

Flavonoids from Almond Skins Are Bioavailable and Act Synergistically with Vitamins C and E to Enhance Hamster and Human LDL Resistance to Oxidation^{1,2}

Chung-Yen Chen, Paul E. Milbury, Karen Lapsley,* and Jeffrey B. Blumberg³

Antioxidants Research Laboratory, Jean Mayer U.S. Department of Agriculture Human Nutrition Research Center on Aging, Tufts University and *The Almond Board of California, Modesto, CA

ABSTRACT Consumption of tree nuts such as almonds has been associated with a reduced risk of coronary heart disease. Flavonoids, found predominantly in the skin of almonds, may contribute to their putative health benefit, but their bioactivity and bioavailability have not previously been studied. Almond skin flavonoids (ASF) were extracted with HCl:H₂O:methanol (1:19:80) and their content of catechins and flavonols identified by HPLC with electrochemical detection. ASF bioactivity was assessed in vitro by their capacity to increase the resistance of human LDL to oxidation induced by 10 $\mu\text{mol/L}$ Cu²⁺. ASF from 0.18 to 1.44 μmol gallic acid equivalent (GAE)/L increased the lag time to LDL oxidation in a dose-dependent manner ($P \leq 0.0001$). Combining ASF with vitamin E or ascorbic acid extended the lag time >200% of the expected additive value ($P \leq 0.05$). The bioavailability and in vivo antioxidant activity of 40 μmol ASF were examined in BioF1B hamsters. Peak plasma concentrations of catechin, epicatechin, and flavonols (quercetin, kaempferol, and isorhamnetin) occurred at 60, 120, and 180 min, respectively. The concentration of isorhamnetin was significantly elevated in liver at 180 min. Absorbed ASF enhanced the ex vivo resistance of hamster LDL collected at 60 min to oxidation by 18.0% ($P = 0.028$), and the in vitro addition of 5.5 $\mu\text{mol/L}$ vitamin E synergistically extended the lag time of the 60-min sample by 52.5% ($P \leq 0.05$). Thus, ASF possess antioxidant capacity in vitro; they are bioavailable and act in synergy with vitamins C and E to protect LDL against oxidation in hamsters. J. Nutr. 135: 1366–1373, 2005.

KEY WORDS: • almonds • antioxidants • bioavailability • flavonoids • synergy

Flavonoids are natural constituents of plant foods and have been carefully studied in fruits and vegetables, but less attention has been paid to their presence in whole grains and tree nuts (1,2). Flavonoids appear to possess a variety of biological activities, including antioxidant, anti-inflammatory, and vasodilatory actions (3). Putative health benefits of flavonoids were suggested by epidemiologic studies showing an inverse association between intake and risk of cardiovascular disease (4,5). A similar relation was observed with tree nuts, leading to the recent approval by the FDA of a qualified health claim for tree nuts, including almonds, that eating 42.6 g/d (1.5 oz/d) as part of a diet low in saturated fat and cholesterol may reduce the risk of heart disease (6). It is worth noting here the randomized clinical trial conducted by Jenkins et al. (7) demonstrating that almond consumption can reduce total and LDL cholesterol and increase the resistance of LDL to oxidation.

Almonds (*Prunus amygdalus* Batsch) rank highest among tree nuts, such as hazelnut, pecan, pistachio, and walnut, in total annual crop production (8), and are a good source of nutrients associated with heart health such as vitamin E, monounsaturated fatty acids, PUFA, arginine, and potassium (9). Almond skins, removed from the nut by hot water blanching during preparation of almond meat, constitute ~4% of the total almond weight and are generally treated as a waste product. However, an array of flavonoids, including catechins, flavonols, and flavanones in their aglycone and glycoside forms, were identified in almond skin (10,11). These compounds may contribute to the health benefits associated with almond consumption. The bioavailability and pharmacokinetics of individual flavonoids were demonstrated in several studies (12–14). Catechins and flavonols from food were also found to be bioavailable in rodent and human studies with the food, although its matrix and preparation may have a significant effect on their bioavailabilities (15–20). However, no studies have explored the bioavailability of flavonoids from almond skins and their effect on antioxidant activity. Therefore, we examined almond skin flavonoids (ASF)⁴ for their in vitro action with and without vitamins C and E on the resistance

¹ Presented in part at Experimental Biology 03, April 2003, San Diego, CA [Chen, C.-Y., Shen, J., Li, T., Milbury, P. & Blumberg, J. (2003) Bioavailability and antioxidant activity of polyphenolics from almond skins in hamsters. FASEB J. 17: A377 (abs.)].

² Supported by the USDA Agricultural Research Service under Cooperative Agreement No. 58-1950-4-401 and the Almond Board of California. The contents of this publication do not necessarily reflect the views or policies of the USDA nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.

³ To whom correspondence should be addressed.
E-mail: jeffrey.blumberg@tufts.edu.

⁴ Abbreviations used: C_{max}, maximal concentration; ECD, electrochemical detection; GAE, gallic acid equivalents, T_{max}, time to maximal concentration.

of human LDL to oxidation as well as their bioavailability, pharmacokinetics, and in vivo antioxidant actions in hamsters.

METHODS AND MATERIALS

Chemicals and reagents. The following reagents were obtained from Sigma Chemical: copper sulfate, vitamin E (α -tocopherol), sodium chloride, quercetin, catechin, (-)-epicatechin, kaempferol, isorhamnetin, sodium phosphate monobasic, sodium phosphate dibasic, BHT, Folin Ciocalteu phenol reagent, and β -glucuronidase type H-2 (containing sulfatase from *Helix pomatia*). All organic solvents, glacial acetic acid, food-grade ascorbic acid, and potassium bromide were purchased from Fisher.

Flavonoid profile of almond skin. Pulverized almond skin powder provided by the Almond Board of California was used for ASF extraction. The powder (0.5 g) was extracted twice with 10 mL acidified methanol solution (HCl:H₂O:methanol, 1:19:80) over 16 h at 4°C. The sample was centrifuged at 1000 \times g for 15 min at 4°C and methanol evaporated with nitrogen gas. The residue was reconstituted with the aqueous mobile phase and the ASF characterized by HPLC with electrochemical detection (ECD) according to a slightly modified method of Milbury (21). The residue was also reconstituted with phosphate buffer (7.79 mmol/L Na₂HPO₄, 2.59 mmol/L NaH₂PO₄, and 150 mmol/L NaCl, pH 7.4) for the in vitro LDL oxidation assay. All HPLC procedures were performed with ESA instruments, including 2 pumps (model 582), autosampler (model 542), and Coularray 5600 A detector. Analyte separation was achieved using a Zorbax ODS C18 column (4.6 \times 150 mm, 3.5 μ m) with a 0.6 mL/min flow rate and mobile phase gradient from mobile phase A (75 mmol/L citric acid and 25 mmol/L ammonium acetate in 90% H₂O and 10% acetonitrile) to mobile phase B (75 mmol/L citric acid and 25 mmol/L ammonium acetate in 50% H₂O and 50% acetonitrile) for 68 min. The following mobile phase gradient profile was used (% solvent B): 1% (0–15 min), 1–10% (15–25 min), 10–80% (25–60 min), 80–10% (60–65 min), and 10–1% (65–68 min). Detection was achieved with potentials applied from 60 to 720 mV with 60-mV increments. The identification of individual ASF was achieved by comparing its retention time and electrochemical response with purified standards obtained from Sigma Chemical. The quantity of individual almond flavonoids was calculated according to concentration curves constructed with purified standards. Total almond skin phenolics were assessed via the Folin-Ciocalteu reaction according to Singleton et al. (22) and expressed as μ mol/L gallic acid equivalents (GAE).

Antioxidant capacity of ASF in human LDL. The antioxidant capacity of ASF was assessed in vitro with human LDL according to a slight modification of the method described by Esterbauer et al. (23). Collection of venous blood, approved by the Institutional Review Board of the Tufts-New England Medical Center, was performed between 1400 and 1430 h in 6 healthy Caucasian women who were not fasting; the women were 28–64 y old with a mean body weight of 63 \pm 15 kg. LDL was separated from plasma according to Chung et al. (24) using a Beckman NVT-90 rotor in a Beckman L8-mol/L centrifuge (329,271 \times g for 90 min). KBr and EDTA were removed from the sample using a PD-10 column (Amersham Pharmacia Biotech). LDL protein was determined using a bicinchoninic acid protein assay kit (Pierce). LDL samples from the first 3 subjects were used to assess the dose-response relation of ASF, and from the last 3 subjects for experiments on the interaction between ASF with vitamins C and E. All LDL experiments were performed in duplicate in each of 3 subjects. LDL (182 nmol/L) were oxidized by 10 μ mol/L CuSO₄ in a total volume of 1.0 mL phosphate buffer (7.79 mmol/L Na₂HPO₄, 2.59 mmol/L NaH₂PO₄, and 150 mmol/L NaCl, pH 7.4). Formation of conjugated dienes was monitored by absorbance at 234 nm at 37°C over 6 h using a Shimadzu UV1601 spectrophotometer (Japan) equipped with a 6-position automated sample changer. The results of the LDL oxidation are expressed as lag time (defined as the intercept at the abscissa in the diene-time plot) (25). An aliquot of ASF in acidified methanol was dried under nitrogen and redissolved in an equal volume of phosphate buffer (pH 7.4) for testing in the assay. Concentrations of ASF from 0.18 to 1.44 μ mol/L were selected

to reflect amounts that can be obtained from dietary intakes (26). Concentrations of vitamin E and C employed in these experiments were 9–18% and 3–6% of plasma concentrations, respectively, levels generally found in healthy people (27). Vitamin E (α -tocopherol) was dissolved in methanol and subsequently diluted with phosphate buffer to obtain the selected concentrations; the final concentration of methanol was 0.5%. ASF and vitamin E were incubated with LDL at 37°C for 30 min before initiation of oxidation, and ascorbic acid was dissolved in PBS and added to the reaction immediately before initiation of oxidation. The effect of ASF plus vitamin C or E on the resistance of LDL to oxidation was expressed as the lag time increase compared with the lag time of LDL without the addition of ASF or vitamin C or E.

Preparation of ASF for hamster gavage. ASF were extracted with acidic aqueous methanol as described above with the substitution of white vinegar for HCl. Methanol was removed using a Rotavapor 134 (Buchi, Brinkmann Instrument) and the resulting slurry used for the hamster gavage.

Animals. BioF1B strain Golden Syrian Hamsters ($n = 22$; Bio-Breeders) were used in the bioavailability experiments due to the similarity of their lipoprotein metabolism to that of humans (28). The 1-y-old hamsters, with a body weight of 136.0 \pm 1.2 g (mean \pm SE), were housed individually in cages with a 10:14 h light:dark cycle. To increase lipoprotein production for subsequent collection, hamsters consumed ad libitum a nonpurified diet (Harlan) enriched with 10 g coconut oil and 0.5 g cholesterol/100 g for 2 wk before the acute administration of the ASF slurry (29).

After overnight food deprivation, hamsters were randomly assigned on the basis of their body weight to 6 time point groups: 0 (baseline), 60, 90, 120, 180, and 300 min ($n = 4, 4, 4, 4, 4, 2$, respectively). Because Kühnau (30) estimated daily intake of flavonoids and other polyphenolics by a person weighing 70 kg at 14 mg/kg, we chose a test dose of 50 mg/kg body weight or 40 μ mol GAE/hamster because rodents consume 5–6 times more food-based energy per unit of body weight than humans (31). Thus, ASF with 40 μ mol GAE (6.8 mg) was delivered in 1.0 mL via stomach gavage to hamsters anesthetized with Aerrane™ (Baxter); 1.0 mL saline was administered to the baseline group. Blood from each hamster was collected from the orbital sinus via micro-hematocrit tubes coated with heparin and stored in microtubes containing ETDA. Plasma samples were collected after the whole blood was centrifuged at 1000 \times g for 15 min at 4°C. An aliquot of plasma was stored at –80°C for determination of flavonoid status with the remainder used immediately for analysis of LDL oxidation. Whole liver was removed, rinsed with saline, and snap-frozen in liquid nitrogen for additional flavonoid analysis. This study was approved by the Animal Care and Use Committee of the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University.

Analysis of plasma and liver flavonoids. ASF in hamster plasma and liver were assessed by the HPLC-ECD as described above. Briefly, 20 μ L vitamin C-EDTA (200 mg ascorbic acid plus 1 mg EDTA in 1.0 mL of 0.4 mol/L NaH₂PO₄, pH 3.6) and 20 μ L β -glucuronidase (98,000 kU/L β -glucuronidase and 2400 kU/L sulfatase) were added to 200 μ L plasma and the mixture was incubated at 37°C for 45 min. ASF were extracted with 500 μ L acetonitrile; 500 μ L of supernatant was removed following a vigorous vortex for 30 s and centrifugation at 14,000 \times g for 5 min, dried under purified nitrogen, and reconstituted in 200 μ L of the aqueous HPLC mobile phase. After centrifugation at 14,000 \times g for 5 min, 100 μ L of supernatant was injected into the HPLC.

Liver samples (0.5 g) frozen in liquid nitrogen were pulverized and extracted twice with 5 mL acetonitrile containing 0.01% BHT. The supernatant was removed after centrifugation at 14,000 \times g for 5 min, combined, dried under nitrogen, and reconstituted in 500 μ L of 1 mol/L sodium acetate buffer, pH 5.5. After incubation with 10 μ L β -glucuronidase (98,000 kU/L β -glucuronidase and 2400 kU/L sulfatase) at 37°C overnight, the mixture was processed in the same manner as plasma samples. Due to a lower volume of β -glucuronidase and different buffer system, deconjugation of flavonoid metabolites took longer in liver than in plasma samples. Spiked, purified stan-

dards, rather than an internal standard, were added at the beginning of sample processing to construct standard curves and account for extraction losses and quantify concentrations (2). The recovery rates of spiked standards ranged from 75 to 80%.

Ex vivo antioxidant capacity of ASF. The ex vivo resistance of hamster LDL to Cu^{2+} -induced oxidation was performed as described above for the in vitro experiments to test antioxidant activity of flavonoids in ASF-gavaged hamsters. Because hamster LDL obtained through ultracentrifugation was lower than human LDL (determined by protein content), 91 nmol/L LDL was oxidized by 5 $\mu\text{mol/L}$ CuSO_4 in a total volume of 1.0 mL phosphate buffer (pH 7.4). Based on the synergistic results obtained with ASF plus vitamin E in the in vitro experiments, vitamin E was added in vitro to hamster LDL to a final concentration of 5.5 $\mu\text{mol/L}$ before initiation of oxidation.

Statistics. All results are reported as means \pm SE. The Tukey-Kramer honestly significant difference test was used in experiments on ASF in hamster plasma, and in vitro hamster and human LDL oxidation after significant differences were obtained by one-way ANOVA. The equal variance assumption was assessed by Hartley's test (32) and data, including epicatechin and isorhamnetin in plasma and catechin, epicatechin, quercetin, and kaempferol in liver, were square root-transformed before ANOVA analysis (2). The difference in lag time of ex vivo hamster LDL oxidation between baseline and ASF administration was determined by a simple *t* test. A paired *t* test was performed to determine the significance of the synergy between ASF and vitamins C or E in the in vitro human LDL oxidation by comparing the observed lag time during co-incubation with the expected (calculated) sums of values observed for ASF and vitamin C or E treatment alone. Data from all time points was included in correlation tests. Differences with $P \leq 0.05$ were considered significant. The JMP IN 4 statistical software package (SAS Institute) was used to perform all statistical analyses.

RESULTS

The total phenolic content in the almond skins as determined by the Folin-Ciocalteu method was 8.2 $\mu\text{mol GAE/g}$. HPLC-ECD chromatography resolved ~ 30 peaks with detectable redox potential, indicating the presence of numerous potential antioxidant compounds in almond skin (Fig. 1). Five aglycone flavonoids were identified and quantified as follows

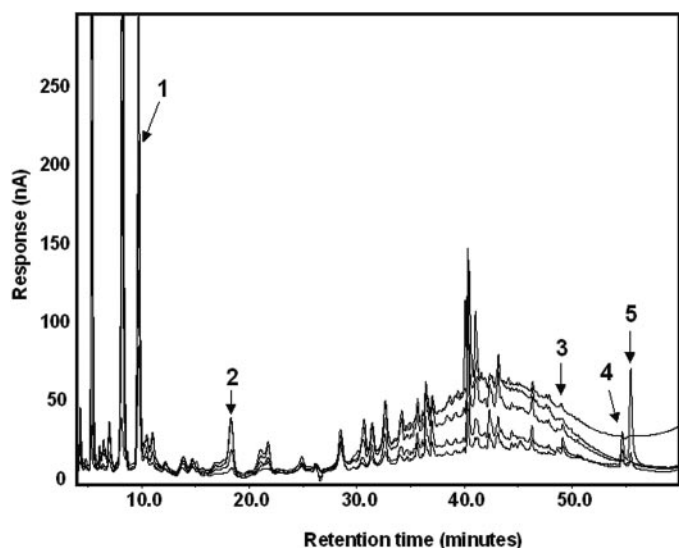


FIGURE 1 HPLC-ECD profile of ASF. The unit of the ordinate is nanoamp (nA). Each trace reflects the electrochemical response of a specific applied potential (as mV) in the ECD. Labeled peaks are: (1) catechin, (2) epicatechin, (3) quercetin, (4) kaempferol, (5) isorhamnetin.

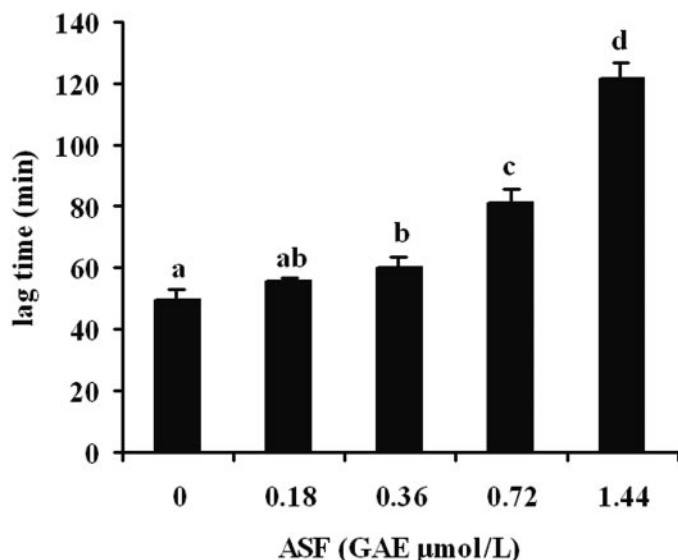


FIGURE 2 Effect of ASF on lag time to Cu^{2+} -induced oxidation of human LDL in vitro. Lag time of control (no added ASF) = 49.3 ± 3.7 min. Values are means \pm SE, $n = 3$. Means without a common letter differ, $P \leq 0.0001$.

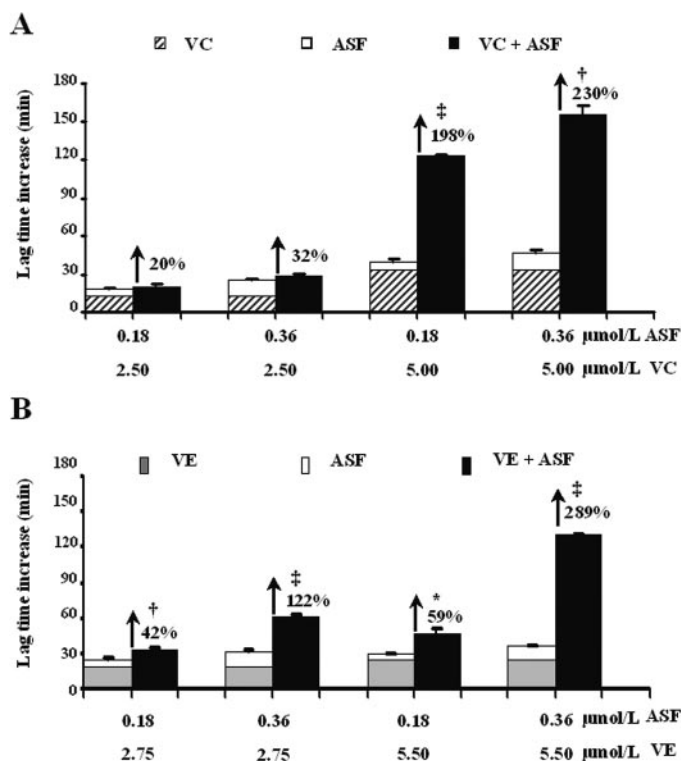
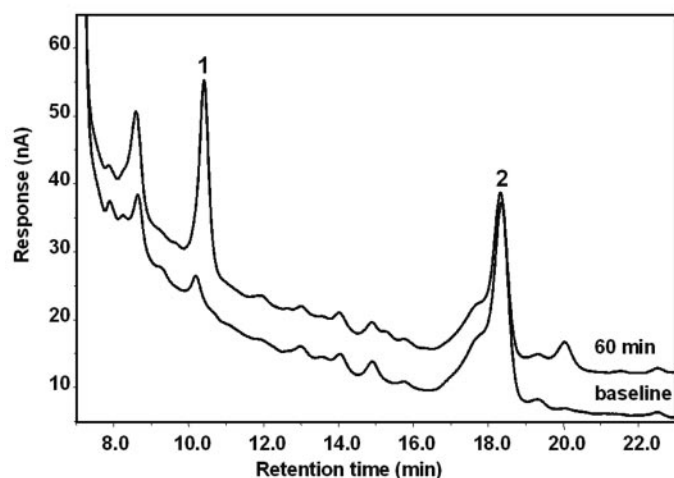


FIGURE 3 The synergistic effect of ASF and vitamin C or E (VC or VE) on the lag time of human LDL oxidation in vitro. LDL (182 $\mu\text{mol/L}$) was oxidized by 10 $\mu\text{mol/L}$ Cu^{2+} with the addition of ASF, VC or VE, or ASF + VC or VE. Values are means \pm SE, $n = 3$. Lag time of control (no added ASF, VC, or VE) = (A) 45.5 ± 0.7 min and (B) 47.7 ± 1.5 min. Bars are stacked to illustrate the calculated additive effect of the 2 treatments. The percentage value above the solid bar indicates the observed synergy greater than the calculated sums of the individual treatments. Symbols indicate different from the calculated sums: * $P \leq 0.05$, † $P \leq 0.01$, ‡ $P \leq 0.005$.

A. 210 nA trace



B. 70 nA trace

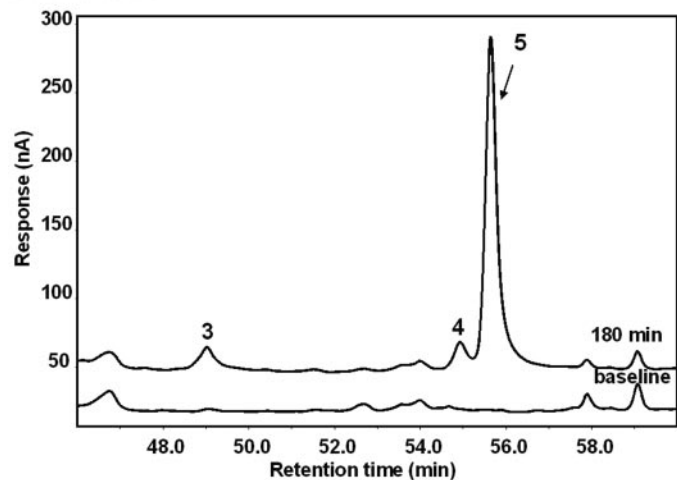


FIGURE 4 HPLC-ECD chromatographs of hamster plasma samples obtained at 60 min (A) and 180 min (B) after administration of 40 μmol GAE ASF and immediately after gavage with saline (baseline). (A) 210 mV ECD trace. (B) 70 mV ECD trace. Labeled peaks are: (1) catechin, (2) epicatechin, (3) quercetin, (4) kaempferol, (5) isorhamnetin.

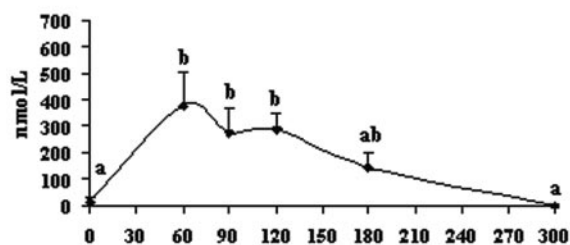
(nmol/g): catechin (186.9), epicatechin (77.5), isorhamnetin (20.8), quercetin (6.0), and kaempferol (3.9). These 5 flavonoids represent 3.6% of the total phenolic content of almond skins.

In vitro, ASF increased the resistance of human LDL against Cu^{2+} -induced oxidation in a dose-dependent manner ($P \leq 0.0001$) (Fig. 2). Ascorbic acid at 2.5 and 5.0 $\mu\text{mol/L}$ increased lag time by 12.9 ± 1.6 and 34 ± 2.7 min, respectively; vitamin E at 2.75 and 5.5 $\mu\text{mol/L}$ increased lag time by 19.1 ± 1.4 and 24.3 ± 0.7 min, respectively. A 1-fold synergy (i.e., an observed value twice the calculated additive value) was observed with 0.18 and 0.36 $\mu\text{mol/L}$ of ASF in combination with 5.0 $\mu\text{mol/L}$ ascorbic acid ($P \leq 0.01$); no synergy was observed with the 2.5 $\mu\text{mol/L}$ dose of vitamin C (Fig. 3A). The lag time was 289% longer than the expected additive value with the combination of 0.36 $\mu\text{mol/L}$ ASF and 5.5 $\mu\text{mol/L}$ vitamin E ($P \leq 0.001$) (Fig. 3B).

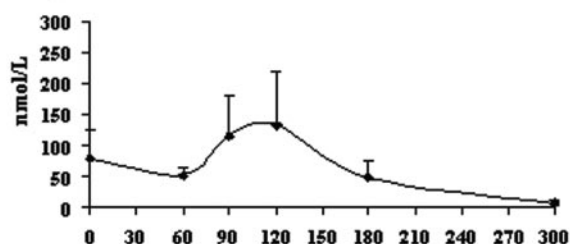
All 5 identified flavonoids from almond skins were bioavailable in hamster plasma (Fig. 4). Based on their pharmacokinetic profile in the plasma, the maximum concentrations (C_{max}) of catechin, epicatechin, kaempferol, quercetin, and

isorhamnetin were 376, 133, 72, 222, and 761 nmol/L, respectively (Fig. 5). The time to reach the C_{max} (T_{max}) was 60 and 120 min for catechin and epicatechin, respectively, and 180

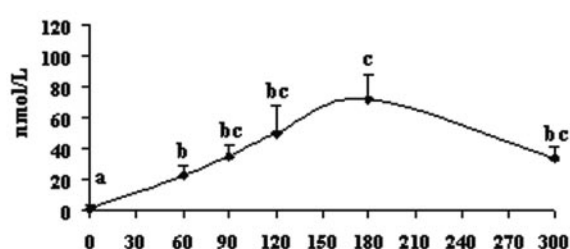
A. Catechin



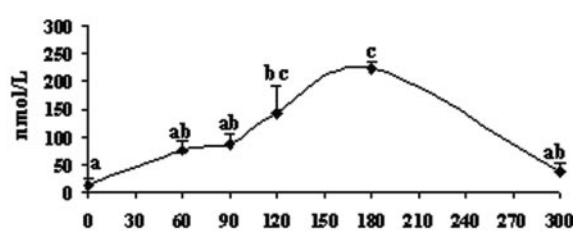
B. Epicatechin



C. Quercetin



D. Kaempferol



E. Isorhamnetin

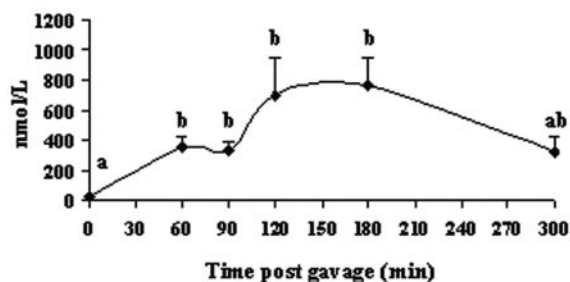


FIGURE 5 Time course of catechin, epicatechin, quercetin, kaempferol, and isorhamnetin in the plasma of hamsters administered 40 μmol GAE ASF. Values are means \pm SE, $n = 4$. Means in each panel without a common letter differ, $P \leq 0.05$.

TABLE 1

Correlation coefficients among flavonoids in the plasma and liver of hamsters administered 40 μmol GAE ASF¹

	Kaempferol		Isorhamnetin		Epicatechin	
	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value	<i>r</i>	<i>P</i> -value
Plasma						
Quercetin	0.78	≤ 0.0001	0.66	≤ 0.0001	—	—
Kaempferol	—	—	0.73	≤ 0.0001	—	—
Catechin	—	—	—	—	0.19	0.2
Liver						
Quercetin	0.62	≤ 0.0001	0.08	0.2	—	—
Kaempferol	—	—	0.34	≤ 0.005	—	—
Catechin	—	—	—	—	0.31	≤ 0.01
Plasma and liver						
Isorhamnetin	—	—	0.54	≤ 0.0001	—	—

¹ Based on one observation in 22 hamsters.

min for the 3 flavonols. At 300 min, the plasma concentrations of these flavonoids were less than half the C_{max} and their concentrations (with the exception of quercetin and isorhamnetin) did not differ from the baseline value. Correlation coefficients between the 3 plasma flavonols ranged from 0.66 to 0.78 although no relation was noted between the catechins (Table 1).

Pharmacokinetic patterns of the flavonoids in liver, except for isorhamnetin, differed from those in the plasma (Fig. 6). Concentrations of catechin, quercetin, and kaempferol in liver were not affected by acute ASF gavage. The epicatechin concentration was significantly elevated at 300 min. The C_{max} of liver quercetin and kaempferol at 73 and 130 nmol/g did not differ from baseline and their T_{max} was 90 min, in contrast to the 180 min observed in plasma. The C_{max} of isorhamnetin in the liver was 698 nmol/g at 180 min after administration; isorhamnetin remained higher than baseline levels after 300 min ($P \leq 0.05$). Concentrations of quercetin and kaempferol were correlated (Table 1). Isorhamnetin pharmacokinetics paralleled those of kaempferol, but not quercetin. Isorhamnetin showed a similar pharmacokinetic pattern in both plasma and liver.

The lag time of ex vivo hamster Cu^{2+} -induced LDL oxidation was 30.8 ± 0.8 , 36.3 ± 1.8 , 34.5 ± 1.4 , 38.1 ± 1.7 , and 38.9 ± 3.9 min at the baseline, 60, 90, 120, and 180 min time points, respectively. Absorbed ASF appeared to induce a small increase of 18.0 and 24.0% in the ex vivo resistance of LDL to oxidation at 60 and 120 min ($P = 0.028$ and 0.008 , respectively). When 5.5 $\mu\text{mol/L}$ vitamin E was added in vitro to the reaction, the lag time of LDL oxidation was 119.9 ± 2.4 , 182.9 ± 13.5 , 152.3 ± 17.0 , 160.1 ± 8.0 , and 160.0 ± 6.7 min, respectively. However, only LDL collected at 60 min showed a synergistic increase with a 52.5% longer lag time than that collected at baseline ($P \leq 0.05$).

DISCUSSION

The putative health benefits of flavonoids have been attributed in part to their antioxidant activity (33,34). However, most of the supportive evidence has been based only on in vitro experiments or in vivo feeding studies with a single flavonoid aglycone or glycoside (26). In contrast, we examined the behavior of a complex array of polyphenolics in almond

skins while measuring several specific flavonoid constituents. Almond skins were previously reported to contain 3 classes of flavonoids, i.e., catechins, flavanones, and flavonols (10,11). Using HPLC-ECD, we quantified 5 aglycones (catechin, epicatechin, quercetin, kaempferol, and isorhamnetin) and measured ~25 other redox compounds (Fig. 1), likely flavonoids or related polyphenolics, such as glycosides of quercetin, kaempferol, isorhamnetin, and naringenin as well as protocatechuic acid, vanillic acid, and *p*-hydroxybenzoic acid (10,11).

The complex of ASF effectively increased the resistance of human LDL to oxidation in vitro in a dose-dependent fashion within physiologically relevant concentrations (Fig. 2) (26). These results are consistent with several reports of the antioxidant activity of flavonoids such as catechin and quercetin with concentrations administered ranging from 0.25 to 10 $\mu\text{mol/L}$ (35–37). In contrast, Filipe et al. (38) reported a prooxidant activity of low concentrations of quercetin (<2 $\mu\text{mol/L}$) indicated by increased malondialdehyde formation during Cu^{2+} -induced LDL oxidation. If a prooxidant action of quercetin occurred in our study, it may have been masked by other constituents of almond skin. It is possible that the potential prooxidant actions of single flavonoids do not occur in natural mixtures of plant polyphenols in which opportunities for recycling oxidized compounds may exist, such as the regeneration of oxidized malvidin 3-glucoside by catechin (39). Such interactions (14,40,41) may also partly account for the synergy between the ASF and vitamins C and E observed here (Fig. 3). Similar synergies were observed between genistein and ascorbic acid (40), oat phenolics and ascorbic acid (2), and quercetin and urate (42). In addition to recycling mechanisms, Hwang et al. (40) suggested that polyphenolics may stabilize the LDL particle structure via an interaction with the apoprotein-B domain. Further, vitamin C may contribute to the synergy by inhibiting the decomposition of lipid peroxides and/or preventing Cu^{2+} from binding to LDL (40).

Extrapolations about flavonoids from in vitro results are limited because of their relatively poor general bioavailability and extensive biotransformation in vivo (14). The bioavailabilities of selected single catechin and flavonol compounds were reported (14,16,19,43,44), but little information is available regarding the concurrent absorption profile of mixtures. Similar to our finding in hamsters, others found a faster plasma T_{max} for catechin than quercetin when single compounds were fed to rats (16,44), suggesting that catechin is rapidly absorbed

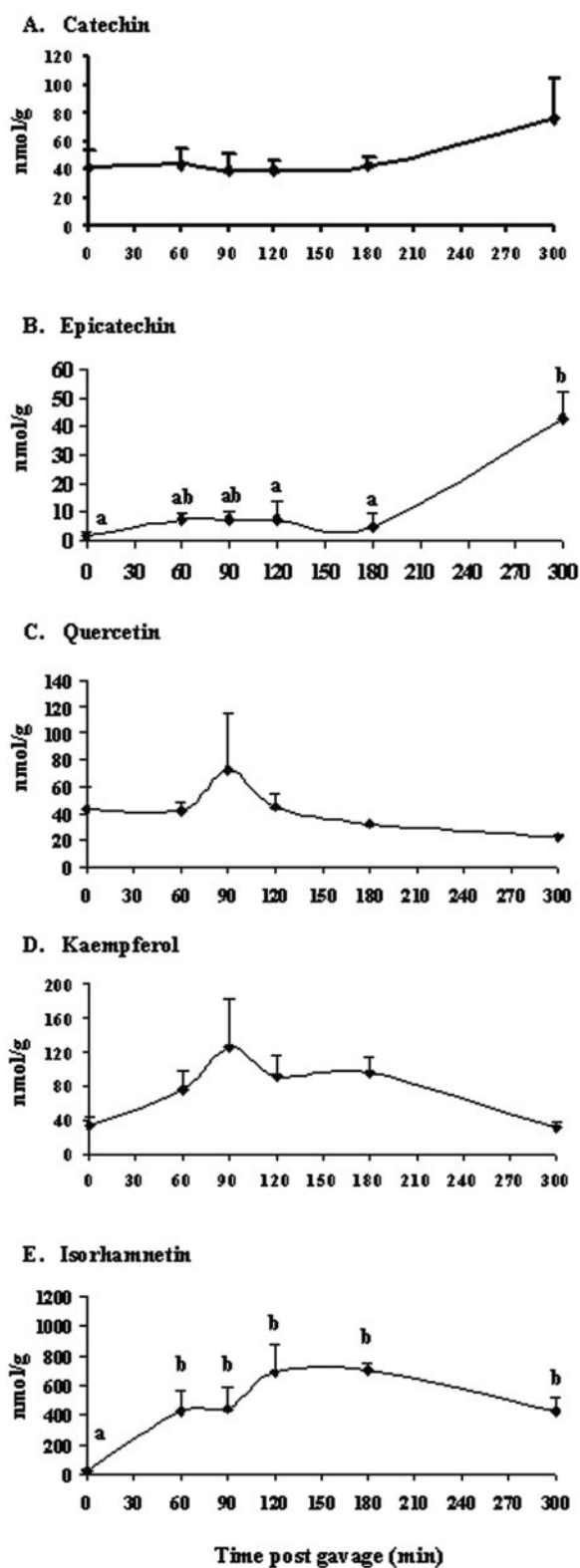


FIGURE 6 Catechin, epicatechin, quercetin, kaempferol, and isorhamnetin in the liver of hamsters administered 40 μ mol GAE ASF. Values are means \pm SE, $n = 4$. Means in each panel without a common letter differ, $P \leq 0.05$.

in the upper gastrointestinal tract and cleared from plasma within several hours. In contrast to a T_{\max} of 120 min for epicatechin in hamsters (Fig. 5), Baba et al. (44) reported a

60-min value in rats. However, caution is warranted when interpreting these values due to pharmacokinetic differences between species as well to other confounding variables such as differing ingredients in the diet fed and potential competition between polyphenolics for absorption (44). In addition, glycoside moieties appear to have a substantial influence on pharmacokinetics, e.g., quercetin-4'-glycoside and rutin (quercetin rutinoside) have T_{\max} of 0.5 and 7 h, respectively, in humans (26). We observed a T_{\max} at 180 min for the 3 almond skin flavonol aglycones after deconjugation by glucuronidase and sulfatase in hamsters (Fig. 5), suggesting the potential contribution to this value from unidentified flavonol glycosides in almond skins, such as quercetin, isorhamnetin, and kaempferol glycosides (10,11). Similar to the relations suggested by Manach et al. (13) for humans, we found a shorter half-life in plasma for the catechins compared with the flavonols in hamsters. The half-life of flavonols in the hamsters (5 h) was shorter than that in rats (11–28 h), although such differences could be accounted for by differences in their glycosides (12).

The plasma pharmacokinetics of the almond flavonols were similar with high correlation coefficients (Table 1). Although isorhamnetin is a metabolite of quercetin (45), the association between their plasma concentrations was low, possibly due to direct contribution of isorhamnetin from almond skins. No association was noted between the plasma concentrations of the 2 catechins.

Although the number of reports is limited, flavonoids were detected in mouse and rat tissues with concentrations from 30 to 3000 ng aglycone equivalents/g (13). In hamsters, after ASF gavage, the 2 catechins and 3 flavonols were detected in liver although, with the exception of isorhamnetin, their pharmacokinetic patterns were quite different from those in plasma (Fig. 6). Noticeable amounts of catechin, quercetin, and kaempferol in hamster liver may be attributed to their presence in the basal nonpurified diet fed to hamsters. It is not clear why ASF administration did not increase catechin, epicatechin, kaempferol, or quercetin in the liver, although it is possible that these flavonoids were quickly redistributed to other tissues. Manach et al. (14) reported that >90% of catechin and quercetin in rat liver was methylated. Similar to their relation in plasma (Table 1), quercetin and kaempferol concentrations in the liver were correlated. Although isorhamnetin status in plasma was correlated with its concentration in liver, it was not associated with other hepatic flavonols. The relatively high level of isorhamnetin in liver may reflect a slow rate of clearance compared with other flavonoids or a gradual contribution from methylation of aglycone and glycone quercetin. The increase in hepatic catechins, especially epicatechin, at 300 min may reflect an unusually slow distribution to this tissue or result from the small sample size employed at this one time point.

Consistent with the antioxidant capacity of almond skins using the LDL oxidation assay, Halvorsen et al. (46) demonstrated the antioxidant potency of whole almonds in vitro with the ferric reducing antioxidant power assay, although the vitamin E content of the nut may have contributed substantially to this result. In vivo, a very modest antioxidant effect of ASF was suggested by the small increase in the resistance of LDL in hamsters treated with the almond skin extract compared with saline. Although a higher dose may have produced a greater effect, the weak direct response elicited in vivo may be a result of a lower potency of glucuronidated, sulfated, and/or methylated metabolites of the ASF. Indeed, Moon et

al. (47) and Cren-Olive et al. (48) both reported that conjugated derivatives of quercetin and catechin provided less antioxidant protection than their parent compounds against LDL oxidation *in vitro*. Further, many flavonoid metabolites appear bound to plasma proteins *in vivo*; thus, they may be less available to interact with LDL (16,20). Nonetheless, despite a weak effect of ASF on the resistance of LDL to oxidation *in vivo*, its antioxidant action was strengthened by their synergy with the vitamin E added *in vitro* in this experiment. A similar synergistic relation was observed between oat phenolics and vitamin C in the same hamster model (2). Interestingly, the ASF-vitamin E synergy appeared in LDL collected at 60 min, the T_{max} for catechin, rather than at 180 min, when the greatest concentration of flavonols was obtained. It may be attributed to the more potent antioxidant activity of catechin against copper-induced LDL oxidation than quercetin (49).

In summary, ASF possess antioxidant capacity and interact with vitamins E and C in a synergistic manner to protect LDL against oxidation *in vitro*. Of the 5 identified flavonoids in the ASF, all appeared in plasma and liver after oral administration. These flavonoids and/or related compounds in the ASF slightly enhanced the resistance of hamster LDL against *in vivo* Cu^{2+} -induced oxidation, and their antioxidant capacity was amplified with the *in vitro* addition of vitamin E. Because almonds represent one of the richest dietary sources of vitamin E, further research is warranted examining the relations between the natural ingredients in this whole food and their potential association with health benefits, such as a reduction in the risk of cardiovascular disease.

ACKNOWLEDGMENTS

We thank Donald Smith for his contributions to establishing the hamster model and Ting Li and Jennifer O'Leary for their excellent technical assistance in the laboratory.

LITERATURE CITED

- Lou, H., Yuan, H., Ma, B., Ren, D., Ji, M. & Oka, S. (2004) Polyphenols from peanut skins and their free radical-scavenging effects. *Phytochemistry* 65: 2391–2399.
- Chen, C. Y., Milbury, P. E., Kwak, H. K., Collins, F. W., Samuel, P. & Blumberg, J. B. (2004) Avenanthramides and phenolic acids from oats are bioavailable and act synergistically with vitamin C to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* 134: 1459–1466.
- Nijveldt, R. J., van Nood, E., van Hoorn, D.E.C., Boelens, P. G., van Norren, K. & van Leeuwen, P. A. (2001) Flavonoids: a review of probable mechanisms of action and potential applications. *Am. J. Clin. Nutr.* 74: 418–425.
- Knekt, P., Kumpulainen, J., Järvinen, R., Rissanen, H., Heliövaara, M., Reunanen, R., Hakulinen, T. & Aromaa, A. (2002) Flavonoid intake and risk of chronic diseases. *Am. J. Clin. Nutr.* 76: 560–568.
- Huxley, R. R. & Neil, H.A.W. (2003) The relation between dietary flavonol intake and coronary heart disease mortality: a meta-analysis of prospective cohort studies. *Eur. J. Clin. Nutr.* 57: 904–908.
- U.S. Food and Drug Administration (2003) FDA Authorizes Heart Disease Health Claim for Nuts. Docket No. 02P-0505.
- Jenkins, D. J., Kendall, C. W., Marchie, A., Parker, T. L., Connelly, P. W., Qian, W., Haight, J. S., Faulkner, D., Vidgen, E., et al. (2002) Dose response of almonds on coronary heart disease risk factors: blood lipids, oxidized low-density lipoproteins, lipoprotein(a), homocysteine, and pulmonary nitric oxide: a randomized, controlled, crossover trial. *Circulation* 106: 1327–1332.
- Fruit and tree nuts outlook, FTS2004 [Online]. Economic Research Service of US Department of Agriculture. <http://www.ers.usda.gov/publications/fts/> [accessed Mar. 9, 2005].
- Nuts, almonds [Online]. USDA National Nutrient Database for Standard Reference, Release 17 (2004) Agricultural Research Service, U.S. Department of Agriculture. <http://www.nal.usda.gov/fnic/foodcomp/search/index.html> [accessed Mar. 9, 2005].
- Sang, S., Lapsley, K., Jeong, W. S., Lachance, P. A., Ho, C. T. & Rosen, R. T. (2002) Antioxidative phenolic compounds isolated from almond skins (*Prunus amygdalus* Batsch). *J. Agric. Food Chem.* 50: 2459–2463.
- Frison-Norrie, S. & Sporns, P. (2002) Identification and quantification of flavonol glycosides in almond seedcoats using MALDI-TOF MS. *J. Agric. Food Chem.* 50: 2782–2787.
- Manach, C., Morand, C., Demigné, C., Texier, O., Regerat, F. & Rémésy, C. (1997) Bioavailability of rutin and quercetin in rats. *FEBS Lett.* 409: 12–16.
- Manach, C., Scalbert, A., Morand, C., Rémésy, C. & Jimenez, L. (2004) Polyphenolics: food sources and bioavailability. *Am. J. Clin. Nutr.* 79: 727–747.
- Manach, C., Texier, O., Morand, C., Crespy, F., Regerat, F., Demigné, C. & Rémésy, C. (1999) Comparison of the bioavailability of quercetin and catechin in rats. *Free Radic. Biol. Med.* 27: 1259–1266.
- Crozier, A., Lean, M.E.J., McDonald, M. S. & Black, C. (1997) Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce and celery. *J. Agric. Food Chem.* 45: 590–595.
- Manach, C., Morand, C., Texier, O., Favier, M. L., Agullo, G., Demigné, C., Regerat, F. & Rémésy, C. (1995) Quercetin metabolites in plasma of rats fed diets containing rutin or quercetin. *J. Nutr.* 125: 1911–1922.
- Hollman, P. C., de Vries, J. H., van Leeuwen, S. D., Mengelers, M. J. & Katan, M. B. (1995) Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* 62: 1276–1282.
- Hollman, P. C., vd Gaag, M., Mengelers, M. J., van Trijp, J. M., de Vries, J. H. & Katan, M. B. (1996) Absorption and disposition kinetics of the dietary antioxidant quercetin in man. *Free Radic. Biol. Med.* 21: 703–707.
- Lesser, S., Cermak, R. & Wolfram, S. (2004) Bioavailability of quercetin in pigs is influenced by the dietary fat content. *J. Nutr.* 134: 1508–1511.
- van het Hof, K. H., Wiseman, S. A., Yang, C. S. & Tijburg, L. B. (1999) Plasma and lipoprotein levels of tea catechins following repeated tea consumption. *Proc. Soc. Exp. Biol. Med.* 220: 203–209.
- Milbury, P. E. (2001) Analysis of complex mixtures of flavonoids and polyphenols by high performance liquid chromatography electrochemical detection methods. *Methods Enzymol.* 335: 15–26.
- Singleton, V. L., Orthofer, R. & Lamuela-Raventos, R. M. (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* 299: 152–178.
- Esterbauer, H., Striegl, G., Puhl, H. & Rotheneder, M. (1989) Continuous monitoring of *in vitro* oxidation of human low-density lipoprotein. *Free Radic. Res. Commun.* 6: 67–75.
- Chung, B. H., Wilkinson, T., Geer, J. C. & Segrest, J. P. (1980) Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* 21: 284–291.
- Esterbauer, H., Gebicki, J., Puhl, H. & Jurgens, G. (1992) The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.* 13: 341–390.
- Manach, C., Williamson, G., Morand, C., Scalbert, A. & Rémésy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* 81: 230S–242S.
- Schunemann, H. J., Grant, B. J., Freudenheim, J. L., Muti, P., Browne, R. W., Drake, J. A., Klocke, R. A. & Trevisan, M. (2001) The relation of serum levels of antioxidant vitamins C and E, retinol and carotenoids with pulmonary function in the general population. *Am. J. Respir. Crit. Care Med.* 163: 1246–1255.
- El-Swefy, S., Schaefer, E. J., Semanm, L. J., van Dongen, D., Sevanian, A., Smith, D. E., Ordovas, J. M., El-Sweidy, M. & Meydani, M. (2000) The effect of vitamin E, probucol, and lovastatin on oxidative status and aortic fatty lesions in hyperlipidemic-diabetic hamsters. *Atherosclerosis* 149: 277–286.
- Dorfman, S. E., Smith, D. E., Osgood, D. P. & Lichtenstein, A. H. (2003) Study of diet-induced changes in lipoprotein metabolism in two strains of Golden-Syrian hamsters. *J. Nutr.* 133: 4183–4188.
- Kühnau, J. (1976) The flavonoids. A class of semi-essential food components: their role in human nutrition. *World Rev. Nutr. Diet.* 24: 117–191.
- Luceri, C., Caderni, G., Sanna, A. & Dolara, P. (2002) Red wine and black tea polyphenolics modulate the expression of cyclooxygenase-2, inducible nitric oxide synthase and glutathione-related enzymes in azoxymethane-induced F344 rat colon tumors. *J. Nutr.* 132: 1376–1379.
- Ott, R. L. (1993) *An Introduction to Statistical Methods and Data Analysis*. Wadsworth Publishing Co., Belmont, CA.
- Hollman, P. C., Hertog, M. G. & Katan, M. B. (1996) Role of dietary flavonoids in protection against cancer and coronary heart disease. *Biochem. Soc. Trans.* 24: 785–789.
- Hollman, P. C., Feskens, E. J. & Katan, M. B. (1999) Tea flavonols in cardiovascular disease and cancer epidemiology. *Proc. Soc. Exp. Biol. Med.* 220: 198–202.
- Janisch, K. M., Williamson, G., Needs, P. & Plumb, G. W. (2004) Properties of quercetin conjugates: modulation of LDL oxidation and binding to human serum albumin. *Free Radic. Res.* 38: 877–884.
- Brown, J. E., Khodr, H., Hider, R. C. & Rice-Evans, C. A. (1998) Structural dependence of flavonoid interactions with Cu^{2+} ions: implications for their antioxidant properties. *Biochem. J.* 330: 1173–1178.
- Zhu, Q. Y., Huang, Y., Tsang, D. & Chen, Z. Y. (1999) Regeneration of alpha-tocopherol in human low-density lipoprotein by green tea catechin. *J. Agric. Food Chem.* 47: 2020–2025.
- Filipe, P., Haigle, J., Silva, J. N., Freitas, J., Fernandes, A., Maziere, J. C., Maziere, C., Santus, R. & Morliere, P. (2004) Anti- and pro-oxidant effects of quercetin in copper-induced low density lipoprotein oxidation. Quercetin as an effective antioxidant against pro-oxidant effects of urate. *Eur. J. Biochem.* 271: 1991–1999.

39. Rossetto, M., Vanzani, P., Mattivi, F., Lunelli, M., Scarpa, M. & Rigo, A. (2002) Synergistic antioxidant effect of catechin and malvidin 3-glucoside on free radical-initiated peroxidation of linoleic acid in micelles. *Arch. Biochem. Biophys.* 408: 239–245.
40. Hwang, J., Sevanian, A., Hodis, H. N. & Ursini, F. (2000) Synergistic inhibition of LDL oxidation by phytoestrogens and ascorbic acid. *Free Radic. Biol. Med.* 29: 79–89.
41. Laranjinha, J., Vieira, O., Madeira, V. & Almeida, L. (1995) Two related phenolic antioxidants with opposite effects on vitamin E content in low density lipoproteins oxidized by ferrylmyoglobin: consumption vs regeneration. *Arch. Biochem. Biophys.* 323: 373–381.
42. Filipe, P., Lanca, V., Silva, J. N., Morliere, P., Santus, R. & Fernandes, A. (2001) Flavonoids and urate antioxidant interplay in plasma oxidative stress. *Mol. Cell. Biochem.* 221: 79–87.
43. Shimoi, K., Yoshizumi, K., Kido, T., Usui, Y. & Yumoto, T. (2003) Absorption and urinary excretion of quercetin, rutin, and alphaG-rutin, a water soluble flavonoid, in rats. *J. Agric. Food Chem.* 51: 2785–2789.
44. Baba, S., Osakabe, N., Natsume, M., Muto, Y., Takizawa, T. & Terao, J. (2001) In vivo comparison of the bioavailability of (+)-catechin, (-)-epicatechin and their mixture in orally administered rats. *J. Nutr.* 131: 2885–2891.
45. Morand, C., Crespy, V., Manach, C., Besson, C., Demigné, C. & Rémésy, C. (1998) Plasma metabolites of quercetin and their antioxidant properties. *Am. J. Physiol.* 275: R212–R219.
46. Halvorsen, B. L., Holte, K., Myhrstad, M.C.W., Barikmo, I., Hvattum, E., Remberg, S. F., Wold, A.-B., Haffner, K., Baugerod, H., et al. (2002) A systematic screening of total antioxidants in dietary plants. *J. Nutr.* 132: 461–471.
47. Moon, J. H., Tsushida, T., Nakahara, K. & Terao, J. (2001) Identification of quercetin 3-O-beta-D-glucuronide as an antioxidative metabolite in rat plasma after oral administration of quercetin. *Free Radic. Biol. Med.* 30: 1274–1285.
48. Cren-Olive, C., Teissier, E., Duriez, P. & Rolando, C. (2003) Effect of catechin O-methylated metabolites and analogues on human LDL oxidation. *Free Radic. Biol. Med.* 34: 850–855.
49. Vaya, J., Mahmood, S., Goldblum, A., Aviram, M., Volkova, N., Shaalan, A., Musa, R. & Tamir, S. (2003) Inhibition of LDL oxidation by flavonoids in relation to their structure and calculated enthalpy. *Phytochemistry* 62: 89–99.