased from Wako Chemicals GmbH. Randomly methylated-β-cyclodextrin (RMCD) was purchased from Cyclolab Ltd. (Budapest, Hungary). All other chemicals used were of analytical grade.

Sample Preparation. Lyophilized oregano (*Origanum vulgare*) and parsley (*Petroselinum crispum*) were purchased from a local market. Lyophilized OMWW was provided by Ebiser (Castellón, Spain). All samples were stored in the dark at room temperature until use.

*Extraction (ORAC, FRAP, and ICA Assays).*Lipophilic and hydrophilic plant extracts were obtained according to the method of Prior et al. (*10*) and in duplicate. Briefly, 1 g of ground sample was weighed in a 20 mL centrifuge tube. Lipophilic extract was obtained by mixing the sample with hexane (2 × 10 mL) and centrifuging at 2500 *g* for 5 min. Supernatants were combined and evaporated to dryness under nitrogen. The lipophilic residue was reconstituted in 10 mL of acetone and filtered. Hydrophilic extract was obtained by mixing the residue from lipophilic extraction with 3 × 5 mL acetone/water/acetic acid (70:28:2 v/v/v) and centrifuging at 2500*g* for 5 min. Supernatants were combined, and acetone/water/acetic acid (70:28:2 v/v/v) was added to constant volume (25 mL) before filteration.

Determination of Total Phenol Content (FC Method). *Sample Preparation in the FC Assay.* To determine total phenol content, samples were extracted according to the method of Georgé et al. (*8*) with minor modification. Briefly, 1 g of sample was extracted with 50 mL of acetone/water/acetic acid 70:28:2 (v/v/v) for 30 min and filtered to obtain the raw extracts (RE). RE were diluted with distilled water (1/10) and loaded (2 mL) onto an Oasis cartridge (Waters SA, Montreux, Switzerland). Interfering water-soluble compounds were eluted with distilled water (2 × 2 mL). The recovered volume of the washed extract (WE) containing the interfering compounds was carefully measured. Both RE and WE were submitted to FC analyses.

FC Analyses. Diluted RE and WE were both analyzed in duplicate using the FC method (*21*), as modified by Georgé et al. (*8*) and adapted to our study with minor changes. Briefly, extract (300 μL) was mixed with diluted (1/10) FC reagent (1.5 mL). The mixture was incubated for 2 min at room temperature, followed by the addition of 1.2 mL of sodium carbonate solution (75 g/L). The mixture was incubated at 50 °C for 15 min, and the absorbance was measured at 760 nm (UV−vis spectrophotometer Lambda 12, Perkin-Elmer AG). Gallic acid standard solutions (5, 10, 20, 30, 40, and 50 mg/L) were used to construct a calibration curve. Results from the diluted RE included the quantification of both phenol compounds and interferences, whereas results from WE included only the interferences results. Thus, total phenol content was calculated by subtracting WE from RE results. Total phenols were expressed as milligrams of gallic acid equivalents per gram of original sample on dry mass basis (mg of GAE/g, db).

ORAC. Hydrophilic ORAC (H-ORAC) assay was performed according to the method of Huang et al. (*11*) with minor modification. Duplicate hydrophilic extracts (0.1, 0.2, and 0.4 g/L) and Trolox calibration solutions (30, 50, 70, 90, and 110 μM) were prepared in phosphate buffer, pH 7 (disodium hydrogen phosphate/potassium dihydrogen phosphate, Merck KGaA). Blank (phosphate buffer, pH 7), hydrophilic extracts and Trolox solutions were submitted to the H-ORAC assay: 20 μL of sample was added to a 96-well microplate followed by the addition of 200 μL of fluorescein solution (1.1 μM). The mixture was incubated at 37 °C for 20 min before the addition of 75 μL of AAPH solution (63 mM). Fluorescence was monitored using

485 nm (excitation) and 525 nm (emission) at 1 min intervals for 90 min (microplate reader fluorimeter FLxTD800, BioTek Instruments, Inc.). Lipophilic ORAC (L-ORAC) assay was performed in duplicate as described above, except that the solvent used to dilute the lipophilic extracts and Trolox solutions was 7% (%w) randomly methylated-β-cyclodextrin (RMCD) in acetone/water (1:1 v/v) solution. The ORAC values were calculated by plotting Trolox concentration (micromolar) versus the net area under the fluorescence curve. All measurements were expressed relative to the initial reading. The area under the curve (AUC) was calculated as

$$\mathrm{AUC} = (1 + f_1/f_0 + f_2/f_0 + f_3 + f_4/f_0 + \ldots f_{44}/f_0 + f_f/f_0) \times \mathrm{CT} \quad (1)$$

where f_0 = initial fluorescence reading, f_i = fluorescence reading at cycle i, and CT = cycle time in minutes.

The net AUC was calculated by subtracting the AUC of the blank from that of the sample, and results were expressed as micromoles of Trolox equivalents (TE) per gram of original sample on a dry mass basis (μmol of TE/g, db). The total ORAC value is considered to be the sum of H-ORAC and L-ORAC.

FRAP. The hydrophilic FRAP (H-FRAP) assay was performed according to the method of Benzie et al. (*22*) with minor modification and in duplicate. Hydrophilic extracts (1, 2, and 4 g/L) and Trolox calibration solutions (90, 180, 270, 360, 450, and 540 μM) were prepared in Milli-Q water. FRAP reagent was prepared by mixing 20 mL of acetate buffer (pH 3.6), 2 mL of TPTZ (10 mM, dissolved in 400 mM HCl), and 2 mL of iron(III) chloride (20 mM). Blank (Milli-Q water), hydrophilic extracts, and Trolox calibration solutions were submitted to FRAP analyses as follows: 25 μL of sample and 250 μL of FRAP reagent were added in a 96-well microplate and incubated at room temperature for 8 min, and absorbance was measured at 593 nm. Lipophilic FRAP (L-FRAP) assay was performed as described above, but dissolving the extracts (10, 20, and 50 g/L) in 7% (%w) RMCD acetone/water (1:1 v/v) and Trolox calibration solutions in acetone/water (1:1 v/v). The FRAP value was calculated by plotting Trolox concentration (micromolar) versus the absorbance, and results were expressed as micromoles of Trolox equivalents (TE) per gram of original sample on a dry mass basis (μmol of TE/g, db). The final FRAP value is considered to be the sum of H-FRAP and L-FRAP.

ICA. Hydrophilic metal chelating capacity assay was performed according to the method of Carter (*20*) with minor modification and in duplicate. EDTA calibration solutions (8, 16, 24, 32, 40, and 48 μM) and hydrophilic extracts (0.5, 1, and 5 g/L) were diluted in acetate buffer (pH 3.6). Blank (acetate buffer, pH 3.6), hydrophilic extracts, and Trolox calibration solutions were submitted to ICA analyses as follows: 135 μL of sample and 15 μL of iron(II) chloride (1 mmol/L in methanol) were added in a 96-well microplate. Following incubation for 20 min at room temperature 150 μL of ferrozine (1 mmol/L in acetate buffer, pH 3.6) was added, and the absorbance was measured at 562 nm after a further 5 min of incubation. The chelating capacity (percent) of antioxidant was calculated as follows:

$$\%\ \text{iron chelated} = \left(1 - \frac{\text{absorbance of sample at 562 nm}}{\text{absorbance of blank at 562 nm}}\right) \times 100 \quad (2)$$

Results were expressed as micromoles of EDTA equivalents per gram of original sample on a dry mass basis (μmol of EDTA equiv/g, db).

Statistical Analyses. ORAC, FRAP, and ICA were validated in terms of linearity and precision with an in-house statistical program making use of the robust-statistics concept of Rousseew and Croux (*23*). Linearity was evaluated by calculating the coefficient of determination (r^2) and the residual standard deviation (SEC), which expresses the variability of the difference between the experimental data and the value predicted by the linear regression model. The precision (repeatability and intermediate reproducibility) of the methods was evaluated by calculating the standard deviation, relative standard deviation, and repeatability/reproducibility limits at 95% confidence level (calculated as 2.772 × standard deviation). Samples were analyzed at least in duplicate. Significance differences between samples were calculated by mean comparison using the test of Aspin−Welch, and significance was declared at $p < 0.05$.

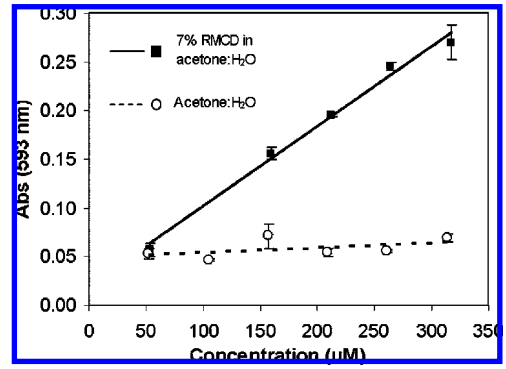

Figure 1. Influence of the dilution solvent on the antioxidant activity of α-tocopherol in the L-FRAP assay. RMCD, randomly methylated β-cyclodextrin. Error bars represent the standard deviation of triplicate analyses. Some error bars may overlap with the data points.

RESULTS AND DISCUSSION

Optimization and Validation of Antioxidant Methods. FC and ORAC analyses were carried out according to previously published methods (*8, 21, 24*), whereas FRAP and ICA methods were modified from the original methods (*20, 22*). Validation was performed for all methods measuring AOC, that is, ORAC, FRAP, and ICA methods.

Optimization of the FRAP Assay. Three aspects of the FRAP method were optimized: (1) the solvent used to assess AOC of lipophilic compounds; (2) temperature; and (3) amount of reactive species required for microplate reader measurements.

A suitable solvent to dilute the lipophilic extracts must allow the reaction between Fe^{3+}−TPTZ and the extracts. Previously, 7% (%w) RMCD in acetone/water (1:1 v/v) has been found to be suitable for assessing the AOC of lipophilic compounds (*11*). Nevertheless, RMCD (a solubility enhancer for lipophilic antioxidants in aqueous solutions) is not easily affordable for small and medium laboratories. To select a convenient solvent for lipophilic measurement, Trolox and α-tocopherol were dissolved in acetone/water (1:1 v/v) and in 7% RMCD (%w) acetone/water (1:1 v/v), and the reducing capacity was measured. The reducing capacities of Trolox dissolved in either 7% RMCD (%w) in acetone/water (1:1 v/v) or acetone/water (1:1 v/v) were compared. No significant difference ($p < 0.05$, results not shown) in the reducing capacity of Trolox was found. Therefore, Trolox calibration solutions were prepared in acetone/water (1:1 v/v). However, α-tocopherol reducing capacity was influenced greatly by the solvent, and solutions diluted in 7% RMCD in acetone/water showed a significantly higher reducing capacity than solutions diluted in acetone/water (1:1 v/v) (**Figure 1**). These results indicate that a solubility enhancer of lipophilic compounds in aqueous solutions such as 7% RMCD (%w) in acetone/water (1:1 v/v) is needed to allow the chemical reaction between lipophilic compounds and the aqueous FRAP reagent. Thus, 7% RMCD (%w) in acetone/water (1:1 v/v) was selected to dilute the lipophilic extracts.

The influence of temperature on L-FRAP and H-FRAP assays was evaluated by measuring the ferric reducing capacities of α-tocopherol and Trolox solutions (concentrations ranging from 90 to 540 μM) at 25 and 37 °C. No significant differences in absorbance were found at the two temperatures ($p < 0.05$, results not shown). Thus, experiments were performed at 25 °C.

The amounts and concentrations of reactants in the original assay (*22*) were varied to obtain high absorbance within the

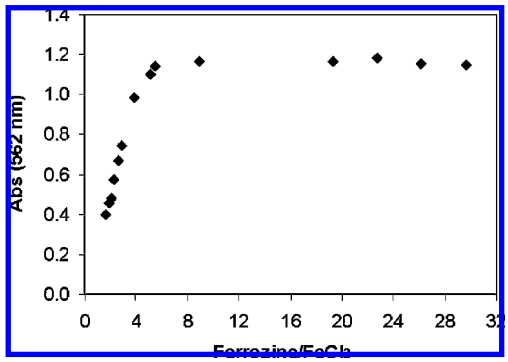

Figure 2. Impact of the ferrozine/$FeCl_2$ ratio (FZ/$FeCl_2$) on the absorbance (λ = 562 nm) in the ICA assay.

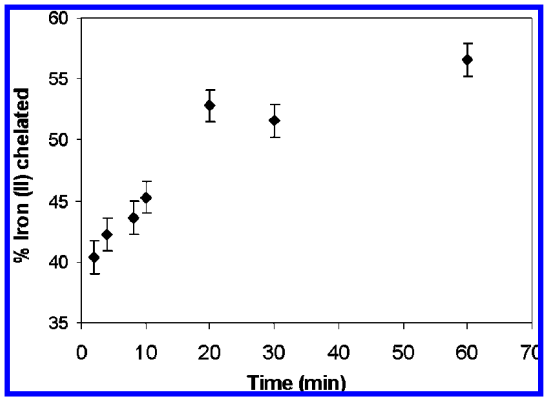

Figure 3. Impact of the incubation time between Fe^{2+} solution (16.7 μM) and EDTA solution (8.6 μM) on the amount of Fe^{2+} chelated.

quantification range of the spectrophotometer and within the limitation of the 96-well microplate volumes (maximum = 300 μL). The amounts of reactant chosen were 25 μL of sample (at the concentrations specified under Materials and Methods) and 250 μL of FRAP reagent.

Optimization of the ICA Assay. The ICA assay was optimized and validated for microplate reader spectrophotometer measurements in acid media (pH 3.6). A low pH was chosen to optimize iron solubility and to provide conditions similar to those found in foods such as fish oil enriched mayonnaise that are susceptible to iron pro-oxidant activity (*17*).

The amount and concentrations of reactants were optimized to obtain a high absorbance within the quantification range of the spectrophotometer. It is important to add a sufficient amount of ferrozine (FZ) to complex all available Fe^{2+}. By stoichiometry, 3 mol of FZ complex 1 mol of Fe^{2+} (*25*). The optimal FZ/$FeCl_2$ ratio was investigated using ratios ranging from 2 to 30 and measuring the absorbance at 562 nm (**Figure 2**). At FZ/$FeCl_2$ ratios > 6 the absorbance was constant, suggesting that all of the iron was complexed by ferrozine. Thus, a FZ/$FeCl_2$ ratio of 10 was chosen to perform the experiments, by adding 150 μL of ferrozine (1 mmol/L) and 15 μL of $FeCl_2$ (1 mmol/L).

Studies in the literature differ considerably in the incubation time between Fe^{2+} and the antioxidant prior to ferrozine addition (*26, 27*). The impact of incubation time on the absorbance was evaluated by measuring absorbance at 562 nm of EDTA solutions (8.6 μM final concentration) containing Fe^{2+} (16.70 μM final concentration) (**Figure 3**) at different incubation times. The rate of complex formation decreased after 20 min of incubation, and the amounts of Fe^{2+} chelated after 20 and 60 min were 53 and 57%, respectively. Such a difference is generally acceptable in the estimation of the chelating activity. Accordingly, an incubation time of 20 min was chosen to give a shorter analysis time.

Table 1. Slope, Intercept, Coefficient of Determination (r^2), and Residual Standard Deviation (SEC) of the Regression Line (y = Slope$x$ + Intercept) in the ORAC, FRAP, and ICA Assays

	H-ORAC	L-ORAC	H-FRAP	L-FRAP	ICA
slope	0.18	0.19	0.0021	0.0026	0.87
intercept	1.4	3.8	0.0167	−0.021	0.04
r^2	0.987	0.991	0.999	0.997	0.994
SEC	0.935	0.572	0.105	0.0259	1.01

Validation of the ORAC, FRAP, and ICA Assays. ORAC, FRAP, and ICA methods were validated by calculating linearity, limit of quantification (LoQ), repeatability, and intermediate reproducibility.

Linearity. Linearity was calculated by measuring the response (at least in triplicate) of five concentration levels ranging from 10 to 110 μM Trolox, from 30 to 110 μM Trolox, from 90 to 540 μmol/L Trolox, and from 8 to 48 μmol/L EDTA, for H-ORAC, L-ORAC, FRAP, and ICA methods, respectively. The analyte concentrations were plotted against either the instrument response (FRAP), net area under the curve (ORAC), or the percentage of iron(II) chelated (ICA), and the fitted line was characterized by a correlation coefficient (r^2), the slope intercept, and the residual standard deviation (**Table 1**). Good correlation coefficients (r^2 ranging from 0.987 to 0.999) were obtained in all of the assays, showing that the methods are linear over the concentration range tested. In the ORAC assay, no significant difference in the linearity of both L-ORAC and H-ORAC assay was found. The differences in the slope and intercept between the hydrophilic and lipophilic FRAP assays (**Figure 4**), may be explained by the influence of the solvents on the redox potential of the reaction mixture (*28*) or by the better solubility

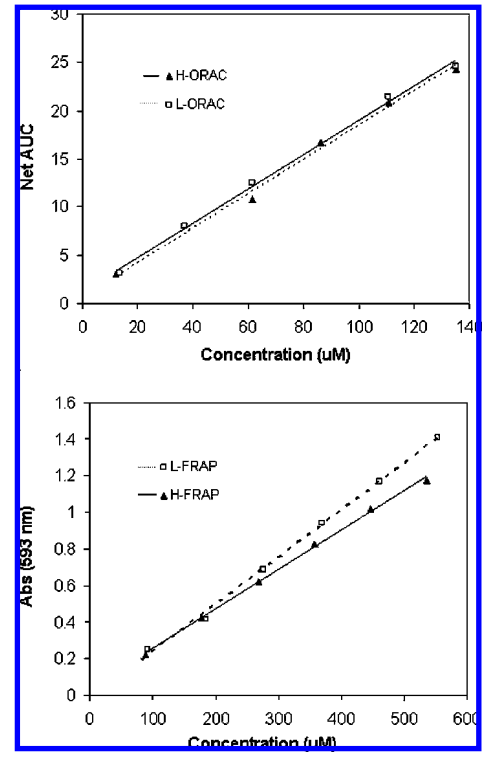

Figure 4. Linearity of the H-ORAC, L-ORAC, H-FRAP, and L-FRAP methods. Each data point represents the average of three replicates.

Table 2. ORAC, FRAP, and ICA Repeatability (**A**) and Intermediate Reproducibility (**B**) Results

(**A**) Repeatability Results[a]

	H-ORAC	L-ORAC	H-FRAP	L-FRAP	ICA
CV (*r*), %	1.2	4.3	4.8	4.9	6.9
r	31.8	4.3	29.0	0.7	0.1

(B) Intermediate Reproducibility Results[b]

	H-ORAC (μmol of TE/g)	L-ORAC (μmol of TE/g)	H-FRAP (μmol of TE/g)	L-FRAP (μmol of TE/g)	ICA (μmol of EDTA equiv/g)
average	1132.6	35.1	254.6	2.0	1.4
SD(iR)	88.0	6.3	14.2	0.3	0.151
CV(iR), %	7.7	18.0	5.6	17.3	11.0
iR	243.9	17.5	39.4	0.9	0.418

[a] The relative standard deviation of repeatability CV(*r*) and the repeatability limit at 95% (*r*) were calculated by analyzing six samples($n = 2$) in the same day. [b] The standard deviation of the intermediate reproducibility [SD(iR)], the relative standard deviation of intermediate reproducibility [CV(iR)], and the intermediate reproducibility limit at 95% [iR, calculated as 2.772 $\times$ SD(*R*)] were calculated by analyzing oregano samples ($n = 2$) on six different days. TE, Trolox equivalents; EDTA equiv, EDTA equivalents; g, grams of original sample on a dry basis.

Table 3. Total Phenols (Folin−Ciocalteu) and ORAC, FRAP, and ICA Values Obtained for Parsley, Oregano, and Olive Mill Wastewaters (OMWW)[a]

	ORAC (μmol of TE/g)			FRAP (μmol of TE/g)				
sample	L-ORAC	H-ORAC	total ORAC	L-FRAP	H-FRAP	total FRAP	ICA (mg of EDTA equiv/g)	total phenols (mg of GAE/g)
parsley ($n = 2$)	2.85 ± 0.1	653 ± 27	656 ± 27	0.60 ± 0.02	17.2 ± 0.8	17.8 ± 0.8	3.11 ± 0.24*	18.0 ± 0.8
oregano ($n = 12$)	35 ± 5	1133 ± 104	1168 ± 105	2.03 ± 0.25	254.6 ± 11.6	256.7 ± 11.8	1.36 ± 0.11*	60.7 ± 2.0
OMWW ($n = 2$)	nd	1723 ± 15	1723 ± 15	nd	319.4 ± 8.6	319.4 ± 8.6	0.47 ± 0.09	87.6 ± 2.5

[a] Results are expressed as the average $\pm$ standard deviation. TE, Trolox equivalents; EDTA equiv, EDTA equivalents; GAE, gallic acid equivalents; g, grams of original sample on a dry basis; nd, not detectable. *, no significant difference between the samples was found ($p < 0.05$). Statistical means comparison was performed by the test of Aspin−Welch.

of Trolox in acetone/water rather than in water. The ICA assay showed a good linearity as indicated by the values of 0.9935 and 1.01 for r^2 and the residual standard deviation (SEC), respectively.

LoQ. The LoQ was considered to be the lowest concentration of the calibration curve, that is, 10 μM Trolox (H-ORAC), 30 μM Trolox (L-ORAC), 90 μM Trolox (FRAP), and 8 μM EDTA (ICA).

Repeatability and Intermediate Reproducibility. The precision of the method was evaluated by calculating the simple repeatability and intermediate reproducibility. The repeatability of the method was evaluated by analyzing six food samples in duplicate, the same day, by the same analyst and with the same equipment. The relative standard deviation of repeatability and repeatability limit at 95% are listed in **Table 2A**. The relative standard deviation of repeatability ranged from 1.2 to 6.9%.

Intermediate reproducibility was calculated by the same analyst analyzing six oregano samples on different days. The average, standard deviation of reproducibility, relative standard deviation of intermediate reproducibility, and intermediate reproducibility limit are shown in **Table 2B**.The relative standard deviation of intermediate reproducibility ranged between 5.6 and 18.0%. Repeatability and intermediate reproducibility results showed the good precision of the methods.

Antioxidant Capacity of OMWW, Oregano, and Parsley. The feasibility of using the validated in vitro ORAC, FRAP, and ICA assays to measure the AOC was evaluated using hydrophilic and lipophilic extracts from oregano, parsley, and OMWW. In addition, total phenol content was measured by using the FC method.

Total Phenols. The FC assay (**Table 3**) showed that OMWW extract is the richest in phenol compounds (87.6 mg of GAE/g) followed by oregano (60.7 mg of GAE/g) and parsley (18.0 mg of GAE/g). The interferences from reducing agents (e.g., ascorbic acid or reducing sugars) was determined as described by Georgé et al. (*8*). No interferences from reducing agents were found in any of the samples.

The phenol content of OMWW has been reported to range between 9 and 10.6% of the mass of dried solids (*29, 30*). Several phenol compounds such as hydroxytyrosol, tyrosol, elenolic acid, gluteolin 7-glycoside, quercetin, and cinnamic acid derivatives have been previously identified in OMWW extracts (*30*). Hydroxytyrosol is the most abundant phenol compound in OMWW (*31*), and its concentration has been previously reported to vary from 1.2 to 9.8% among different OMWW extracts (*30*). The hydroxytyrosol content of the OMWW extract used in our study was 3.4% dry solids (information provided by the supplier).

Oregano (*O. vulgare*) phenols have been identified previously (*32, 33*) and comprise phenolic acids (rosmarinic acid, caffeic acid, *p*-coumaric acid, caffeoyl derivatives), flavones (apigenin, luteolin), and flavanols (myricetin, quercetin). The content of phenol compounds determined in lyophilized oregano in our study (60.7 mg of GAE/g) is similar to the level reported by Wu et al. (*34*) (72.82 mg of GAE/ofg). The content in parsley (*P. crispum*; 18.0 mg of GAE/g) compares well with a value determined previously (*34*), 22.4 mg of GAE/g, and was the lowest of the three samples analyzed. The main phenol compounds in parsley have been identified as apigenin and caffeic acid (*33, 35*).

ORAC Results. OMWW extract showed the highest radical scavenger capacity, 1723 μmol of TE/g, (**Table 3**), probably due to the high content of phenols, which has been reported to correlate well with hydrogen-donating capacity (*7, 36*). Hydroxytyrosol, the main phenol compound found in OMWW, has been reported previously as an efficient radical scavenger

Table 4. ORAC and FRAP Values Expressed per Unit of Total Phenols[a]

sample	ORAC/TP (μmol of TE/mg of GAE)		FRAP/TP (μmol of TE/mg of GAE)
	this study	Wu et al. (*34*)	
parsley	36.44	33.17	0.99
oregano	19.24	27.49	4.23
OMWW	19.67		3.65

[a] The ORAC/TP values of oregano and parsley determined by Wu et al. (*34*) are also shown. TE, Trolox equivalents; GAE, gallic acid equivalents.

(*37*). Only aqueous extracts of OMWW showed hydrogen-donating activity, whereas the AOC of lipophilic extracts was not detectable. These results indicate that the antioxidant compounds were extracted with acetone/water/acetic acid (70:28:2 v/v/v) rather than in hexane. It has been reported previously that even if olive fruit phenols are amphiphilic in nature, they are more soluble in water than in oil (*38*), confirming our findings.

Oregano and parsley ORAC results were 1168 and 656 μmol of TE/g, respectively (**Table 3**) and differed significantly ($p < 0.05$). These results differed from those published by Wu et al. (*34*), who found ORAC values of 2001 and 743 μmol of TE/g for oregano and parsley, respectively. The difference may be explained by the variation in the content of phenol compounds of the samples due to differences in the genotype, environmental, and agronomic factors (such as water availability, soil composition, and UV radiation) in which the samples were grown (*39*). To check this hypothesis, the ratio between the ORAC results and total phenols (ORAC/TP) of both studies was calculated and compared (**Table 4**). In both studies, ORAC/TP was similar for parsley but differed substantially for oregano. The higher ORAC/TP ratio found in oregano by Wu et al. (*34*) suggests that their sample was richer in phenol compounds with an efficient structure to scavenge free radicals, such as catechol groups (two *o*-dihydroxy groups), which are the most important structural feature for strong antioxidant activity in phenols (*33*). In our study, parsley showed the highest ORAC/TP ratio, whereas oregano and OMWW showed comparable results (**Table 4**). These results indicate that not only the total level of phenols but also the phenol structure determines the radical scavenging capacity of food extracts.

Oregano and parsley lipophilic extracts both showed lower antioxidant activity than their hydrophilic counterparts, representing 3 and 0.4% of the total ORAC value, respectively.

FRAP Results. OMMW showed the highest reducing capacity (319.4 μmol of TE/g), followed by oregano (256.7 μmol of TE/g) and parsley (17.8 μmol of TE/g) (**Table 3**). The reducing capacities of all the samples were significantly different ($p < 0.05$). Several studies have shown good correlation (coefficient of correlation, $r > 0.95$) between the reducing capacity determined by FRAP and the phenol content measured by the FC assay (*5, 36*). Thus, the reducing capacity of the extracts is most likely due to the presence of phenol compounds. In our study, the reducing capacities per unit of phenol (FRAP/TP value, **Table 4**) were similar for OMWW and oregano extracts, whereas parsley reducing capacity was smaller than expected from the level of phenols. These results are opposite that found in the ORAC assay, by which parsley showed the highest AOC per unit of phenols. The lack of correlation between parsley FRAP/TP and ORAC/TP may indicate that phenols are not the only compounds with AOC in the parsley extracts. Proteins with radical scavenging and chelating properties have been reported previously (*40, 41*). Dried parsley and oregano protein contents have been evaluated at 22.42 and 11% (%w), respectively (*35*); therefore, the presence of proteins in the aqueous extracts could explain the lack of correlation between FRAP/TP and ORAC/TP. Nevertheless, this hypothesis needs to be addressed and clarified in further research. In oregano, the lipophilic fraction represented 0.8% of the total reducing capacity, whereas the OMWW lipophilic fraction did not show reducing capacity. Parsley's reducing capacity was 17.8 μmol of TE/g, comparable to the one (17 μmol of TE/g) reported by Halvorsen et al. (*42*). In our study, the lipophilic fraction of parsley (L-FRAP) accounted for 3.5% of the total reducing capacity.

ICA. No significant differences ($p < 0.05$) in the chelating capacity of parsley (3.11 mg of EDTA equiv/g) and oregano (1.36 mg of EDTA equiv/g) were found, whereas OMWW showed a significantly lower chelating capacity (0.47 mg of EDTA equiv/g) (**Table 3**). The chelating properties of parsley have been previously reported by Hinneburg et al. (*26*), who found considerably better chelating properties for parsley (178 mg of Na_2EDTA equiv/g) than we did. The use of the salt Na_2EDTA as reference can only account for part of the difference in the results from the studies. The chelating activities of EDTA and Na_2EDTA are the same, and the effect of the difference in molecular mass on expressing the results as equivalents is small. The difference in the phenol content of the samples used in the two studies will also account in part for the difference in the Fe^{2+} chelating capacities. Nevertheless, the use of different assay conditions could contribute the most to the discrepancies between our study and Hinnerburg's results (*26*). The iron chelating activity of oregano was lower (1.36 vs and 2.93 mg of EDTA equiv/g) than that reported previously (*43*). The value for OMWW (0.15 mg of EDTA equiv/g) represents, to our knowledge, the first report of its Fe^{2+} chelating activity.

The results showed that a high content of phenol compounds does not link directly to a high Fe^{2+} chelating activity (**Table 3**). Nevertheless, several previous studies have reported chelating properties for phenol compounds (*14*). The metal-chelating properties of phenol compounds are attributed to specific structural features, requiring two points of coordination between metal and the phenol compound. Thus, *o*-diphenol (3′,4′-diOH-) in ring B and ketol (3-OH-4-keto or 5-OH-4-keto) structures in ring C show good chelating activity (*14*). Caffeic acid, the main phenol compound found in parsley and also present is oregano, has an *o*-diphenol structure and can account for the good chelating properties of parsley and oregano. Hydroxytyrosol, the main phenol compound in OMWW, is also an *o*-diphenol substituted compound and may account for the chelating properties of the OMWW extract.

In general, the results obtained with the ICA method indicate the difficulties in obtaining reproducible interlaboratory results. The differences in extraction solvent, assay conditions, and phenol content of the samples may explain the differences found between the various studies. Standardization of the ICA method is necessary to allow better comparison and interpretation of results obtained by different laboratories.

In summary, the use of a common format for ORAC, FRAP, and ICA assays provided a comprehensive, precise, and high-throughput assessment of the antioxidant capacity of food extracts. Measurements of the AOC of oregano, parsley, and OMWW using the validated methods showed agreement with published results for ORAC and FRAP assays. On the other hand, ICA results differed substantially from those previously published, indicating the need of standardization of the assay conditions to obtain similar interlaboratory results.

OMWW showed the highest radical scavenging and reducing capacity, determined by the ORAC and FRAP assays, respectively, followed by oregano and parsley. Parsley showed an iron chelating activity similar to that of oregano, whereas OMMW chelating properties were the lowest of the tested extracts. In addition, total phenols were measured and correlated well with ORAC and FRAP results, but no correlation was found in the ICA assay.

Finally, the use of several antioxidant assays with differing reaction mechanisms is necessary to give an overall understanding of the mechanisms of action of an antioxidant.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AOC, antioxidant capacity; AUC, area under the curve; EDTA, ethylenediaminetetraacetic acid; FC, Folin−Ciocalteu; FZ, ferrozine; FRAP, ferric reducing antioxidant power; GAE, gallic acid equivalents; HAT, hydrogen atom transfer; ICA, iron(II) chelating assay; OMWW, olive mill wastewaters; ORAC, oxygen radical absorbance capacity; RE, raw extracts; RMCD, randomly methylated-β-cyclodextrin; SET, single electron transfer; TE, Trolox equivalents; TEAC, Trolox equivalent antioxidant capacity; TPTZ, tri(2-pyridyl)-*s*-triazine; WE, washed extracts.

SAFETY

Apart from standard caution with all solvents and acids, there are no specific safety criteria for this work.

LITERATURE CITED

(1) Decker, E. A.; Warner, K.; Richards, M. P.; Shahidi, F. Measuring antioxidant effectiveness in food. *J. Agric. Food Chem.* **2005**, *53*, 4303–4310.

(2) Donald-Wicks, L. K.; Wood, L. G.; Garg, M. L. Methodology for the determination of biological antioxidant capacity in vitro: a review. *J. Sci. Food Agric.* **2006**, *86*, 2046–2056.

(3) Frankel, E. N.; Meyer, A. S. The problems of using one-dimenional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.

(4) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841–1856.

(5) Stratil, P.; Klejdus, B.; Kuban, V. Determination of total content of phenolic compounds and their antioxidant activity in vegetables—evaluation of spectrophotometric methods. *J. Agric. Food Chem.* **2006**, *54*, 607–616.

(6) Introduction: White papers from the 'First International Congress on Antioxidant Methods'. *J. Agric. Food Chem.* **2005**, *53*, 4288−4289.

(7) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290–4302.

(8) George, S.; Brat, P.; Alter, P.; Amiot, M. J. Rapid determination of polyphenols and vitamin C in plant-derived products. *J. Agric. Food Chem.* **2005**, *53*, 1370–1373.

(9) Asami, D. K.; Hong, Y. J.; Barrett, D. M.; Mitchell, A. E. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. *J. Agric. Food Chem.* **2003**, *51*, 1237–1241.

(10) Prior, R. L.; Hoang, H.; Gu, L.; Wu, X.; Bacchiocca, M.; Howard, L.; Hampsch-Woodill, M.; Huang, D.; Ou, B.; Jacob, R. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL)) of plasma and other biological and food samples. *J. Agric. Food Chem.* **2003**, *51*, 3273–3279.

(11) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Development and validation of oxygen radical absorbance capacity assay for lipophilic antioxidants using randomly methylated beta-cyclodextrin as the solubility enhancer. *J. Agric. Food Chem.* **2002**, *50*, 1815–1821.

(12) Arnao, M. B.; Cano, A.; Acosta, M. The hydrophilic and lipophilic contribution to total antioxidant activity. *Food Chem.* **2001**, *73*, 239–244.

(13) Cano, A.; Arnao, M. B. Hydrophilic and lipophilic antioxidant activity in different leaves of three lettuce varieties. *Int. J. Food Prop.* **2005**, *8*, 521–528.

(14) Frankel, E. N. *Lipid Oxidation*; Oily Press: Dundee, Scotland, 1998.

(15) Jacobsen, C.; Hartvigsen, K.; Lund, P.; Meyer, A. S.; Adler-Nissen, J.; Holstborg, J.; Holmer, G. Oxidation in fish-oil-enriched mayonnaise. *Eur. Food Res. Technol.* **1999**, *210*, 13–30.

(16) Jacobsen, C.; Hartvigsen, K.; Thomsen, M. K.; Hansen, L. F.; Lund, P.; Skibsted, L. H.; Holmer, G.; Adler-Nissen, J.; Meyer, A. S. Lipid oxidation in fish oil enriched mayonnaise: calcium disodium ethylenediaminetetraacetate, but not gallic acid, strongly inhibited oxidative deterioration. *J. Agric. Food Chem.* **2001**, *49*, 1009–1019.

(17) Jacobsen, C.; Timm, M.; Meyer, A. S. Oxidation in fish oil enriched mayonnaise: ascorbic acid and low pH increase oxidative deterioration. *J. Agric. Food Chem.* **2001**, *49*, 3947–3956.

(18) Decker, E. A.; Welch, B. Role of ferritin as a lipid oxidation catalyst in muscle food. *J. Agric. Food Chem.* **1990**, *38*, 674–677.

(19) Yamaguchi, F.; Ariga, T.; Yoshimura, Y.; Nakazawa, H. Antioxidative and anti-glycation activity of garcinol from *Garcinia indica* fruit rind. *J. Agric. Food Chem.* **2000**, *48*, 180–185.

(20) Carter, P. Spectrophotometric determination of serum iron at the submicrogram level with a new reagent (ferrozine). *Anal. Biochem.* **1971**, *40*, 450–458.

(21) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158.

(22) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76.

(23) Rouseeuw, P. J.; Croux, C. Alternatives to the median absolute deviation. *J. Am. Stat. Assoc.* **1993**, *88*, 1273–1283.

(24) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619–4626.

(25) Sarradin, P. M.; Le Bris, N.; Le Gall, C.; Rodier, R. Fe analysis by the ferrozine method: adaptation to FIA towards in situ analysis in hydrothermal environment. *Talanta* **2005**, *66*, 1131–1138.

(26) Hinneburg, I.; Damien Dorman, H. J.; Hiltunen, R. Antioxidant activities of extracts from selected culinary herbs and spices. *Food Chem.* **2006**, *97*, 122–129.

(27) Mao, L. C.; Pan, X.; Que, F.; Fang, X. H. Antioxidant properties of water and ethanol extracts from hot air-dried and freeze-dried daylily flowers. *Eur. Food Res. Technol.* **2006**, *236*, 241.

(28) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396–3402.

(29) Visioli, F.; Vinceri, F. F.; Galli, C. 'Waste waters' from olive oil production are rich in natural antioxidants. *Experientia* **1995**, *51*, 32–34.

(30) Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive mill waste waters. *J. Agric. Food Chem.* **1999**, *47*, 3397–3401.

(31) Capasso, R.; Evidente, A.; Avolio, S.; Solla, F. A highly convenient synthesis of hydroxytyrosol and its recovery from agricultural waste waters. *J. Agric. Food Chem.* **1999**, *47*, 1745–1748.

(32) Mathew, S.; Abraham, T. E. In vitro antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extract assayed by different methodologies. *Food Chem. Toxicol.* **2006**, *44*, 198–206.
(33) Shan, B.; Cai, Y. Z.; Sun, M.; Corke, H. Antioxidant capacity of 26 spice extracts and characterization of their phenolic constituents. *J. Agric. Food Chem.* **2005**, *53*, 7749–7759.
(34) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026–4037.
(35) USDA National Nutrient Database, U.S. Department of Agriculture, http://www.ars.usda.gov/, 2007.
(36) Proteggente, A. R.; Pannala, A. S.; Paganga, G.; Van Buren, L.; Wagner, E.; Wiseman, S.; Van De Put, F.; Dacombe, C.; Rice-Evans, C. A. The antioxidant activity of regularly consumed fruit and vegetables reflects their phenolic and vitamin C composition. *Free Radical Res.* **2002**, *36*, 217–233.
(37) Rietjens, S. J.; Bast, A.; Haenen, G. M. M. New insight into controversies on the antioxidant potential of the olive oil antioxidant hydroxytyrosol. *J. Agric. Food Chem.* **2007**, *55*, 7609–7614.
(38) Panayotis, S. R.; Karathanos, V. T.; Antzavinou, A. Partitioning of olive oil antioxidants between oil and water phases. *J. Agric. Food Chem.* **2002**, *50*, 596–601.
(39) Parr, A. J.; Bolwell, G. P. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenols content or profile. *J. Sci. Food Agric.* **2000**, *80*, 985–1012.
(40) Medina, I.; Tombo, I.; Satue-Gracia, M. T.; German, J. B.; Frankel, E. N. Effects of natural phenolic compounds on the antioxidant activity of lactoferrin in liposomes and oil-in-water emulsions. *J. Agric. Food Chem.* **2002**, *50*, 2392–2399.
(41) Almajano, M. P.; Delgado, M. E.; Gordon, M. H. Changes in the antioxidant properties of proteins solutions in the presence of epigallocatechin gallate. *Food Chem.* **2006**, *101*, 126–130.
(42) Halvorsen, B. L.; Holte, K.; Myhrstad, M. C.; Barikmo, I.; Hvattum, E.; Remberg, S. F.; Wold, A. B.; Haffner, K.; Baugerod, H.; Andersen, L. F.; Moskaug, O.; Blomhoff, R. A systematic screening of total antioxidants in dietary plants. *J. Nutr.* **2002**, *132*, 461–471.
(43) Su, L.; Yin, J. J.; Charles, D.; Zhou, K.; Moore, J.; Yu, L. Total phenolic content, chelating capacities, and radical-scavenging properties of black peppercorn, nutmeg, roseship, cinnamon and oregano leaf. *Food Chem.* **2007**, *100*, 990–997.

Received for review December 20, 2007. Revised manuscript received February 13, 2008. Accepted March 5, 2008.

JF703723S