JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Alkylperoxyl Radical Scavenging Activity of Red Leaf Lettuce (*Lactuca sativa* L.) Phenolics

CHARLES R. CALDWELL*

Phytonutrients Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, United States Department of Agriculture, Beltsville, Maryland 20705

Although lettuce may provide relatively low levels of antioxidative phytochemicals which may contribute to human health, lettuce leaf extracts in fact contained compounds with high specific peroxyl radical scavenging activities. After determining the extraction conditions that minimized phenolic oxidation and produced the highest oxygen radical absorbance capacity (ORAC) values, the phenolic compounds from red leaf lettuce were separated by reverse-phase high-performance liquid chromatography (HPLC). The primary phenolic compounds in the leaf tissue extracts were mono- and dicaffeoyltartaric acid (CTA and DCTA), mono- and dicaffeoylquinic acid (CQA and DCQA), quercetin 3-malonylglucoside (QMG), quercetin 3-glucoside (QG), cyanidin 3-malonylglucoside (CMG), and an unknown phenolic ester (UPE). Significant levels of DCQA were only found after wounding. Using the new fluorescein-based ORAC assay procedures, fractions from the HPLC analyses were assayed for peroxyl radical absorbance capacity. Using absorbance to estimate concentration, the decreasing order of contribution to the total ORAC value of an extract from wounded tissue was QMG > DCQA > CMG > DCTA > UPE > QG > CTA. The decreasing order of the specific peroxyl radical scavenging activities was CMG > QG > DCTA > DCQA > QMG > UPE > CQA > CTA. Since the concentrations of plant flavonoid and phenolic acid esters are sensitive to environmental factors, this information may be used to develop pre- and postharvest conditions which increase the dietary benefits of leaf lettuce.

KEYWORDS: Antioxidants, *Lactuca sativa*, lettuce, ORAC, peroxyl radical, phenolics

INTRODUCTION

The antioxidative activity of dietary phytochemicals has been linked to reductions in human degenerative diseases in populations that consume high amounts of fruits and vegetables (*1*−*3*). In particular, the ability of plant phenolic compounds or their possible human metabolites to scavenge various oxygen and nitrogen free radicals has suggested mechanisms for the human health benefits of diets rich in fruits and vegetables (*4*, *5*). Using in vitro analytical procedures, the relative antioxidative abilities of fruit and vegetable extracts and some of their chemical components have been estimated and used to suggest certain health consequences of increased consumption of specific agricultural products (*6*−*9*). Although they may have relatively high in vitro antioxidative activities, the chemical composition of many fruit and vegetable extracts are often unknown. Therefore, many free radical scavenging plant components remain to be identified and their potential health advantages assessed.

The oxygen radical absorbance capacity (ORAC) assay has frequently been utilized to assess the relative antioxidative activities of different fruits and vegetables (*10*−*13*). In general, the ORAC assay is based on the reactivity of B-phycoerythrin (B-PE) with peroxyl radicals produced by the thermal decomposition of a free radical generator, 2,2′-azo-bis(2-amidinopropane)dihydrochloride (AAPH). The decay in B-PE fluorescence induced by AAPH decomposition is measured in the presence and absence of plant extracts or individual phytochemicals. Differences in the rate of fluorescence decay relative to the controls are indicative of the anti- or prooxidative activity of the test material. Although not intended as a precise measure of changes in free radical reaction rates, the ORAC assay is a convenient and widely accepted method to estimate the relative free radical scavenging capacities of dietary phytochemicals. However, the use of a protein reporter molecule, B-PE, in the ORAC assay may result in anomalous results. Phytochemical−phycoerythrin interactions or changes in B-PE conformation induced by cations or other assay components could alter the protein reactivity with peroxyl radicals or change the protein's fluorescent properties. The recent identification of fluorescein as a low-cost alternative to B-PE in ORAC analyses (*14*) could have the added advantage of low fluorescein reactivity with assay components other than the peroxyl free radicals.

ORAC and other methods to estimate antioxidative potential have identified certain fruits and vegetables with high antioxidative activities and, therefore, possible dietary benefits. Varia-

* To whom correspondence should be addressed. Tel: 301-504-5146. Fax: 301-504-9456. E-mail: crc@erols.com.

10.1021/jf030005q
Published on Web 07/04/2003

tions in extraction conditions and antioxidative capacity measurements as well as the frequent absence of compositional analyses have made it difficult to compare the results from the different reports. Furthermore, many of the phenolic phytochemicals believed to have high antioxidative activities are synthesized by the plant in response to environmental signals (*15*−*18*). Therefore, differences in both pre- and postharvest conditions could alter significantly the chemical composition of the fruit and vegetable material (*19*, *20*). This situation may result in significant variations in antioxidative potential for the same plant material obtained from different sources and complicate attempts to set dietary standards for fruits and vegetables based on phenolic phytochemical bioactivity measurements (*1*, *21*).

To estimate the relative contributions of the different phytochemicals in plant extracts to the total antioxidative activity, a method was developed that utilized the B-PE-based ORAC analyses with samples obtained from the fractionation of plant extracts by HPLC (*22*). Considering the recent changes in the ORAC assay procedures (*14*) and the need to evaluate the effect of extraction conditions on the ORAC measurements, a study was initiated to evaluate the relative contributions of individual lettuce leaf phenolics to the total antioxidative activity. Red leaf lettuce was chosen as the experimental material because of its commercial availability and known phenolic composition (*15*, *19*, *20*). Although lettuce leaf extracts have relatively low antioxidative activity when expressed in terms of sample weight or serving size (*9*), the peroxyl radical scavenging activity of the lettuce leaf extracts is higher than many of the high antioxidant fruits and vegetables when expressed on the basis of total phenolic concentration. The per capita rate of lettuce consumption (*23*) combined with the high specific antioxidative activities of the lettuce phenolics suggest that lettuce may provide a significant source of dietary antioxidants.

MATERIALS AND METHODS

Chemicals. All chemicals except 2,2′-azo-bis(2-amidinopropane)-dihydrochloride (AAPH, Wako Chemicals, Richmond, VA) were obtained from Sigma/Aldrich Chemical Co. (St. Louis, MO).

Plant Material and Sample Preparation. Leaf disks (7 mm) were cut randomly with a number 2 cork borer from the red pigmented portion of leaves of commercially grown lettuce (*Lactuca sativa* L. cv Lollo Rosso) purchased at local grocery stores and used immediately. Unless otherwise noted, five leaf disks were ground with a glass homogenizer in 500 μL of cold (4 °C) 50% (v/v) methanol containing 1% (v/v) formic acid. The homogenized tissues were centrifuged at 14000*g* for 5 min and the supernatants stored at −20 °C. Samples of other commercially grown lettuce types and an *Echinacea* preparation were treated similarly. To determine any effects of tissue wounding on the red leaf lettuce phenolics, leaf disks were prepared and floated, adaxial side up, on distilled water at 27 °C in the dark for 6 h prior to extraction.

HPLC Analytical Procedures. All HPLC procedures were performed with a Waters 600 series pump and controller and a Waters 990 photodiode array detector. For analysis of the aqueous methanol extracts, 200 μL of the extract was injected into a 25 cm × 4.6 mm Spherisorb ODS-2 (octadecylsilyl, 5μm particle size) column (Sigma Chemical Co., St. Louis, MO) and eluted with a 30 min linear gradient from 25% to 60% (v/v) methanol in 0.1% (v/v) aqueous formic acid. Spectra from 250 to 600 nm were stored. Since the phenolic compounds in the lettuce extracts had reasonable absorbances at 280 nm, the separation was monitored at 280 nm. After conversion of chromatograms or spectra into ASCII text files using the Waters 990 software, a series of WordPerfect macros were used to modify the ASCII files into SigmaPlot-compatible format. All chromatograms represent data obtained at 1-s intervals, and all spectra have 0.2-nm resolution.

During the HPLC analysis, fractions were collected at 30-s intervals. Since the longest HPLC analysis was 30 min at 1.5 mL/min, a maximum of 60 fractions of 750 μL each were collected. After measuring the absorbances of the fractions at 280 nm with a Shimadzu (Kyoto, Japan) model UV-160A spectrophotometer, the fractions were dried using a Savant (Farmingdale, NY) SpeedVac Concentrator and stored dry at −20 °C. Immediately before ORAC analyses, the dried fractions were reconstituted in 50 μL of 25% (v/v) aqueous acetone and sonicated for 30 s. To reduce the possibility of sample photodegradation, the fractions were collected in amber microcentrifuge tubes and all manipulations performed under reduced light.

ORAC Assay Conditions. ORAC assays were performed using a dedicated instrument capable of 45 simultaneous assays (*24*). To allow the measurement of ORAC using fluorescein (*14*), the "true-green" light-emitting diode (LED) was replaced with a blue-green LED (LED1052, Nichia mdl. NSPE590S) and the orange OG570 long-pass filter replaced with an orange OG550 long-pass filter (L32−754, Edmund Optics, Barrington, NJ). The ORAC assay conditions were also modified. The AAPH stock concentration was increased 2.5-fold, resulting in 10 mM AAPH in the assay solution. The final concentration of fluorescein was 2.5 μM. The 75 mM potassium phosphate (pH 7.4) assay buffer was filtered (0.45 μm) prior to daily reagent preparation. All assays were performed at 37 °C for a maximum of 90 min and the data acquired and analyzed as previously described (*22*). Decay curves which did not reach completion were discarded and the assays repeated at lower sample concentrations. Since the lot-to-lot variability and stability in solution of the standard antioxidant Trolox precluded its use as a reliable standard for the comparison of ORAC results from different laboratories, the ORAC values were computed from the area under the curve (AUC) of the samples, subtracting the AUC of the controls. Statistical analyses were performed using the resident SigmaPlot column statistics.

RESULTS AND DISCUSSION

Identification of Lettuce Leaf Phenolics. Homogenization of fresh lettuce leaf tissues in acidified 50% (v/v) methanol resulted in the extraction of a variety of UV-absorbing compounds (**Figure 1**) with spectral characteristics consistent with either flavonol glycosides (**Figure 2E**) or phenolic acids (**Figure 2A**−**D**).Using similar extraction and HPLC procedures, the primary UV light-absorbing compounds from lettuce separated by HPLC have been identified (*15*, *19*, *20*). Consistent with these reports, the primary flavonol glycoside in the red leaf lettuce was quercetin 3-malonylglucoside (QMG) (**Figure 1**, peak i) with smaller amounts of quercetin 3-glucuronide (**Figure 1**, peak g) and quercetin 3-glucoside (**Figure 1**, peak h). On the basis of spectral characteristics (**Figure 2C**), peak e of **Figure 1** was identified as cyanidin 3-malonylglucoside (CMG). Unlike many other leafy vegetables that contain high levels of flavonoids (*2*, *5*, *25*), the primary UV-absorbing components in the leaf lettuce extracts are phenolic acids (*15*, *19*, *20*). Considering the published analyses (*15*, *19*, *20*), phenolic acid standards, and known lettuce responses to wounding (*20*), the major phenolic acids in these lettuce leaf extracts were identified tentatively as caffeoyltartaric acid (CTA, peak a), caffeoylquinic acid (CQA, peak b), dicaffeoyltartaric acid (DCTA, peak d), and dicaffeoylquinic acid (DCQA, peak f). CQA and DCQA are also known as chlorogenic and isochlorgenic acids, respectively. CTA and DCTA are also referred to as caftaric and chicoric acids, respectively. Peak c of **Figure 1** remains unknown. Using different HPLC conditions, Bennett et al. (*15*) reported a major unknown caffeoyl ester eluting between CQA and DCTA in 70% aqueous methanol extracts of lettuce cotyledons.

Comparison of both red and green leaf lettuce phenolic content with that of other major lettuce types supports the

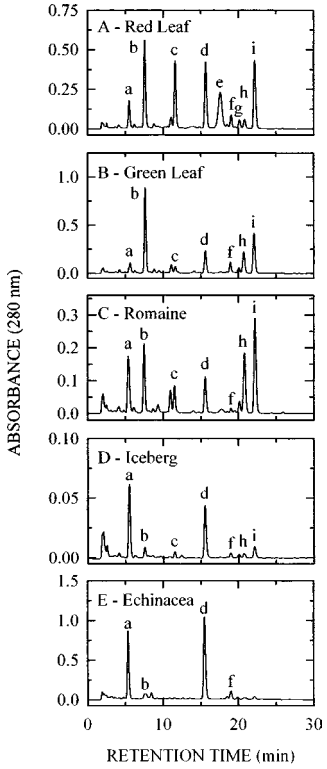

Figure 1. Representative reverse-phase HPLC analysis of acidified, aqueous methanol [50% (v/v), containing 1% (v/v) formic acid] extracts of lettuce leaves and dried *Echinacea purpurea*. Using standards and spectral analyses, peaks were identified as caffeoyltartaric acid (a), caffeoylquinic acid (b), dicaffeoyltartaric acid (d), cyanidin 3-malonylglucoside (e), dicaffeoylquinic acid (f), quercetin 3-glucuronide (g), quercetin 3-glucoside (h), and quercetin 3-malonylglucoside (i). Peak c is an unknown compound, possibly a caffeoyl ester. Extractions of all lettuce types were based on equal surface areas rather that wet weight of the tissues.

suggestion that leaf lettuce generally contains larger amounts of leaf phenolics than romaine or iceberg lettuce (**Figure 1**). However, unlike studies with other lettuce types, Ferreres et al. (*20*) also indicated that red leaf lettuce (cv. Lollo Rosso) contained large amounts of isochlorogenic acid (DCQA) that did not result from wounding or ethylene treatments. Apparently there has been some controversy about the presence of DCQA in fresh lettuce leaf tissues. Since standards for most of the lettuce leaf phenolic acids were not readily available, a 50% aqueous methanol extract of dried *Echinacea pupurea* flowers was analyzed under identical HPLC conditions as the various lettuce leaf extracts. As shown in **Figure 1E**, the *Echinacea* extract contained two major compounds with the same spectral characteristics and retention times as peaks a and d of the lettuce extracts. *Echinacea* flowers contain primarily CTA and DCTA (*26*), confirming the identification of these compounds in red leaf lettuce. Since the retention time of the unknown compound (**Figure 1**, peak c) is too short relative to the known phenolic acids to be DCQA, the red leaf lettuce purchased locally apparently did not contain large amounts of DCQA. This conclusion is supported by the effects of wounding on the red leaf lettuce phenolics. As noted by Ferreres et al. (*20*), DCQA concentrations increase in lettuce leaves after wounding. Comparison of the HPLC profiles of red leaf lettuce control and wounded tissues (**Figure 3**) clearly shows a significant increase in the level of compound f 6 h after the leaf disks were prepared. Therefore, compound f can be tentatively identified as DCQA.

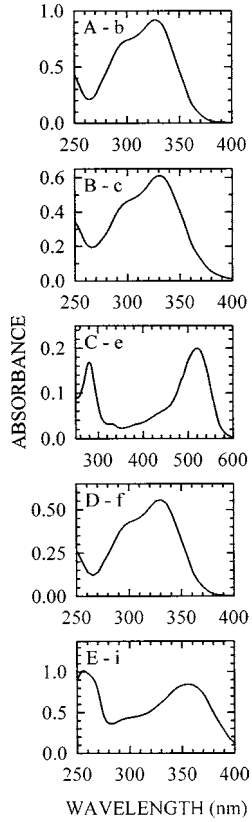

Figure 2. Absorbance spectra of selected phenolic compounds found in red leaf lettuce. Each section is labeled with a capital identification letter followed by a lower case letter that refers to the peak designations presented in **Figure 1**: caffeoylquinic acid (b), putative caffeoyl ester (c), cyanidin 3-malonylglucoside (e), dicaffeoylquinic acid (f), and quercetin 3-malonylglucoside (i).

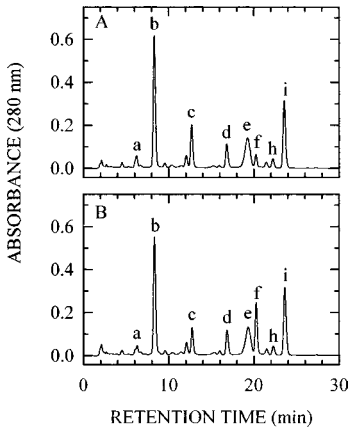

Figure 3. Effect of wounding on the phenolic compounds of red leaf lettuce. Leaf disks were punched from the red pigmented portions of the lettuce leaves and either extracted immediately (**A**) in 50% (v/v) aqueous methanol, containing 1% (v/v) formic acid, or after a 6 h incubation on distilled water at 27 °C in the dark (**B**). Peak designations are the same as those presented in **Figure 1**.

Effect of Extraction Conditions on ORAC Activity. On occasion, ORAC analyses of fruits and vegetables have utilized simple aqueous extracts (*27*). Although aqueous extracts may be suitable for plant material not subject to enzymatic browning, the use of these procedures with red leaf lettuce samples resulted in the rapid browning of the extracts. These results are consistent with the rapid enzymatic and nonenzymatic oxidation of lettuce phenolic compounds during extraction at neutral pH (*28*).

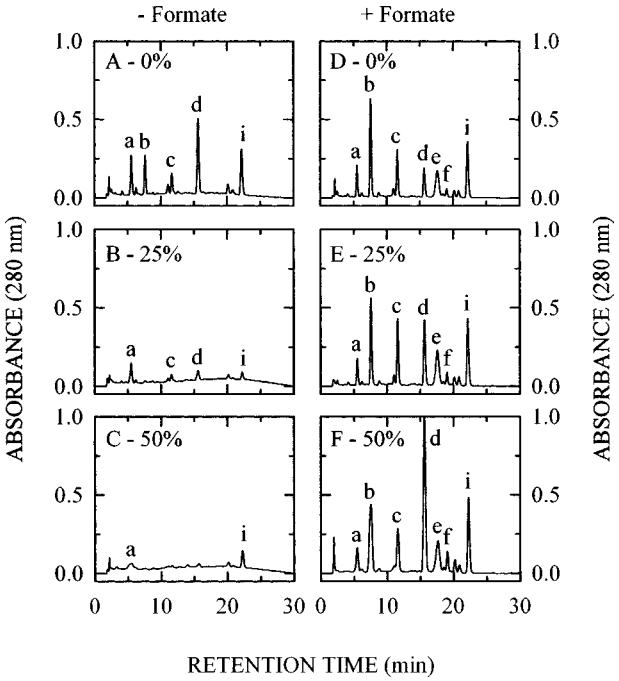

Figure 4. Effect of methanol concentration and the presence of formic acid on the extraction of phenolic compounds from the leaves of red leaf lettuce. Equal surface areas of red pigmented lettuce leaf disks were homogenized in the given concentration (% v/v) of aqueous methanol with and without 1% (v/v) formic acid and the composition of the extracts analyzed by reverse-phase HPLC. Peak designations are the same as those presented in **Figure 1**.

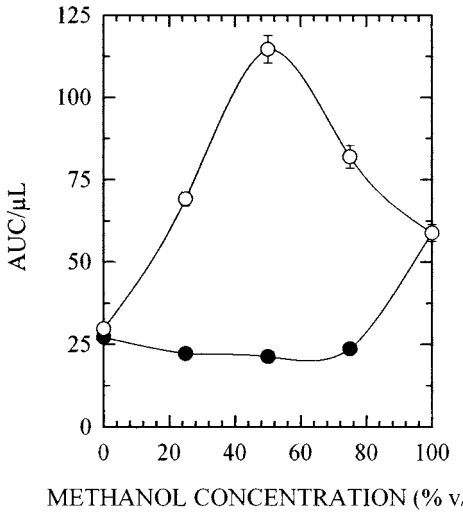

Figure 5. Effect of methanol concentration and the presence (○) or absence (●) of formic acid on the ORAC values of red leaf lettuce extracts. The extracts used to obtain the results presented in **Figure 2** as well as extracts prepared with higher methanol concentrations were used in the fluorescein-based ORAC assay. The results are presented as the means with standard errors of triplicate determinations of the area under the curve (AUC) for the fluorescence decays after subtraction of the control AUC per microliter of extract.

Inclusion of 1% (v/v) formic acid in the extraction solutions prevented the browning. At acidic pH, polyphenol oxidase [EC 1.14.18.1] (PPO) activity is inhibited and the stability of phenolic compounds increased (*29*, *30*). Therefore, the effects of the different extraction conditions on the phenolic content and antioxidant activity of red lettuce leaf phenolic compounds was assessed. As shown in **Figure 4**, homogenization of the lettuce leaf disks in water resulted in the extraction of phenolic compounds. However, in the absence of formic acid, CMG (peak e) was not present in the extracts. Increasing the methanol concentration in the absence of formic acid decreased the levels of all lettuce leaf phenolics and caused a baseline shift that may represent increased levels of phenolic oxidation products (**Figure 4B**,**C**). This result is consistent with increased PPO activity in solutions of decreased polarity (*29*). Extracting at neutral pH and methanol concentrations above 50% (v/v), the brown product of phenolic acid oxidation tended to clog the HPLC column, indicating the formation of polymeric material and precluding HPLC analyses. Oxidation of lettuce phenolic compounds by PPO produces *o*-quinones (*28*), which can participate in the formation of high-molecular weight polymers and degrade CMG (*31*).

ORAC analyses of the different aqueous methanol extracts indicated that homogenization in 50% (v/v) aqueous methanol, containing 1% formic acid, produced the highest ORAC values (**Figure 5**). Even though the amounts of known phenolics separated by HPLC decreased with increasing methanol levels in the extraction solution at neutral pH (**Figure 4**), the ORAC values did not decrease significantly (**Figure 5**). This suggests that the brown oxidation products possess antioxidative activity. In the absence of formic acid, the AUC/μL for the red leaf lettuce tissue increased in 100% (v/v) methanol extracts (**Figure 5**), indicating the denaturation of PPO at high alcohol concentrations (*29*). However, the 100% (v/v) methanol lettuce leaf extracts were green, suggesting the presence of chlorophyll and other hydrophobic phytochemicals. Therefore, comparison of ORAC results for total fruit and vegetable extracts requires some consideration of the extraction conditions. Depending upon the plant material, ORAC values for simple aqueous extracts may reflect the antioxidative capacity of polymeric phenolic compounds. Extraction in high levels of alcohol or acetone may decrease phenolic oxidation but may also solubilize more hydrophobic phytochemicals. Although the use of acidified methanol extraction solutions may minimize oxidative degradation of the leaf phenolics in vitro, determination of the actual phytonutritional relevance of these compounds will require consideration of their stability during ingestion and digestion. Considering the variation in extraction procedures, the utility of ORAC measurements in estimating the potential dietary antioxidative activities of fruits and vegetables may be enhanced by measurements of the specific phytochemical composition of the extracts.

Relative ORAC Values For Lettuce Leaf Phenolics Separated By HPLC. Since wounded red leaf lettuce tissues had elevated levels of DCQA (**Figure 3B**), leaf disks were preincubated for 6 h prior to extraction in 50% aqueous methanol, containing 1% formic acid. During the HPLC analysis, 750 μL fractions were collected, the absorbance at 280 nm measured, and then dried. The drying step was necessary to remove both the formic acid and methanol, which might interfere with the ORAC analyses. The volatility of formic acid allows its removal without hydrolyzing the phenolic phytochemicals (*22*). After reconstitution in 50 μL of 25% (v/v) aqueous acetone, the fluorescein-based ORAC assays were performed.

As shown in **Figure 6A**, the wounded tissues had significant quantities of DCQA (peak f). However, when compared to the results presented in **Figure 3**, the relative amounts of both CTA (peak a) and the unknown caffeoyl ester (peak c) were also increased. Although the extracts used to obtain the chromatograms presented in both **Figures 3B** and **6A** were obtained from the same leaf and treated identically, there is considerable

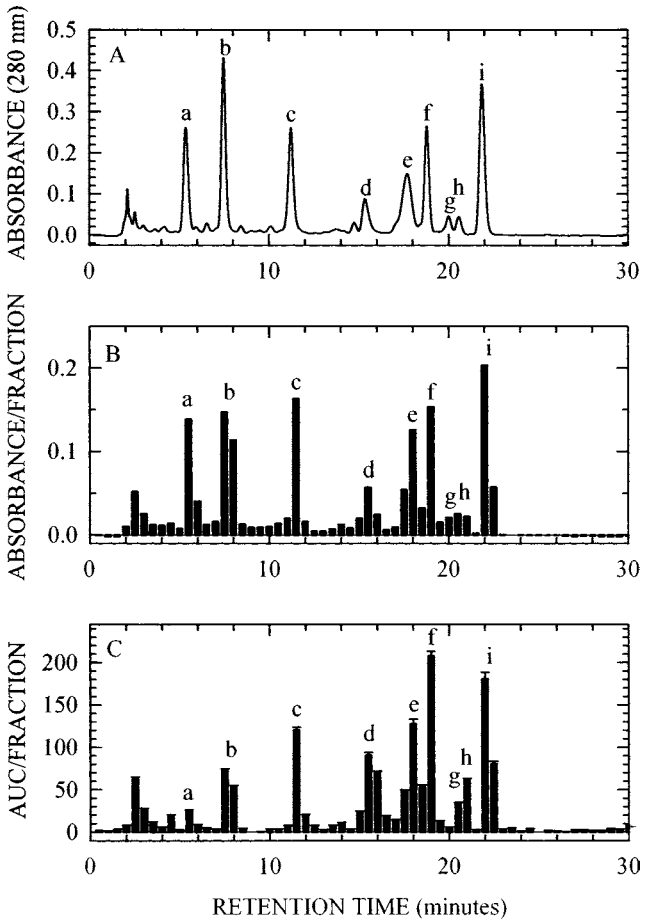

Figure 6. ORAC analyses of the components of a red leaf lettuce extract separated by HPLC. Wounded, red pigmented leaf tissue was extracted with 50% (v/v) aqueous methanol, containing 1% (v/v) formic acid, and the extract analyzed by reverse-phase HPLC monitored at 280 nm (**A**). Fractions (750 μL) were collected at 30 s intervals and their absorbance at 280 nm measured (**B**). After drying and reconsititution in 50 μL of 25% (v/v) aqueous acetone, the ORAC values in AUC units were determined for 10 μL aliquots of each fraction. In fractions with high ORAC values, the samples were diluted and reanalyzed to permit accurate measurement of the complete fluorescence decay. At least duplicate ORAC measurements were performed for each fraction and the results presented as the means with standard errors of the total ORAC in the fractions (**C**). Peak designations are the same as those presented in **Figure 1**.

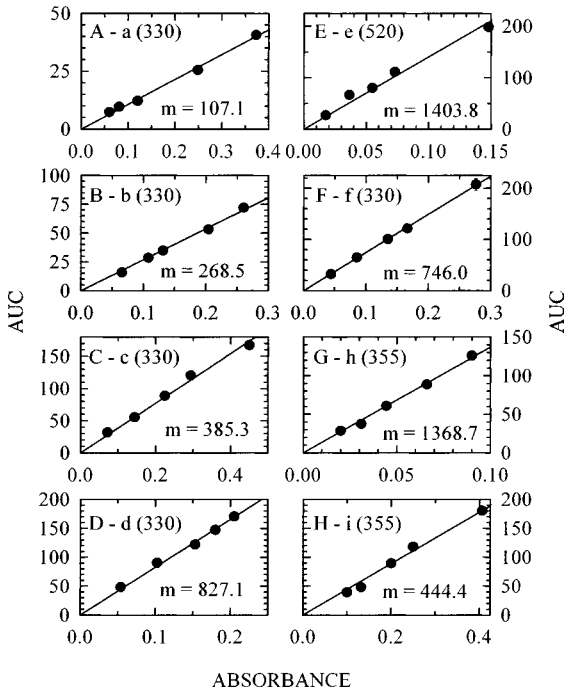

Figure 7. Dose–response relations for the peroxyl radical scavenging capacities of red leaf lettuce phenolics. Using the results presented in **Figure 6** as well as those from other extracts, HPLC and ORAC analyses, duplicate measurements at different sample concentrations were performed. Absorbance at the peak's absorbance maximum was used to estimate concentration. Only ORAC values from fluorescence decays that reached completion were used. Plotting ORAC values (AUC) as a function of absorbance resulted in linear ($r > 0.98$) relations with slopes (m) proportional to the relative antioxidant activity of the samples. Each section is labeled with a capital identification letter followed by a lower case letter that refers to the peak designations presented in **Figure 1** and the wavelength in nanometers of the absorbance measurements.

difference in the amount of CTA in the sample. Since reanalyses of these samples 1 week after preparation as well as following drying and reconstitution produced identical results, the differences in phenolic concentration may reflect variations in the leaf content of these phenolics rather than sample degradation.

Examination of the absorbances (280 nm) of the HPLC fractions clearly indicate the fractions which contain specific phytochemicals (**Figure 6B**). To allow the identification of any phytochemicals in the extract which might have low 280 nm absorbance and high ORAC values, all the fractions were initially analyzed for their antioxidative activity. As shown in **Figure 6C**, elevated ORAC values seem to correspond to the presence of the known lettuce leaf phenolics. Unlike similar analyses with spinach extracts (*22*), the antioxidant activity at the injection peak was relatively low, indicating that most of the activity of the extract resulted from the antioxidative capacities of the phenolics initially retained on the column. Even though some of the minor components of the extract appear to have high ORAC specific activities (i.e., quercetin-3-glucoside, peak h), the total ORAC activity in the extract can be attributed primarily to the major phenolic phytochemicals in the extract. Even though no new compound with high ORAC activity was found, the results demonstrated considerable differences in the specific peroxyl radical scavenging capacities of the various phenolic compounds. On the basis of peak area (**Figure 6A**), CTA (peak a), the unknown compound (peak c), and DCQA (peak f) were present in about the same amounts in the extract. However, the fractions containing CTA had significantly lower ORAC values than those containing the other phytochemicals.

Using the results shown in **Figure 6C** as well as ORAC assays performed with different sample concentrations, dose–response relationships for the major phenolic antioxidants in the red lettuce leaf extract were determined. Plotting the AUC as a function of absorbance at the compound's absorbance maximum resulted in linear relations with slopes proportional to the relative antioxidant specific activities (**Figure 7**). Although some variations in the specific activity may be attributed to the differences in the extinction coefficients for these phenolic compounds, the addition of a second caffeoyl moiety to the monocaffeoyl esters significantly increased the ORAC values. Cyanidin 3-malonylglucoside had high ORAC values, contributing about 15% of the total antioxidant activity in the extract (**Figure 7E**). Although quercetin 3-malonylglucoside was the predominant flavonoid in the lettuce extracts, quercetin-3-glucoside has higher antioxidant specific activity (**Figure 7G**).

During plant evolution, development of the antioxidative properties of flavonoids may have been secondary to some other property, such as effectiveness as screening pigments. Regardless of their value to the plant, the beneficial role to human health of phenolic phytochemicals with antioxidative activity has gained wide acceptance, even though the bioavailability of these chemicals may be unknown. Using methods similar to those employed in this study, continued investigations of the antioxidant activities of dietary phytochemicals may allow the identification of compounds with high free radical scavenging activity to facilitate the development of pre- and postharvest agricultural practices to increase the levels of these compounds in fruits and vegetables.

CONCLUSIONS

Unlike some other vegetables which contain high levels of flavonoids, the majority of the antioxidative activity in leaf lettuce extracts can be attributed to the presence of mono- and dicaffeoyl esters of tartaric and quinic acid. Depending upon the source of the lettuce samples and extraction conditions, significant quantitative and qualitative variations in lettuce leaf phenolic composition and antioxidative activity may be obtained, complicating the development of dietary standards based on phytochemical content and activity.

ABBREVIATIONS USED

AAPH, 2,2′-azo-bis(2-amidinopropane)dihydrochloride; AUC, area under the curve; B-PE, B-phycoerythrin; CQA, caffeoylquinic acid; CTA, caffeoyltartaric acid; CMG, cyanidin 3-malonylglucoside; DCQA, dicaffeoylquinic acid; DCTA, dicaffeoyltartaric acid; HPLC, high-performance liquid chromatography; QG, quercetin 3-glucoside; ORAC, oxygen radical absorbance capacity; QMG, quercetin 3-malonylglucoside; UPE, unidentified phenolic ester.

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Received for review January 6, 2003. Accepted May 14, 2003.

JF030005Q