JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of the Total Antioxidant Activity of Fruits and Vegetables by a Liposome Assay

WILLIAM G. ROBERTS AND MICHAEL H. GORDON*

Hugh Sinclair Unit of Human Nutrition, School of Food Biosciences, The University of Reading, PO Box 226, Whiteknights, Reading, RG6 6AP, UK

The effects of mixtures of antioxidants on the oxidation of phospholipids have been investigated in large unilamellar liposomes following initiation by 2,2′-azobis(2-aminopropane) dihydrochloride. The lag phase increased linearly with antioxidant concentration. The lag phases of mixtures containing α-tocopherol with ascorbic acid showed synergy between the antioxidants, but mixtures of β-carotene with α-tocopherol or ascorbic acid were not synergistic. The liposome system was used to investigate the total antioxidant activity of lipid- and water-soluble extracts from 16 samples of fruits, vegetables, and related food products. The water-soluble extracts caused greater increases in lag phase than the lipid-soluble extracts. The lag phase of liposomes containing the water-soluble extracts from fruits and vegetables increased linearly with the total phenolic concentration, with the continental salad extract having the longest lag phase. The lipid-soluble extract from apples caused the largest increase in lag phase of the lipid-soluble extracts. The lag phases of the lipid-soluble and water-soluble extracts of all fruits and vegetables studied were additive, but no synergy was detected. The lag phase of the liposomes containing both the water-soluble and lipid-soluble extracts varied from 611.5 min for the continental salad extracts to 47.5 min for the cauliflower extracts.

KEYWORDS: Liposomes; antioxidants; fruits; vegetables

INTRODUCTION

There is considerable epidemiological evidence that a diet rich in fruit and vegetables may be protective against coronary heart disease (*1*, *2*). The observed protective effect of fruits and vegetables has prompted national bodies to recommend consumption of 5 portions of fruit and vegetables per day (*3*).

Oxidative stress leads to oxidation of low-density lipoproteins (LDL), which plays a key role in the pathogenesis of atherosclerosis, which is the primary cause of coronary heart disease (*4*). Much attention has been paid to the activity of the natural antioxidants present in fruit and vegetables, since potentially these components may reduce the level of oxidative stress. Fruit and vegetables are a rich source of a wide range of antioxidant compounds such as ascorbic acid, carotenoids, flavonoids, and other phenolics. These compounds have been shown to effectively scavenge reactive oxygen species and inhibit lipid peroxidation in vitro (*5*−*7*). A wide range of methods has been described in the literature for assessing antioxidant activity. These include radical scavenging assays, ferric reducing assay, or inhibition of the oxidation of oils, emulsions, low-density lipoproteins (LDL), or liposomes (*7*−*13*). Several of these methods have been criticized because there is no oxidizable substrate being protected by the antioxidant (*14*). Methods which involve homogeneous solutions can only be adapted for antioxidants with a wide range of polarity by the use of a cosolvent such as dimethyl sulfoxide, which presumably forms a complex with, and may modify the activity of, nonpolar antioxidants. Some success has been achieved in predicting the antioxidant activity of natural extracts from fruits and vegetables from their composition, when the antioxidant activity is assessed by the TEAC, ORAC, or FRAP methods (*8*). However, the use of LDL or liposomes appears to be most promising as a method of assessing antioxidant properties relevant to human nutrition, since these systems allow investigation of the protection of a substrate by an antioxidant in a model biological membrane or a lipoprotein. Assessing antioxidants by their ability to inhibit oxidation of LDL is relevant to physiological effects of water-soluble extracts from foods (*7*), but this assay appears to be mainly suitable for water-soluble extracts, since addition of water-insoluble antioxidants faces the same problems as in homogeneous solutions. Comparison of the antioxidant activity of water-soluble samples by a liposome assay provides similar orders of activity to those from other radical-scavenging assays (*15*). However, assessment of the activity of mixtures of lipid-soluble and water-soluble antioxidants in liposomes has clear advantages over other commonly used methods. The liposome system allows the lipid-soluble components to be present in the lipid phase without the presence of a cosolvent, while the water-soluble antioxidants can be added to the aqueous phase of the liposome. The liposome system also allows synergy between tocopherols and ascorbate or other water-soluble antioxidants to be demonstrated (*16*, *17*), whereas synergy is not normally observed if these components are present in homogeneous

* To whom correspondence should be addressed. Tel: +44-118-378-6723; FAX: +44-118-9310080; e-mail: m.h.gordon@reading.ac.uk.

10.1021/jf025983t CCC: $25.00
Published on Web 01/24/2003

Table 1. Concentrations of Polar Antioxidants in Fruits and Vegetables

	mean concentration (mg/100 g fresh weight ± range; $n = 2$)							
fruit/vegetable	chlorogenic acid	(–)-epicatechin	hesperitin	naringenin	cyanidin	pelargonidin	quercetin	ascorbic acid
broccoli								64.3 (1.2)
carrot								2.8 (0.0)
cauliflower								29.7 (0.2)
clementine			36.6 (1.37)	10.6 (0.20)				49.3 (0.1)
continental salad	21.6 (0.64)				7.4 (0.23)		24.0 (0.19)	ND[a]
gala apple	12.7 (0.43)	4.15 (0.36)						ND
nectarine					1.5 (0.01)			ND
onion							17.5 (0.08)	3.0 (0.0)
orange juice			21.1 (0.33)	7.8 (0.15)				31.1 (0.2)
red pepper								168.5 (0.3)
snap pea							4.2 (0.06)	20.0 (0.1)
strawberry					0.6 (0.05)	20.7 (0.72)		45.9 (0.1)
sweet corn	9.3 (0.46)							2.5 (0.0)
tomato soup								0.5 (0.0)
Victoria plum	17.4 (0.06)				6.3 (0.30)			ND
white grape								ND

[a] ND = not detected (below detection limit).

solution. This paper describes the use of a liposome system for the assessment of the total antioxidant activity of fruits and vegetables arising from both lipid-soluble and water-soluble antioxidants. Previous studies (*18*−*23*) investigating the antioxidant effects of extracts from fruit and vegetables in liposomes have used a more limited range of samples, and they have not investigated synergy between the lipid-soluble and water-soluble components. A limited number of studies have attempted to simultaneously extract the water-soluble and lipid-soluble antioxidants from fruits and vegetables in order to determine their total antioxidant capacity. A simple extraction procedure with ethanol and hexane was used to obtain water-soluble and lipid-soluble extracts from Sea Buckthorn berries (*24*). Another study extracted antioxidants from a wide range of fruits and fruit juices using water to extract water-soluble components followed by acetone to extract lipid-soluble components (*25*). However, these simple extraction procedures used water or buffer solutions to extract the water-soluble antioxidants, but these are not suitable for the efficient extraction of ascorbic acid or all flavonoids. Fresh plant material and metaphosphoric or a similar acid must be used in the extraction solution to extract ascorbic acid without degradation (*26*). Aqueous methanol is normally used to extract flavonoid glycosides.

MATERIALS AND METHODS

β-Cryptoxanthin, apigenin, kaempferol, and cyanidin chloride were from Extrasynthese (Lyon-Nord, France). 2,2′-Azobis(2-aminopropane) dihydrochloride (AAPH) was from Sigma-Aldrich (Gillingham, U.K.). L-α-Phosphatidylcholine (PC) from soybean (99%) and all other chemicals were purchased from Sigma- Aldrich. All solvents were supplied by Rathburn Chemical Company (Walkerburn, Scotland) and were of HPLC grade.

The fruits and vegetables were purchased on the day before analysis from a local supermarket and kept refrigerated until needed. Frozen vegetables were defrosted at 4 °C for 12 h prior to analysis.

Extraction of the Water-Soluble Antioxidants from Fruit and Vegetables. Fruit or vegetable (100−200 g) was homogenized for 1 min under argon with a Waring industrial blender. Homogenate (5 g) was transferred to a flask, and 5% metaphosphoric acid (MPA):methanol (50 mL, 70:30) was added immediately. The flask was then flushed with argon for 20 s. and sealed. After being shaken for 10 min on a mechanical shaker, the extract solution was filtered under vacuum using a Whatman no. 1 filter paper. The residue was then washed with the MPA−methanol mixture (10 mL). The methanol was removed from the filtrate using a rotary evaporator with the water bath set at 40 °C.

The pulp and filter paper from the first extraction were then extracted using a solution of water:methanol (50:50, 10 mL). The two aqueous fractions obtained after rotary evaporation of the methanol were then mixed together and made up to 75 mL with water to produce the water-soluble extract.

Extraction of Lipid-Soluble Antioxidants from Fruit and Vegetables. The remaining residue and filter paper were mixed with anhydrous sodium sulfate (15 g) and extracted twice with acetone (50 mL). These acetone extracts were combined and evaporated to dryness on a rotary evaporator with the water bath set at 40 °C. The dry solid was redissolved in methanol (75 mL) to produce the lipid-soluble extract.

Storage of Extracts. The water-soluble and lipid-soluble extract solutions were filtered through a Whatman 0.1 μm polycarbonate cyclopore membrane (13 mm diameter) using a glass syringe fitted with a stainless steel filter holder. The antioxidant activity of the extract was assessed on the same day as extraction using the liposome peroxidation assay. Extract samples for HPLC and total phenol assays were stored under argon at −80 °C until needed.

HPLC Apparatus and Conditions. The HPLC apparatus for all analyses consisted of a Dionex P580 pumping system, a Dionex ASI-100T automated sample injector and a Dionex PDA-100 photodiode array detector (Sunnyvale, CA). The HPLC apparatus was connected to an IBM-compatible personal computer. All analyses were performed in duplicate.

Analysis of Ascorbic Acid. Plant extracts were diluted with mobile phase (1:1), and the solution (20 μL) was injected onto a 5 μm Nucleosil (250 mm × 4.6 mm I.D) C_{18} analytical column protected by a C_{18} guard column (Hichrom, Reading, U.K.). The column was in a column thermostat set at 15 °C, and samples within the autosampler were kept at 4 °C to prevent degradation. The mobile phase was ammonium dihydrogen phosphate buffer (pH 2.4, 20 mM, 1.0 mL/min) containing 0.15% (w/v) MPA with detection at 245 nm. Quantification was by an external standard method.

Determination of the Phenolic Concentrations of Water-Soluble Extracts. The reversed phase HPLC method described by Merken and Beecher (*27*) was used to measure the flavonoid and hydroxycinnamic acid concentrations within the water-soluble extracts of fruit and vegetables. The water-soluble extracts were analyzed before and after acid hydrolysis in order to quantify as many different classes of phenolics as possible. All phenolics in **Table 1** were identified by retention time and UV spectrum compared with standards.

Hydrolysis of Glycosides. Acid-hydrolysis of water-soluble extracts (1 mL) was performed as described previously (*28*). Peaks in the fruit and vegetable extract chromatograms were identified by comparison of UV spectra and retention times with those of standards. Catechins and hydroxycinnamic acids were quantified from the chromatograms of unhydrolyzed extracts. Flavonol glycosides and anthocyanins in extracts were quantified as the respective flavonol aglycones and

anthocyanidins after acid hydrolysis. Naringenin glycosides in citrus fruits were also quantified as naringenin after acid hydrolysis. Hesperitin glycosides were quantified as the glycoside due to an interfering peak in the acid hydrolyzed extract.

Analysis of Water-Soluble Phenolics. Unhydrolyzed sample or standard (50 μL) or hydrolyzed sample (150 μL) was injected onto a 5 μm Nucleosil (250 mm × 4.6 mm I.D.) C_{18} analytical column. This was protected by a C_{18} guard column (Hichrom, Reading) and placed in a Dionex STH 585 column thermostat set at 30 °C. The solvents used to make up the mobile phase were water, methanol, and acetonitrile, each containing 0.1% formic acid. Mobile phase was pumped through at 1.0 mL/min. The gradient was water: methanol: acetonitrile 90:6:4 (0 min), 85:9:6 (5 min), 71:17.4:11.6 (30 min), 0:85:15 (60 min), 90:6:4 (61−70 min), The photodiode array detector monitored all wavelengths from 200 to 800 nm to assist in peak identification by comparison of the UV/visible spectra with standards. Wavelengths of 210 nm, 260 nm, 278, 370, and 520 nm were used for the quantification of the phenolics. An external standard method was used to quantify each of the phenolic components listed in **Table 1**.

Analysis of Total Phenolics in Water-Soluble Extracts. Total soluble phenolics in the water-soluble extracts were determined with the Folin-Ciocalteu reagent by the method of Singleton et al. (*29*), using gallic acid as the standard.

Ascorbic acid in the extracts also reacts in this assay to produce an absorbance at 760 nm. Therefore, standard solutions (0−60 μg/mL) of ascorbic acid were also analyzed. The absorbance at 760 nm due to ascorbic acid in the extract samples was then calculated using the ascorbic acid concentrations determined by HPLC. The absorbance due to ascorbic acid was then subtracted from the original extract value. Corrected extract absorbances were then converted to gallic acid equivalents.

Analysis of Lipid-Soluble Extracts. Stored extract samples in methanol (1000 μL containing extract form 0.07 g plant material) were evaporated to dryness under nitrogen and then redissolved in 400 μL of HPLC mobile phase. Sample in the mobile phase (100 μL) was injected onto a 5 μm Nucleosil (250 mm × 4.6 mm I.D) C_{18} analytical column protected by a C_{18} guard column (Hichrom, Reading). The column was in a Dionex STH 585 column thermostat set at 30 °C, and mobile phase was pumped through at 1.6 mL/min. The mobile phase consisted of acetonitrile (68.5%), tetrahydrofuran (22%), methanol (7%), and 1% ammonium acetate solution (2.5%). The photodiode array detector monitored all wavelengths from 200 to 800 nm for each 20 min run. This allowed peaks to be identified by comparison of the UV/visible spectra with standards. However, four different wavelengths were used to quantify the antioxidants. (290 nm for α-tocopherol, 325 nm for retinol, 450 nm for lutein, β-cryptoxanthin, α-carotene, and β-carotene, and 472 nm for lycopene).

Preparation of Liposomes. Liposomes were prepared by an extrusion method based on a literature procedure (*30*). Liposome suspension was prepared by adding soybean PC solution (1.35 mg in 50 μL) and chloroform (2 mL) to a 25 mL round-bottomed flask. This solution contained 1.5 μmol of soybean PC if the relative molecular mass of soybean PC is taken as 900. Methanol or hexane (1 mL) was then added to the flask. This solvent addition was replaced with a volume of lipid-soluble antioxidant solutions (0.2−1 mL) or lipid-soluble extract (0.5 mL from 33.3 mg fruit or vegetable) when required. Solvent was removed under reduced pressure on a rotary evaporator with the water bath set at 30 °C. Argon was introduced to reestablish atmospheric pressure and the flask covered in aluminum foil. An oil pump was then used to keep the flask under a vacuum of <0.5 mmHg for at least 1 h.

After the vacuum was released while introducing argon, the lipid residue was rehydrated with sodium phosphate buffer solution (10 mL, 10 mM, pH 7.4). This buffer solution was replaced with water-soluble antioxidant or plant extracts (0.05 mL) in buffer (10 mL) when required. The flask, under argon atmosphere, was vortexed for 10 min, producing a homogeneous white suspension of multilamellar liposomes. The multilamellar suspension was then shaken while being sonicated for 30 s in an ultrasonic bath. This ensured complete recovery of the lipid from the flask wall. Large unilamellar liposomes were obtained by transferring the liposome suspension to an Avestin Liposofast Basic (Avestin Inc. Ottawa, Canada) small volume extrusion device. The suspension was passed 21 times through a double layer of polycarbonate membranes (200 nm pore size for fruit and vegetable extracts; 100 nm pore size for liposomes containing ascorbic acid, α-tocopherol, and β-carotene).

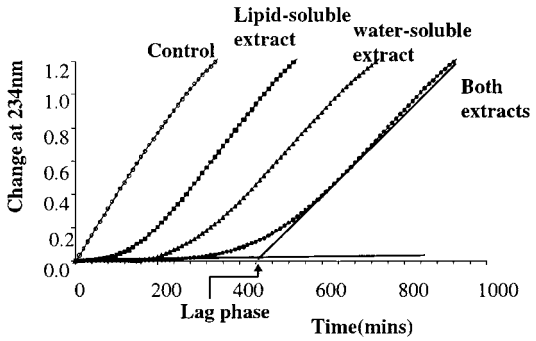

Figure 1. Change in absorbance at 234 nm of liposomes containing lipid-soluble extract (from 3.33 mg apple per mL liposome suspension) and water-soluble extract (from 0.33 mg apple per mL liposome suspension) after the addition of AAPH.

Peroxidation of Liposomes. Unilamellar liposome suspension (2 mL) was pipetted into a quartz cuvette and incubated for 10 min at 37 °C within the water-jacket regulated cell holder of a Perkin-Elmer Lamda Bio 20 UV/Vis Spectrometer with automatic cell changer, connected to an IBM-compatible personal computer running UV Winlab software. Up to six sample liposome suspensions and a blank liposome suspension were measured in one run. An additional quartz cuvette, containing 2 mL of sodium phosphate buffer, was incubated to measure the AAPH absorbance at each time point.

Lipid peroxidation was initiated by the addition of AAPH (20 μL) in sodium phosphate buffer (pH 7.4, final concentration 0.25 mM) to each cuvette. The cuvettes were then sealed to prevent evaporation and inverted five times. The absorbance at 234 nm was recorded every 10 min for each cuvette against a blank of sodium phosphate buffer. Each antioxidant or extract solution was assessed using liposomes prepared on two different occasions. The lag phase was measured as the time in minutes to the point where a tangent to the propagation phase and a tangent to the lag phase intercepted (see **Figure 1**). Blank liposome suspensions produced no lag phase when subjected to AAPH-induced peroxidation.

Statistics. Two-way analysis of variance was used to test for synergy in antioxidant mixtures. The nonparametric Spearman correlation test was used to test the correlation between lag phase and total phenol concentration, since the total phenol concentrations and lag phases were not normally distributed.

RESULTS AND DISCUSSION

Extraction of Antioxidants from Fruits and Vegetables. Although extraction of single classes of antioxidants can be done efficiently, the optimal conditions required for each class are different, and consequently the development of a procedure suitable for extracting all antioxidants is not easy. Ascorbic acid is commonly extracted in aqueous acid, whereas flavonols and flavones can be extracted as aglycones from fruit and vegetables by refluxing freeze-dried material for up to 4 h with methanol/water (1:1) containing hydrochloric acid (*28*). The same hydrolysis procedure has also been used to quantify hydroxycinnamic acids in berries by extending the reflux period (*31*). However, flavanols were found to be rapidly degraded under these conditions (*27*), and aglycones will differ in antioxidant properties from glycosides. In addition, ascorbic acid would be degraded during freeze-drying.

The major flavanols present within fruits and vegetables are the catechins, and an optimized extraction procedure for this class of components has been described (*32*). Optimum extrac-

Table 2. α-Tocopherol and Carotenoid Concentrations in Fruits and Vegetables

	mean antioxidant concentration (mg/100 g fresh weight) ± (range) $n = 2$					
fruit/vegetable	α-tocopherol	lutein/zeaxanthin	β-cryptoxanthin	lycopene	α-carotene	β-carotene
broccoli		423 (7)				359 (13)
carrot					1481 (60)	4618 (37)
cauliflower						
clementine			61 (1)			95 (14)
continental salad		669 (21)				328 (13)
gala apple	608 (66)					
nectarine	579 (10)					57 (2)
onion						
orange juice						
red pepper	1417 (40)	120 (3)	63 (1)		69 (1)	68 (1)
snap pea						
strawberry						
sweet corn						
tomato soup	615 (6)			2829 (27)	396 (9)	1356 (16)
Victoria plum	368 (18)					101 (11)
white grape	312 (19)					

tion occurred when freeze-dried material was shaken with 70% methanol/water for at least 10 min. Lipid-soluble antioxidants require hexane, chloroform, acetone, or similar solvents for extraction.

Therefore, extraction conditions must be carefully chosen to isolate all antioxidant components from whole fruits and vegetables, which include skin, flesh, and juice. Aqueous methanol (10%) was used to extract antioxidants from homogenized fruits or vegetables (*8*). This extraction procedure is suitable for extraction of phenolic components but it will not extract lipid-soluble antioxidants and is likely to allow some loss of vitamin C.

The extraction of active antioxidants from fruits and vegetables was optimized initially, since most techniques employed for the extraction of antioxidants have focused on the extraction of one particular antioxidant or class of antioxidants from a small range of similar fruits or vegetables. The foods used for extraction contained a wide range of antioxidant components (**Tables1** and **2**). Additional unidentified components were also present in some samples. An unidentified compound eluted close to (+)-catechin after 11.0 min in the HPLC chromatograms of all extracts apart from orange juice, apple, plum, and grape, with a UV/vis spectrum with λ_{max} at 197, 220, and 280 nm which was different from (+)-catechin. An additional peak at 9.9 min was present in the extract of broccoli and cauliflower. This peak had the same UV/vis spectrum as the peak seen at 11.0 min. These peaks eluted well before (−)- epicatechin, which had a retention time of 17.2 min.

The total phenolics present within the water-soluble extracts were measured using the Folin-Ciocalteu reagent and corrected for ascorbic acid content. In general, the total phenolic concentrations of the fruit and vegetable extracts correlated with the sum of the individual phenolics identified using HPLC. However, the total phenolic concentrations measured using the Folin-Ciocalteu method cannot all be accounted for by the phenolics identified using HPLC. Even extracts in which no peaks were detected by HPLC showed total phenolic concentrations of up to 20 mg GAE/100 g. Caution must be used in interpreting the results from the Folin-Ciocalteu method as other substances in the extract such as sugars have been shown to interfere with the assay. Also, different classes of phenolics contribute to different extents to the absorbance produced by reaction with the Folin-Ciocalteu reagent when compared to gallic acid (*29*). Nevertheless, the results suggest that the extracts may have contained phenolic compounds that were not detected by HPLC.

The efficiency of the extraction procedure was tested by adding a known mass of antioxidant standard, at a level in the range found in fruits and vegetables, to the extraction flask. The concentrations of antioxidant in the extracts with and without added standard were determined by HPLC, and the percentage recovery of the added antioxidant was calculated. Recovery (for extractions performed in triplicate) of ascorbic acid (1000 μg) added to strawberry was 93.7 ± 5.9%; rutin (400 μg) added to onion was 88.3 ± 2.0%; and β-carotene (20 μg) added to tomato was 92.4 ± 0.7%.

It can be concluded that a wide range of antioxidants were extracted by the procedure adopted, although further work is required to assess the efficiency of the extraction for a wider range of phenolic components. It should be noted that the requirement to prepare an extract without methanol for the aqueous phase of the liposomes required evaporation of methanol from the aqueous methanol extracts, and some polar components may have been lost by precipitation at this stage.

Assessment of Antioxidant Activity by the Liposome Method. There are several methods of generating liposomes (*33*). For this study a multiple extrusion technique was used because this produces large unilamellar vesicles, which are reasonably uniform in size, with curvature in the same range as lipoproteins. The liposomes are not contaminated excessively by metal ions that may be introduced if an ultrasonic probe is inserted into the sample. The method of generation of radicals also had to be selected. Water-soluble antioxidants including ascorbic acid can become pro-oxidant in the presence of metal ions, depending on the antioxidant:metal ratio, so it was not considered desirable to use this method of generating radicals for the assessment of natural extracts where the concentration of different antioxidants will vary widely. A commonly used water soluble radical initiator, AAPH, was added to generate radicals in the aqueous phase of the liposome, and EDTA was added to chelate metal ions.

The end of the lag phase of liposomes containing added antioxidants was readily measured due to the sharp change in gradient at this point (**Figure 1**). When individual antioxidants were used, the lag phase prior to oxidation increased with concentration of antioxidants for α-tocopherol or β-carotene added to the phospholipid phase or for ascorbic acid added to the aqueous phase (**Figure 2**). The increase in activity with concentration was steepest for α-tocopherol, which is an active radical scavenger with the hydroxyl group located near the surface of the phospholipid bilayer, where it is ideally located to react with radicals generated in the aqueous phase before

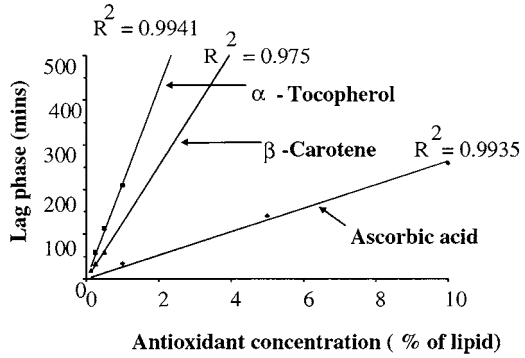

Figure 2. The effect of antioxidant concentration on liposome lag phase.

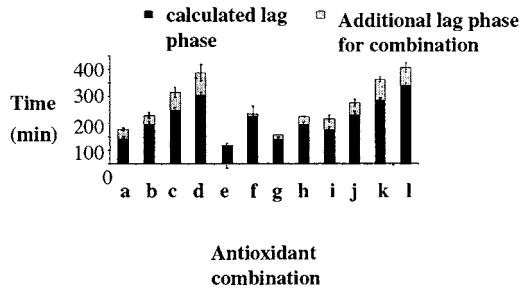

Figure 3. Comparison of observed lag phases for combinations of antioxidants with lag phases calculated by addition from experiments with single antioxidants for liposomes containing combinations of α-tocopherol (αT), β-carotene (βC), and ascorbic acid (AA) (*a* = 0.25% αT + 1% AA; *b* = 0.5% αT + 1% AA; *c* = 0.25% αT + 5% AA; *d* = 0.5% αT + 5% AA; *e* = 0.25% βC + 1% AA; *f* = 0.25% βC + 5% AA; *g* = 0.25% βC + 0.25% αT; *h* = 0.25% βC + 0.5% αT; *i* = 0.25% βC + 0.25% αT + 1% AA; *j* = 0.25% βC + 0.5% αT + 1% AA; *k* = 0.25% βC + 0.25% αT + 5% AA; *l* = 0.25% βC + 0.5% αT + 5% AA).

Table 3. Lag Times for the Oxidation of Liposomes Containing Antioxidants

antioxidant	concentration (mol % relative to lipid)	mean lag phase (min) ± (range) *n* = 2
β-carotene	0.25	34.4 (2.3)
β-cryptoxanthin	0.25	49.7 (0.2)
lutein	0.25	56.9 (6.1)
lycopene	0.25	18.4 (1.2)
ascorbic acid	1.0	34.0 (5.0)
(+)-catechin	1.0	545.1 (33.0)
chlorogenic acid	1.0	542.8 (8.0)
cyanidin	1.0	543.6 (3.9)
pelargonidin	1.0	200.1 (10.6)
rutin	1.0	410.0 (7.9)

they can react with a polyunsaturated fatty acid residue in the phospholipid molecules. The gradient was lowest for ascorbic acid, which is nonphenolic and was diluted by the aqueous phase. Synergy between samples containing α-tocopherol and ascorbic acid was clearly demonstrated ($P = 0.002$), whereas synergy was not shown by mixtures of β-carotene with either α-tocopherol ($P = 0.675$) or ascorbic acid ($P = 0.461$) (**Figure 3**). Phenolic antioxidants were much more effective radical scavengers than ascorbic acid in the aqueous phase of the liposome system (**Table 3**). (+)- Catechin, chlorogenic acid, cyanidin, pelargonidin, and rutin, which are all phenolic compounds, inhibited the lag phase of liposomes to a much greater extent than ascorbic acid. The anthocyanidins both exhibited strong antioxidant activity, but the lag phase for cyanidin chloride (544 min) was much greater than that observed for pelargonidin chloride (200 min). This result is consistent with the antioxidant activity of the anthocyanidins assessed by the TEAC assay (*34*). Cyanidin had a TEAC value of 4.4 whereas pelargonidin was found to possess a TEAC value of only 1.3. This difference in antioxidant activity was attributed to the absence of a second hydroxyl group on the B ring of pelargonidin.

Table 4. Lag Phases of Liposomes Containing Extracts of Fruit and Vegetables

	mean lag phase (min) (± range) (*n* = 2)		
fruit/vegetable	lipid-soluble extract[a]	water-soluble extract[b]	both extracts
broccoli	27.9 (1.3)	98.2 (4.8)	127.4 (1.0)
carrot	61.5 (3.1)	25.3 (0.6)	85.3 (4.2)
cauliflower	0	46.6 (0.7)	47.5 (1.4)
clementine	53.7 (4.3)	119.3 (6.1)	170.6 (4.1)
continental salad	41.5 (1.4)	567.4 (7.2)	611.5 (12.2)
gala apple	177.8 (11.9)	276.4 (20.2)	439.2 (2.7)
nectarine	29.3 (9.0)	72.9 (7)	85.4 (7.6)
onion	0	49.5 (2.7)	50.5 (0.3)
orange juice	34.1 (1.8)	68.0 (0.7)	92.9 (2.6)
red pepper	73.2 (2.0)	89.1 (16.2)	163.2 (3.9)
snap pea	0	59.5 (2.4)	62.6 (3.1)
strawberry	65.8 (5.2)	499.6 (10.7)	566.9 (6.0)
sweet corn	63.4 (4.0)	96.5 (9.4)	159.8 (0.4)
tomato soup	51.3 (1.4)	72.9 (12.5)	96.4 (5.6)
Victoria plum	55.2 (4.5)	274.4 (7.6)	323.7 (4.5)
white grape	46.6 (1.1)	127.1 (3.1)	163.6 (4.1)

[a] Extract from 33.3 mg fruit in 10 mL of liposome suspension. [b] Extract from 3.3 mg fruit in 10 mL of liposome suspension.

When individual carotenoids were studied at 0.25% molar concentration (relative to the phospholipid component), the order of activity was lutein > β-cryptoxanthin > β-carotene > lycopene (**Table 3**). The order of activity of carotenoids is very dependent on the method and medium used to assess activity. However, the observed order of activity is in agreement with previous work for the effects of xanthophylls and carotenes on oxidation in liposomes, when oxidation was initiated in the aqueous phase (*35*).

The lag phases of the lipid-soluble extracts of fruits and vegetables were generally much shorter than those observed for the water-soluble extracts, and therefore the mass of fruit used was 10 times greater for the lipid-soluble extract in order to allow a comparison of the plant sources and to detect any synergy when used in a mixture with water-soluble extracts (**Table 4**). The greater activity of the water-soluble extracts agrees with previous studies examining fruit extracts (*24*, *25*). The low concentrations of tocopherols (in the range of μg/100 g) present in fruits and vegetables are not sufficient to cause detectable synergistic effects. In comparison, levels of ascorbic acid and phenolics found within fruits and vegetables are in the mg/100 g range. The peroxidation curves generally obtained for the liposomes containing lipid-soluble extracts were similar to those observed for liposomes containing β-carotene, with a significant oxidation rate observed even before the end of the lag phase. One notable exception was the lipid-soluble extract from apple. For example the oxidation rate increased from 0 to 0.004 min^{-1} before the end of the lag phase for the red pepper extract, whereas the initial rate of change of absorbance remained at <0.0005 min^{-1} for > 90 min for the apple extract, before it started to increase. The mean lag phase for liposomes containing the lipid-soluble extract of apple was 178 min, which was significantly longer than for all other lipid-soluble extracts, with the second highest lag phase being 74 min for the lipid-soluble extract of red pepper.

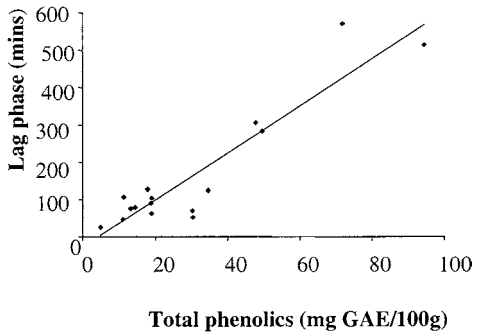

Figure 4. Effect of total phenolic concentration in fruit and vegetables upon the lag phase of liposomes containing water-soluble extracts.

Apples contain dihydrochalcones of which phloridzin (phloretin 2′-glucoside) is the most studied. Phloridzin has been detected at levels of 14−35 mg/kg in four different apple varieties after extraction with aqueous methanol (*36*). In the present study phloridzin was not detected in the water-soluble apple extract, but it may have passed into the acetone-soluble fraction. The presence of dihydrochalcones in the lipid-soluble extract may contribute to the long lag phase of the lipid-soluble apple extract compared to those from other fruits and vegetables, although phloridzin has only a moderate antioxidant activity compared with water-soluble extracts when assessed by the TEAC assay (*34*). Calculation of the lag phases expected for the concentrations of α-tocopherol and carotenoids present in the lipid-soluble extracts confirmed that other unidentified antioxidants were contributing >50% of the antioxidant activity for lipid-soluble extracts from all fruits and vegetables except for the carrot and tomato samples.

Flavanones present within clementine and orange juice water-soluble extracts have been shown to precipitate out of aqueous extraction solutions (*37*). Also, strawberries contain ellagitannins which are highly insoluble in water (*38*). Therefore, it is possible that some of these compounds were extracted in the acetone extract. However, with the exception of the apple extract, the lag phases of the lipid-soluble extracts were much lower than those observed for the water-soluble extracts.

The water-soluble extracts of the fruits and vegetables studied exhibited a wide range of antioxidant activities (**Table 4**). The water-soluble extracts of salad and strawberry produced lag phases of >500 min which were nearly twice as high as those for extracts from apples and plums (~275 min). The next highest lag phases of approximately 120 min were produced by the water-soluble extracts of clementines and grapes. Lag phases for liposomes containing the remaining water-soluble extracts were <100 min, with carrots producing the lowest lag phase observed.

Almost all the lag phase (82%) of the water-soluble extract of red pepper can be attributed to the ascorbic acid concentration of the extract (equivalent to 165 mg/100 g of red pepper). Smaller contributions toward the total lag phase were made by the ascorbic acid present in broccoli (30%), cauliflower (28%), clementine (19%), orange juice (20%), snap pea (15%), and strawberry (4%) extracts. However, the contribution of ascorbic acid toward the observed lag phases of the water-soluble extracts from most samples was small, suggesting that other extract constituents were responsible for most of the antioxidant activity.

Lag phases increased with increasing total phenol concentrations determined by the Folin-Ciocalteu method (**Figure 4**). The correlation between lag phase and total phenol concentration was highly significant ($P = 0.0025$, $R^2 = 0.7$). The Folin-Ciocalteu test has limitations due to the range of sensitivity to different phenolics and the possibility of reactions with non-phenolic substances, but it allows an estimate of the total phenolic content (*29*). Therefore, the result indicates that phenolic components within the water-soluble extract were responsible for most of the extract antioxidant activity.

Some of the major hydroxycinnamic acids and flavonoids were identified using HPLC (see **Table 1**). The water-soluble extracts of strawberry and salad exhibited significantly greater antioxidant activity than the other extracts tested. The major phenolic antioxidants identified by HPLC in extracts from these two foods were cyanidin (both), pelargonidin (strawberry), quercetin (salad), and hydroxycinnamic acids (salad). Hydroxycinnamic acids and cyanidin were also observed in the extracts of plum, and chlorogenic acid was found in the extract of apple. Both these extracts also exhibited high antioxidant activity. Therefore, it was thought that these compounds contributed significantly to the antioxidant activity of the extracts.

Rutin (quercetin 3-*O*-rutinoside) was assessed in the liposome peroxidation assay in order to estimate the effectiveness of the quercetin glycosides identified in the extracts of onion, salad, and snap pea. Rutin present at a concentration of 1% of the PC concentration gave a lag phase of 410 min, indicating effective inhibition of liposome peroxidation. Chlorogenic acid and (+)-catechin also effectively inhibited liposome peroxidation with lag phases of 543 and 545 min at 1% concentration, respectively.

The incorporation of tea catechins into egg yolk liposomes was shown to increase with increasing hydrophobicity (*40*).

One noticeable result from the current study was the low lag phase of 50 min observed for the water-soluble extract of onion. This was significantly lower than the lag phase observed for salad (567 min) yet the concentration of quercetin detected by HPLC in the hydrolyzed extracts was similar (17.5 mg/100 g vs 24.5 mg/100 g). However, the extracts used in the liposome assay contained quercetin glycosides. Onions have been shown to contain high levels of quercetin 4′-*O*-glucosides (*41*) whereas the main quercetin glycoside in lollo rosso lettuce has been identified as a quercetin 3-*O*-glycoside (*42*). Quercetin 4′-*O*-glucoside lacks the *ortho*-dihydroxy structure in the B ring, which has been shown to be an important feature with regards to hydrogen-donating ability. Therefore the quercetin glycoside can make a major contribution to the antioxidant activity of lollo rosso lettuce but a much smaller contribution to that of onions.

Phenolic compounds present within the water-soluble extracts were far more effective than ascorbic acid in protecting liposomes from AAPH-induced peroxidation. This has also been observed in a study of fruit juices (*43*). However, calculation of the expected lag phase due to the identified hydroxycinnamic acid and flavonoids cannot be reliably carried out as the forms present within the extracts are significantly different from the standards tested. More liposome peroxidation studies using pure flavonoid glycosides need to be carried out before such calculations can be performed. It also important to emphasize that a large proportion of the antioxidant activity of the extracts may be due to phenolic compounds that have not been identified in the present study. Fruits such as strawberries, apples, and grapes have been found to contain proanthocyanidins, which have been shown to be effective in inhibiting AAPH-induced lipid peroxidation (*44*), but these components were not investigated in the present study.

The order of the total antioxidant activity was continental salad > strawberry > Gala apple > Victoria plum > clementine, white grape, red pepper, sweet corn > broccoli > tomato soup, orange juice, nectarine, carrot > snap pea > onion, cauliflower.

Four of these fruits (strawberry, plum, white grape, apple) were investigated in the study of Wang et al. (*25*) using the ORAC method, but while the order of activity for three of the fruits was in agreement, the apple sample was significantly more effective as a source of antioxidants compared to the other fruits in the present study. Proteggente et al. (*8*) investigated the order of antioxidant activity for fruits and vegetables using the TEAC, FRAP, and ORAC methods. The order of activity varied somewhat depending on the method used. The high activity for extracts from strawberry and plum, and the low activity for cauliflower was confirmed by all methods. Again the high activity of extracts from apples found in the current study is in contrast to the low activity found previously (*8*). Broccoli, onion, and green grapes were intermediate in activity with all methods, although the relative order of activity varied according to the method used. The results found for Gala apples in the current study appear to be most out of step with the previous studies. Van der Sluis et al (*36*) found that four varieties of apple varied by 80% in total polyphenol content. However, further investigation of the effects of variety of apple, extraction procedure, and method of investigation are required to decide which of these variables are making the main contribution to the increased activity of apple extracts in the present study.

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Received for review September 24, 2002. Revised manuscript received December 16, 2002. Accepted December 17, 2002. The authors thank the Ministry of Agriculture, Fisheries and Food for financial support for the project.

JF025983T