# Antioxidant Capacity of Oat (*Avena sativa* L.) Extracts. 1. Inhibition of Low-Density Lipoprotein Oxidation and Oxygen Radical Absorbance Capacity

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Milled oat groat pearlings, trichomes, flour, and bran were extracted with methanol and the fractions tested in vitro for antioxidant capacity against low-density lipoprotein (LDL) oxidation and R-phycoerythrin protein oxidation in the oxygen radical absorbance capacity (ORAC) assay. The oxidative reactions were generated by 2,2′-azobis(2-amidinopropane) HCl (AAPH) or $Cu^{2+}$ in the LDL assay and by AAPH or $Cu^{2+} + H_2O_2$ in the ORAC assay and calibrated against a Trolox standard to calculate Trolox equivalents (1 Trolox equivalent = 1 TE = activity of 1 μmol of Trolox). The antioxidant capacity of the oat fractions was generally consistent with a potency rank of pearlings (2.89−8.58 TE/g) > flour (1.00−3.54 TE/g) > trichome (1.74 TE/g) = bran (1.02−1.62 TE/g) in both LDL and ORAC assays regardless of the free radical generator employed. A portion of the oat antioxidant constituents may be heat labile as the greatest activity was found among non-steam-treated pearlings. The contribution of oat tocols from the fractions accounted for <5% of the measured antioxidant capacity. AAPH-initiated oxidation of LDL was inhibited by the oat fractions in a dose-dependent manner, although complete suppression was not achieved with the highest doses tested. In contrast, $Cu^{2+}$-initiated oxidation of LDL stimulated peroxide formation with low oat concentrations but completely inhibited oxidation with higher doses. Thus, oats possess antioxidant capacity most of which is likely derived from polar phenolic compounds in the aleurone.

**Keywords:** *Avena sativa; oat; antioxidant capacity; phenolics; radical scavengers; oxygen radical absorbance capacity (ORAC); low-density lipoprotein (LDL) oxidation; aleurone*

## INTRODUCTION

Several classes of compounds with antioxidant activity have been identified in oat (*Avena sativa* L.) including vitamin E tocols, flavonoids, and non-flavonoid phenolic acids (Collins, 1986; Collins et al., 1989, 1991; Peterson and Qureshi, 1993; Shahidi and Naczk, 1995). Additional investigations have further characterized the antioxidant capacity of oat fractions, including hulls, bran, and endosperm (Duve and White, 1991; Dimberg et al., 1993; Xing and White, 1997). These compounds appear to function in growth regulation and disease resistance processes in the plant, in part, through their antioxidant capacity. These oat components have been added to food and beverage products to preserve quality and are associated with flavor, color, and/or aroma (Dimberg et al., 1993). Antioxidant phytochemicals may also play an important role in human health via scavenging reactive oxygen and nitrogen species (Thompson, 1994; Slavin et al., 1997; Bravo, 1998) and modulating several enzyme systems, such as lipoxygenases (de la Puerta et al., 1999). However, the localization of these compounds within the oat and their antioxidant potential have not been well characterized in vitro or in vivo.

Several approaches have been developed to assess the antioxidant potential of different compounds in vitro (Bors et al., 1984; Burton and Ingold, 1984; Stocker et al., 1987; Esterbauer et al., 1992). These models for screening antioxidant activity typically employ the test compound in a reaction between a reactive oxygen species (ROS), such as hydroxyl (•OH) or peroxyl (ROO•) radicals, and a target molecule, such as low-density lipoprotein (LDL) or R-phycoerythrin (R-PE), which can be monitored for oxidative changes. We compare here the antioxidant capacities of methanolic extracts from different oat fractions using two established screening assays: (1) the inhibition of the oxidative modification of LDL and (2) the oxygen radical absorbance capacity (ORAC).

## MATERIALS AND METHODS

**Oat Fractions.** Ten milling products were prepared from oat groats by The Quaker Oats Co. as listed in Table 1. These products were obtained as aliquots from a blend of four to eight varieties of oats grown in Saskatchewan and Manitoba in 1997. A Satake laboratory pearler was employed for abrasion, and sieving was accomplished with a Retsch mill and a 0.5 mm screen. Heat-treated oat groats were steamed and kilned.

**Solvent Extraction of Oat Fractions.** Batches (∼75−215 g) of each milled product were stirred with 500 mL of methanol

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10.1021/jf990529j CCC: $18.00 © 1999 American Chemical Society
Published on Web 11/17/1999

**Table 1. Oat Groat Fractions**

| | |
|---|---|
| F1 | heat-treated oat groats were abraded to yield pearlings equal to 1.6% of the starting material; this abrasion technique releases primarily the surface coat of the groat |
| F2 | like F1, but abraded to release pearlings equal to 3% of the starting material |
| F3 | like F1, but abraded to release pearlings equal to 7% of the starting material |
| F4 | non-heated-treated green oats were abraded to release pearlings equal to 5% of the starting material |
| F5 | trichomes: steam-treated oats were abraded to remove the outer (hairy) surface of the groat |
| F6 | heat-treated oats were ground in a Retsch mill to obtain oat flour |
| F7 | non-heat-treated oats were ground in a Retsch mill to obtain green oat flour |
| F8 | heat-treated oats were milled and air-classified to obtain the bran |
| F9 | heat-treated groats were milled and air-classified to concentrate the bran fraction |
| F10 | groats were steamed and conditioned to allow partial removal of the bran layer by abrasion to obtain aspirations from the flaking process (oat flaking filter fines) |

for 2 h in an extraction flask at ambient temperature. The sample was passed through Whatman No. 1 paper on a Büchner filter, and the filtrate was collected. The extraction flask was washed with 200 mL of methanol, and the solution was passed through the sample residue on the filter. The pooled filtrate from both steps was concentrated with a rotary evaporator to bring the volume to 80 mL. Extracts were stored at 40 °C in dark brown bottles and analyzed for antioxidant activity within 90 days of preparation. Following the preparation of the methanol extracts, the residual oat fractions were extracted with hexane to collect antioxidant compounds not isolated during the initial methanol extraction. Final data were normalized to the original weight of the oat sample, that is, oat weight per milliliter of solvent.

**LDL Oxidation Assay.** Blood was drawn from healthy adults into tubes with sodium ethylene glycol bis($\beta$-aminoethyl ether) *N,N,N′,N′*-tetraacetic acid (EDTA) anticoagulant. LDL were isolated by ultracentrifugation at 40 °C (Schumaker and Puppione, 1986) and dialyzed against Dulbecco's phosphate-buffered saline (PBS) to remove EDTA. The protein content was determined according to the bicinchonic acid method (Smith et al., 1985). Purified LDL was stored at 40 °C and used within 10 days of preparation. LDL were diluted in 400 $\mu$L of PBS, pH 7.4, to a final protein concentration of 100 $\mu$g/mL. LDL oxidation was induced by either 10 $\mu$M $Cu^{2+}$, a transition element, or 10 mM 2,2′-azobis(2−amidinopropane) HCl (AAPH; Wako, DeKalb, IL), a water-soluble diazo $ROO^{\bullet}$ initiator. Dilutions of the oat extracts were added to the LDL samples in 5 $\mu$L of methanol.

LDL samples were incubated under air at 37 °C for 60 min, and the reaction was terminated with 1 mM EDTA and 10 $\mu$M butylated hydroxytoluene. An internal standard (1 nmol of conjugated diene fatty acid ester) was added to the sample, which was then mixed with 500 $\mu$L of ethanol, and extracted with 2 mL of hexane according to Handelman et al. (1993). The hexane layer was collected, evaporated, and dissolved in 100 $\mu$L of 2-propanol for HPLC analysis of cholesterol linoleate hydroperoxide (chol-18:2-OOH).

Analysis of chol-18:2-OOH was performed on 50 $\mu$L aliquots with a Hewlett-Packard 1100 HPLC using a Rainin Microsorb C18 column (150 × 4.6 mm, 5 $\mu$M particle size; Woburn, MA) and diode array detection at 234 nm according to the method of Handelman et al. (1993) and Handelman (1999). The mobile phase was 50% 2-propanol, 40% acetonitrile, and 10% methanol with 0.1% ammonium acetate and run at a 1 mL/min flow rate. The internal standard was prepared by reaction of conjugated diene linoleic acid and a saturated fatty acid (C20:0). Chol-18:2−OOH was prepared via autoxidation of 1 mg of cholesterol linoleate/mL of hexane incubated at 37 °C for 48 h in a sealed vial under air atmosphere.

**ORAC Assay.** An automated ORAC assay was performed on a COBAS FARA II spectrofluorometric centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ) with fluorescence excitation at 540 nm, fluorescence emission at 565 nm, and cuvette temperature at 37 °C according to the method of Cao et al. (1993, 1995). Briefly, 16.7 nmol of R-PE (Sigma), a protein target of oxidant attack, and 20 $\mu$L of oat extract were placed in a 0.4 mL assay volume and tested against either (a) 4 mM AAPH or (b) $Cu^{2+}$ (9 $\mu$M $CuSO_4$) + 0.3% $H_2O_2$ to generate $OH^{\bullet}$. The fluorescence of R−PE was recorded every 2 min after addition of the radical generator; measurements are expressed relative to the initial reading. Final results were calculated from the difference of areas under the R-PE decay curves (AUC) between the blank and sample and expressed as micromoles of Trolox equivalents (TE) per gram.

**Calculation of TE of Oat Fractions.** For the LDL oxidation experiments, a series of dilutions of each sample was tested to identify the amount of oat extract required to achieve 50% inhibition ($IC_{50}$) of the baseline level of chol-18:2-OOH formation. A parallel experiment with different amounts of Trolox was conducted to determine the amount of Trolox needed for $IC_{50}$. Comparison of the amount of oat extract and Trolox required for the same degree of inhibition allowed the calculation of TE per gram of oat fraction used to prepare the methanol extracts. For the ORAC experiments, the AUC was determined with different amounts of oat extract and with Trolox, and a similar calculation was performed as noted above.

**Oat Tocol Determination.** Analysis of oat tocols, that is, tocopherols and tocotrienols, was performed by direct injection of the methanol extract onto a Rainin HPLC column (described above) with fluorescence measured by a Perkin-Elmer 650−15 detector (Norwalk, CT) set at 295 nm excitation and 330 nm emission wavelengths according to the method of Handelman et al. (1985) and Podda et al. (1996). The mobile phase was 95% methanol/5% water, at 1 mL/min flow rate. $\alpha$- and $\gamma$-tocopherol were assessed against calibrated standards (with concentrations determined by UV spectrophotometry), whereas tocotrienols were assumed to have the same fluorescence yield as their tocopherol equivalents. Calibration standards of $\alpha$-tocopherol, $\gamma$-tocopherol, and mixed tocopherols−tocotrienols were obtained from Eastman Chemical Products, Inc. (Kingsport, TN).

**Calculation of Fraction of Antioxidant Capacity Derived from Tocols.** The analysis of tocols allowed for the determination of their contribution to the total antioxidant capacity of each fraction. This determination is based upon the presumption that, as tocols and Trolox each possess a single aromatic −OH, they quench ROS on an equimolar basis in vitro. Therefore, the fraction of TE attributable to the tocols was calculated by comparing the sum of oat tocols with the total TE in each sample and expressing the result as a percentage.

## RESULTS

**Oat Antioxidant Capacity.** The antioxidant capacity of the methanol extracts of oat expressed in micromoles of TE per gram for LDL oxidation ($IC_{50}$) and ORAC (AUC) are presented in Table 2. A general agreement in rank order with regard to relative antioxidant potency is noted between the LDL oxidation and ORAC assays using both AAPH and $Cu^{2+}$ as radical generators. The green oat pearlings (F4) and 1.6% pearlings (F1) generally exhibit the highest antioxidant activity in these tests, whereas the oat bran (F8) and oat flour (F6) have the least.

The inhibition of AAPH-initiated (Figure 1) and $Cu^{2+}$-initiated (Figure 2) LDL oxidations by F4 and F7

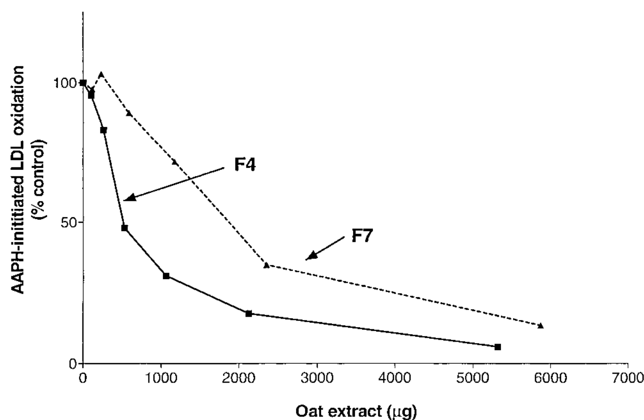


**Figure 1.** Inhibition of AAPH-stimulated LDL oxidation by F4 (green oat pearlings) and F7 (green oat flour). Samples were incubated for 60 min and then assayed for chol-18:2-OOH by HPLC. Control (100%) is value for LDL sample without addition of oat fraction.

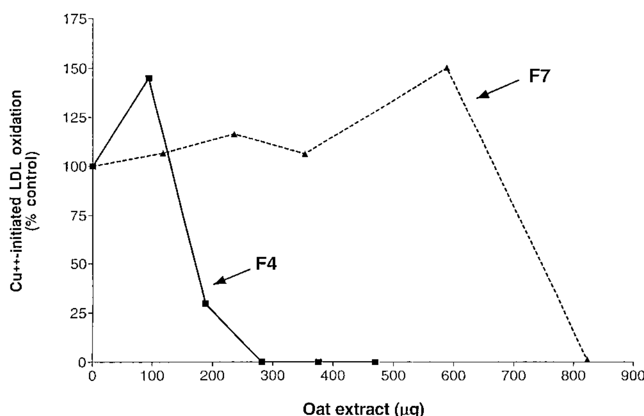


**Figure 2.** Inhibition of $Cu^{2+}$-stimulated LDL oxidation by F4 (green oat pearlings) and F7 (green oat flour). Samples were incubated for 60 min and then assayed for chol-18:2-OOH by HPLC. Control (100%) is value for LDL sample without addition of oat fraction.

**Table 2. Antioxidant Capacity of Oat Fractions Determined by LDL Oxidation ($IC_{50}$) and ORAC Assays (UAC)**

| | μmol of TE/g | | | | | tocol activity |
|---|---|---|---|---|---|---|
| | LDL oxidation | | ORAC | | | |
| extract | AAPH | $Cu^{2+}$ | AAPH | $Cu^{2+} + H_2O_2$ | av | (% of TE) |
| F1 | 3.76 | 4.18 | 6.70 | 5.94 | 5.15 ± 0.70 | 3.1 |
| F2 | 3.76 | 3.94 | 4.00 | 1.32 | 3.26 ± 0.65 | 3.6 |
| F3 | 1.68 | 3.19 | 4.43 | 2.26 | 2.89 ± 0.60 | 3.5 |
| F4 | 6.4 | 3.94 | 8.13 | 15.85 | 8.58 ± 2.57 | 1.6 |
| F5 | 1.52 | 1.57 | 2.74 | 1.11 | 1.74 ± 0.35 | 0.6 |
| F6 | 0.78 | 0.52 | 2.24 | 0.47 | 1.00 ± 0.42 | 4.2 |
| F7 | 1.77 | 0.91 | 4.55 | 6.92 | 3.54 ± 1.37 | 2.0 |
| F8 | 0.58 | 1.11 | 2.08 | 0.31 | 1.02 ± 0.39 | 2.6 |
| F9 | 1.03 | 0.81 | 2.66 | 0.33 | 1.21 ± 0.51 | 4.5 |
| F10 | 1.45 | 0.51 | 2.71 | 1.80 | 1.62 ± 0.45 | 1.1 |

illustrate the magnitude of difference in potency between oat pearling and flour, respectively. Among each sample test with AAPH-initiated reactions, greater inhibition of LDL oxidation was noted with increasing quantity of added oat fraction. In contrast, with $Cu^{2+}$-initiated reactions, low concentrations of oat extracts stimulated LDL oxidation, although complete inhibition of LDL oxidation was achieved with higher doses. A similar pro-oxidant effect on $Cu^{2+}$-initiated LDL oxidation was observed with low concentrations of Trolox (data not shown). The oat extracts were capable of completely suppressing $Cu^{2+}$-initiated LDL oxidation at

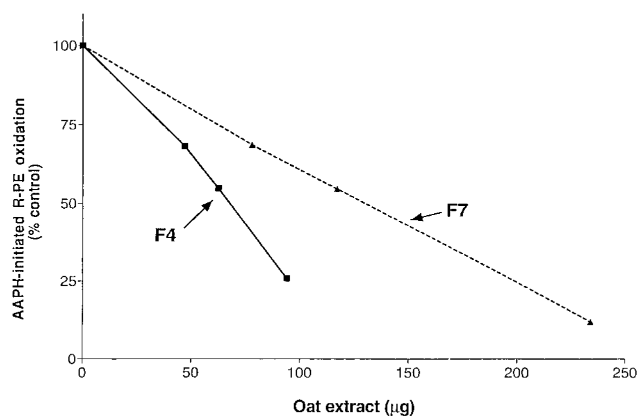


**Figure 3.** Inhibition of AAPH-stimulated oxidation of R-PE by F4 (green oat pearlings) and F7 (green oat flour). R-PE oxidation was determined by fluorescence after 30 min of incubation.

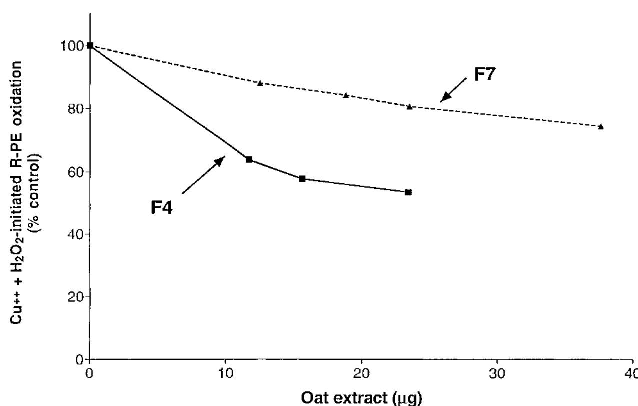


**Figure 4.** Inhibition of $Cu^{2+} + H_2O_2$-stimulated oxidation of R-PE by F4 (green oat pearlings) and F7 (green oat flour). R-PE oxidation was determined by fluorescence after 30 min of incubation.

doses only 20−50% greater than the $IC_{50}$. In contrast, even the highest extract concentrations employed failed to suppress AAPH-initiated oxidation to <10% of control values.

Relative dose−response relationships in the ORAC assay can be illustrated by examining R-PE oxidation at the midpoint (30 min) of the assay, before the complete oxidation of the protein. The inhibition of AAPH-initiated (Figure 3) and $Cu^{2+} + H_2O_2$-initiated (Figure 4) R-PE oxidation by F4 and F7 illustrates the magnitude of difference in potency between oat pearling and flour, respectively. ORAC AUC values (Table 2) were calculated from R-PE oxidations run to completion (70 min) as shown with dose−response curves (200−500-fold dilutions of the original extract) of F4 (Figure 5).

Some of the antioxidant constituents in the oat pearlings may be heat labile to treatment with steam as the non-steam-treated F4 from green oats was the most active extract in three of the four assays compared to the steam-treated F1, F2, and F3 pearlings (Table 2). Similarly, the non-steam-treated F7 green oat flour was more active than steam-treated F6 flour in each assay system. The bran fractions F8, F9, and F10 had low to intermediate activity compared to the other extracts in both LDL oxidation and ORAC models. The F5 oat trichomes generally had antioxidant activity comparable to or somewhat higher than that of the bran fractions.

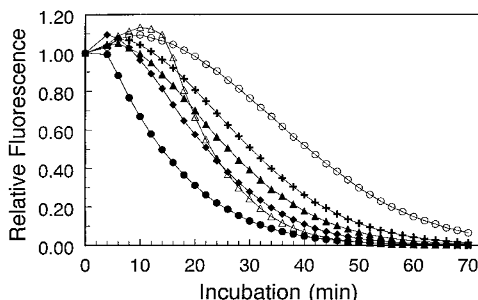


**Figure 5.** Dose response curves of F4 (green oat pearlings) in an AAPH-initiated ORAC assay: (●) blank; (△) 20 $\mu$M Trolox; (○) 200× dilution; (+) 300× dilution; (▲) 400× dilution; (◆) 500× dilution.

**Table 3. Total Tocols in Oat Groat Fractions**

| extract | total tocols ($\mu$mol/g) | extract | total tocols ($\mu$mol/g) |
|---|---|---|---|
| F1 | 0.161 | F6 | 0.047 |
| F2 | 0.100 | F7 | 0.072 |
| F3 | 0.116 | F8 | 0.030 |
| F4 | 0.148 | F9 | 0.054 |
| F5 | 0.011 | F10 | 0.017 |

Following methanol extraction of each oat fraction, the residues were re-extracted with hexane. These hexane extracts contained <1% of the activity of the methanol extracts (data not shown).

**Oat Tocol Antioxidant Capacity.** In the 10 oat fractions, α-tocopherol and α-tocotrienol comprised >90% of the tocols, consistent with the observations by Peterson and Quereshi (1993) and Peterson (1995). The amount of tocols in the extracts ranged from 0.6 to 4.5% of total TE (Table 2). Consistent with the data on total antioxidant capacity, pearlings also had higher levels of tocols than bran or flour (Table 3).

## DISCUSSION

The aleurone or outermost layer contains the greatest concentration of antioxidants in the oat groat (Dimberg et al., 1993). The great preponderance of the oat antioxidants (>99%) were soluble in the initial methanol extraction. As methanol is a polar organic solvent, most of the antioxidant compounds tested here are likely polar molecules. The distribution of antioxidants in the aleurone is consistent with the highest average radical scavenging activity of F4, the 5% pearlings from green oats. Fractions F1, F2, and F3, also derived from the aleurone layer, were strongly active in the LDL oxidation and ORAC assays. Oat bran consists principally of the outer aleurone layer and, thus, might be expected to possess an antioxidant capacity comparable to that of pearlings. However, commercial preparations of oat bran are often mixed with the starchy endosperm, a mixture that could dilute the total antioxidant capacity of these fractions (Wood et al., 1991; Wood and Beer, 1998).

A portion of the oat antioxidant constituents may be heat labile as suggested by the greater activity of the non-steam-treated F4. Rolled oats and other oat products prepared for human consumption are steam-treated to inactivate lipases and retard peroxidative degradation (rancidity) of the processed material (Hutchinson et al., 1951; Youngs, 1978; Deane and Commers, 1986). Lipases require inactivation due, in part, to the high content of linoleic acid (36% of total fatty acids) in oat lipids (Sahasrabhude, 1979). Our results suggest that most of the antioxidant activity in oats survives heat treatment and, thus, is likely to be abundant in commercial oat products. This observation is consistent with the findings of Dimberg et al. (1996), who report that <20% of polar phenolics in oats are degraded during heat processing.

The relative antioxidant capacities among fractions obtained from the LDL oxidation and ORAC assays, each involving free radical generation by AAPH or $Cu^{2+}$, were remarkably consistent considering the differences between the two models. AAPH-initiated oxidation of LDL was inhibited by the oat fractions in a dose-dependent manner, although complete suppression was not achieved with the highest doses tested here. In contrast, $Cu^{2+}$-initiated oxidation of LDL stimulated peroxide formation with low oat concentrations but completely inhibited oxidation with higher doses.

The antioxidant capacity of oat extracts may depend on the nature of the peroxidation reaction with which they interact. The strong agreement ($r = 0.907$) between the LDL oxidation and ORAC values generated with AAPH (Table 2) could be anticipated as the chemistry in both assays is similar, with the phenolics acting principally to scavenge $ROO^{\bullet}$ generated in the aqueous phase and preventing radical attack on the target protein. In the ORAC assay, phenolics may act to scavenge the $^{\bullet}OH$ formed in the aqueous phase by dismutation of $H_2O_2$.

In contrast to AAPH-initiated oxidations, the correlation between the two assays is weaker ($r = 0.505$) when they are initiated by $Cu^{2+}$. In the LDL oxidation assay, phenolics may act at low concentrations through an initial reduction of $Cu^{2+}$ to $Cu^{+}$ by tyrosine residues on the LDL surface and a subsequent reaction with pre-existing hydroperoxides in the LDL particle. This reaction could generate alkoxyl radicals ($RO^{\bullet}$) and propagate LDL peroxidation (Patel et al., 1997). Thus, at low concentrations, oat phenolics (and Trolox) could contribute to $Cu^{2+}$ reduction and promote LDL oxidation. However, at higher concentrations, oat phenolics may suppress lipid peroxidation via chelating $Cu^{2+}$ and quenching $ROO^{\bullet}$ at the surface of the LDL particle. The oat phenolics may directly inhibit $^{\bullet}OH$ produced from the $Cu^{2+} + H_2O_2$ reaction or prevent the reaction by chelating $Cu^{2+}$ (Hanna and Mason, 1992). A similar dose-dependent pro-/anti-oxidant effect of ascorbate and plant phenolics has been reported in other systems employing transition elements (Miller and Aust, 1989). For example, both caffeic acid and mixed flavonoids can be pro-oxidants in a $Cu^{2+}$-stimulated LDL oxidation system (Yamanaka et al., 1997; Otero et al., 1997) and in the ORAC assay (Cao et al., 1997). Similarly, tea extracts have a pro-oxidant effect at low concentrations but an antioxidant effect at high doses against DNA oxidation initiated by $Fe^{2+}$ + bleomycin + $H_2O_2$ (Yen et al., 1997).

The 2−8 $\mu$mol of TE/g range determined for the oat fractions is similar to the 5 $\mu$mol of phenolic equivalents/g reported by Xing and White (1997) for oat groats and hulls. The contribution of oat tocols from any of the fractions accounted for <5% of the measured antioxidant capacity. Thus, the majority of the antioxidant activity is probably derived from caffeic acid, ferulic acids, and other oat phenolics. Dimberg et al. (1996) measured caffeic acid and other phenolics in oats and found 80% of the polar phenolics were in the avenanthramide fraction. The amide linkage of aventhramides may confer resistance to enzymatic hydrolysis in the

gastrointestinal tract and improve potential bioavailability. The antioxidant activity of aventhramides in vivo might complement the cholesterol-lowering property of oat fiber in the prevention of heart disease (Braaten et al., 1994; Onning et al., 1998).

## CONCLUSION

Phenolic-rich fractions of oats possess an antioxidant capacity that can be assessed quantitatively through their ability to inhibit LDL oxidation and protein oxidation (R-PE in the ORAC assay). The greatest degree of antioxidant capacity was associated with compounds extracted with methanol from the aleurone. The identification of the oat constituents from these fractions should be investigated, although candidate compounds would include caffeic acid, ferulic acid, and avenanthramides. These compounds may be bioavailable and contribute to the health effects associated with dietary antioxidants and oats.

## ABBREVIATIONS USED

RO•, alkoxyl radical; AUC, area under the curve; AAPH, 2,2′-azobis(2-amidinopropane) HCl; chol-18:2-OOH, cholesterol linoleate hydroperoxide; EDTA, sodium ethylene glycol bis($\beta$-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high-performance liquid chromatography; •OH, hydroxyl radical; LDL, low-density lipoprotein; ORAC, oxygen radical absorbance capacity; ROO•, peroxyl radical; PBS, phosphate-buffered saline; ROS, reactive oxygen species; R-PE, R-phycoerythrin; TE, Trolox equivalents; T3, tocotrienol; TOC, tocopherol.

## ACKNOWLEDGMENT

We thank Jim Hansa, Alice Hibbs, and Steve Jacobs of The Quaker Oats Co. for preparation of the oat fractions and extracts and Andreas Papas of Eastman Chemical Products for the vitamin E calibration standards.

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Received for review May 17, 1999. Revised manuscript received September 28, 1999. Accepted October 6, 1999. Research support was provided by a contract from The Quaker Oats Co. and by the U.S. Department of Agriculture (USDA) Agricultural Research Service under Cooperative Agreement 58-1950-9-001. 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.

JF990529J