# Using a Modified Ferrous Oxidation–Xylenol Orange (FOX) Assay for Detection of Lipid Hydroperoxides in Plant Tissue

JOHN M. DELONG,*,† ROBERT K. PRANGE,† D. MARK HODGES,†
CHARLES F. FORNEY,† M. CONNY BISHOP,† AND MICHAEL QUILLIAM‡

Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, 32 Main Street,
Kentville, Nova Scotia B4N 1J5, Canada, and Institute for Marine Biosciences, National Research
Council of Canada, Halifax, Nova Scotia B3L 3V2, Canada

The ferrous oxidation–xylenol orange (FOX) assay was adapted for quantifying lipid hydroperoxides (LOOHs) in plant extracts. Excised pieces of several fruit and vegetable species were exposed to 83 kJ m$^{-2}$ day$^{-1}$ of biologically effective ultraviolet B irradiance (UV-B$_{BE}$) for 10–12 days to induce cellular oxidation. The LOOH and thiobarbituric acid reactive substance (TBARS) concentrations of these plant tissues were assessed with the FOX and iodometric assays for the former and a modified TBARS assay for the latter. There was generally good agreement between the FOX and iodometric methods both prior to and following the UV exposure. However, the iodometric assay appeared to have some difficulty in consistently quantifying lower LOOH levels (<11 $\mu$M), whereas the FOX assay measured LOOH concentrations as low as 5 $\mu$M. All tissues exhibited UV-induced increases in TBARS, indicating a marked degree of cellular oxidation in the exposed tissue segments. Compared with the iodometric assay, the FOX method consistently generated less variable LOOH values. The presence of authentic linoleic acid–OOHs in spiked avocado and spinach samples (11 $\mu$M) was identified with liquid chromatography–mass spectrometry techniques, which validated corresponding FOX assay results. The FOX method is inexpensive, is not sensitive to ambient O$_2$ or light levels, and can rapidly generate LOOH measurements. The physiological value of the FOX assay resides in its ability to measure initial rather than more advanced fatty acid oxidation; hence, early membrane-associated stress events in plant tissue can be detected.

KEYWORDS: Fatty acid oxidation; ferrous oxidation–xylenol orange (FOX) assay; lipid hydroperoxides; oxidative stress

## INTRODUCTION

Fatty acid oxidation has been implicated in normal and stress-induced plant metabolism (*1–4*), in plant senescence mechanisms (*5, 6*), in the etiology of various animal-based diseases such as atherosclerosis (*7*), coronary heart disease (*8*), and cancer (*9, 10*), and in inflammation disorders such as arthritis (*11*) and asthma (*12*).

Membrane fatty acids are susceptible to oxidative degradation under a variety of cellular stress conditions often resulting in the eventual breakdown of membrane structure and function (*13, 14*). As polyunsaturated fatty acids (PUFA) undergo nonenzymic peroxidative reactions or enzyme-catalyzed oxidation, molecular (O$_2$) or singlet ($^1$O$_2$) oxygen is incorporated into the hydrocarbon skeleton, resulting in the formation of lipid hydroperoxides (LOOHs) (*15–17*).

Hence, the formation of hydroperoxide moieties is an initial event in the oxidative degradation of fatty acid molecules.

Hydroperoxides can then undergo various decomposition pathways often involving transition metals (notably Fe$^{2+}$) or enzyme-mediated reactions that yield various products such as alkenals, hydroxyalkenals, alkanes, and jasmonic acid (*2, 18*).

Lipid hydroperoxides have been measured by using a variety of techniques including high-performance liquid chromatography (HPLC) (*19*), gas chromatography (GC) (*20*), electrospray mass spectrometry (*21*), iodide oxidation (*22*), heme degradation of peroxides (*23*), cylco-oxygenase activation (*24*), and micro-peroxidase–luminol chemiluminescence (*25*). These methods are time-consuming or costly or require strict control of ambient oxygen levels. In recent years, another method has been developed that is based upon the oxidation of ferrous (Fe$^{2+}$) to ferric (Fe$^{3+}$) ions by LOOHs with the subsequent binding of the Fe$^{3+}$ ion to the ferric-sensitive dye xylenol orange (*26–28*). Known as the ferrous oxidation–xylenol orange (FOX) assay (versions I and II), the technique is sensitive (nanomole to micromole levels of LOOHs), inexpensive, and not affected by ambient oxygen concentrations. As version II permits the quantification of low concentrations of LOOHs in the presence

* Author to whom correspondence should be addressed [telephone (902) 679-5765; fax (902) 679-2311; e-mail DeLongJ@em.agr.ca].
† Agriculture and Agri-Food Canada.
‡ National Research Council of Canada.

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of high background levels of nonperoxidized fatty acids, it is the preferred method.

To date, the FOX assay has been mainly used to measure the presence of LOOHs in reconstructed membrane systems and in mammalian tissue and serum extracts (*26, 27, 29, 30*). It has the potential to be a rapid and inexpensive method for detecting incipient lipid peroxidation in plant tissue. To our knowledge, the FOX assay has been reported by only one other group for LOOH measurement in plant tissue (*31, 57*), although it has been utilized for LOOH detection in edible vegetable oils (*32, 33*). The main objective of this study was to adapt the FOX assay to more accurately detect the presence of LOOHs in plant tissue.

## MATERIALS AND METHODS

**Chemicals.** Boric acid, methanol (HPLC grade), potassium iodide, and trichloroacetic acid (TCA) were purchased from Fisher Scientific, Pittsburgh, PA, whereas the sulfuric acid ($H_2SO_4$; reagent grade) and glacial acetic acid were obtained from Anachemia Science, Montreal, PQ. Bovine serum albumin (BSA, fraction V), butylated hydroxytoluene (BHT), 3,3′-bis[*N,N*-di(carboxyethyl)aminomethyl]-*o*-cresolsulfone-pthalein sodium salt (xylenol orange), ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA), ferrous ammonium sulfate hexahydrate, *n*-heptane, hydrogen peroxide ($H_2O_2$; 30.4%), linoleic acid (*cis*-9,*cis*-12-octadecadienoic acid), lipoxygenase [(LOX) EC 1.13.11.12], thiobarbituric acid (TBA), triphenylphosphine (TPP), and polyoxy-ethylenesorbitan monolaurate (Tween-20) were purchased from Sigma, St. Louis, MO. The double-distilled water was generated by a Millipore QUF water purification system (Millipore, Danvers, MA). The 95% ethanol was purchased from Alcools De Commerce Inc., Boucherville, PQ.

**Plant Material.** All plant material used was obtained from local supermarkets or from the Atlantic Food and Horticulture Research Centre of Agriculture and Agri-Food Canada in Kentville, NS. Tissue segments of avocado (*Persea americana* Mill.) flesh, potato (*Solanum tuberosum* L.) cortex, red cabbage (*Brassica oleracea* L.) and spinach (*Spinacia oleracea* L.) leaves, pear (*Pyrus communis* L.) skin, and red bell pepper (*Capsicum annuum* L.) skin and flesh were used and ranged in mass from 0.2 to 1.1 g. All tissue was hand ground with a mortar and pestle using ∼0.5−1.0 g of inert sand in 10 mL of 80:20 ethanol/water with 0.01% (w/v) BHT added to arrest any further oxidation (*34*). Each sample was then centrifuged at 3000*g* for 10 min with supernatant aliquots being used for LOOH and TBARS determination. The blanks were processed identically except for the absence of the tissue segments. The 80:20 ethanol/water solution was chosen as the superior extracting solvent for both the FOX and TBARS assays compared with either 100% ethanol or ethyl acetate (data not shown).

**FOX Assay.** The FOX version II assay (*47*) was used to quantify the presence of LOOHs in artificial systems and plant tissue extracts. The simplified reaction sequence involves the oxidation of ferrous ($Fe^{2+}$) to ferric ($Fe^{3+}$) ions by LOOHs with the subsequent binding of the $Fe^{3+}$ ion to the ferric-sensitive dye xylenol orange, yielding an orange to purple complex (color dependent on the amount of −OOHs present), which is measured at 560 nm (*27, 28, 35*).

For each 1000 mL volume, the FOX reagent consisted of 90% methanol (v/v), 10% 250 mM $H_2SO_4$ (v/v) (25 mM final concentration), 880 mg of BHT (4 mM), 98 mg of ferrous ammonium sulfate hexahydrate (250 $\mu$M), and 76 mg of xylenol orange (100 $\mu$M). The methanol, $H_2SO_4$, and BHT were mixed and stored at 4 °C, whereas the iron and xylenol orange were added just prior to the addition of reagent to the samples. The complete reagent (with iron and xylenol orange) was used within 24 h or a new batch was made.

For some samples, 100 $\mu$L of the plant extract was combined with 100 $\mu$L of 10 mM TPP (a LOOH reducing agent) in methanol,. The mixture was momentarily stirred with a Vortex-Genie 2 (VWR Scientific, Mississauga, ON) and then incubated for 30 min to allow for the complete reduction of any present −OOHs by TPP (+TPP). Samples without TPP (−TPP) addition were treated identically except that the TPP aliquot was substituted with methanol or ethanol. Following the 30-min TPP incubation, 2000 $\mu$L of FOX reagent was added to
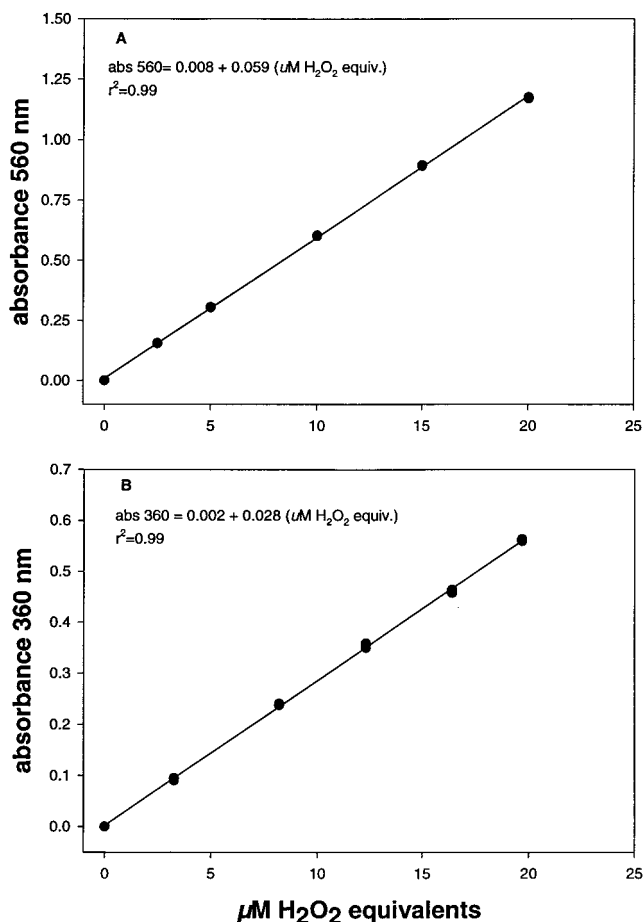


**Figure 1.** Standard curves relating $\mu$M $H_2O_2$ equiv (*X*) to (A) absorbance units at 560 nm (*Y*) as determined by the FOX assay or (B) absorbance units at 360 nm (*Y*) as determined by the iodometric assay.

each sample with the absorbance at 560 nm being recorded exactly 10 min after reagent addition on an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Cambridge, U.K.). The absorbance difference between the samples without and with TPP indicated the presence of LOOHs; −OOH values were then expressed as micromolar $H_2O_2$ equivalents using a standard curve spanning a 0−20 $\mu$M $H_2O_2$ range (Figure 1A). The relationship between the 560 nm absorbance values and $H_2O_2$ concentration became curvilinear around 30 $\mu$M $H_2O_2$ (data not shown).

**Iodometric Assay.** The iodometric assay (*27, 36*) was used to verify the presence of LOOHs in reconstructed LOOH systems and plant tissue extracts. The LOOHs are reduced to alcohols by iodide ($2I^-$) resulting in the conversion of $2I^-$ to the triiodide anion ($I_3^-$), which is measured spectrophotometrically at 290 or 360 nm. Reagent A consisted of a 1:1 (v/v) mixture of methanol/glacial acetic acid, whereas reagent B was a 10% (w/v) solution of KI in methanol with 1 mg·mL$^{-1}$ of EDTA. Reagents A and B were deoxygenated by sparging with high-purity (>99%) $N_2$ for 10 min prior to any sample introduction. Reagent B was stirred for 30 min before deoxygenation to facilitate thorough mixing of the KI into solution, followed by an $N_2$ sparging for 10 min. For each sample, 1.2 mL of reagent A was mixed with 1.8 mL of reagent B in a quartz cuvette and quickly sealed with a threaded cap having a gas-impermeable Teflon septum. The combined reagent solution was deoxygenated by bubbling with $N_2$ gas for 1 min via insertion of a hypodermic needle through the septum, after which 50 $\mu$L of sample extract was introduced by the same method as the $N_2$ gas, followed by inversion of the cuvette to ensure adequate mixing. The sample/reagent absorbances were then measured every 6 min at 360 nm to determine the point of maximum color development. The sample concentrations of LOOHs were calculated as micromolar $H_2O_2$ equivalents based upon a standard curve spanning a 0−20 $\mu$M $H_2O_2$ range (Figure 1B).

**TBARS Assay.** A modified TBARS assay was used as an alternative assessment of lipid oxidation (*34*). The TBARS reagent consisted of 20% (w/v) TCA, 0.65% (w/v) TBA, and 0.01% (w/v) BHT in double-distilled $H_2O$. Two hundred microliters of 80:20 ethanol/water (v/v) was combined with 800 $\mu$L of $H_2O$ and either 1000 $\mu$L of reagent with TBA added (+TBA) or 1000 $\mu$L of reagent without TBA (−TBA). Samples were then mixed vigorously, incubated at 95 °C in a block heater (Multiblok, Lab-Line Instruments) for 25 min, cooled, and centrifuged at 3000*g* for 10 min. Sample absorbances for the malondialdehyde (MDA)−TBA adduct were then measured spectrophotometrically at 532 nm; nonspecific turbidity and total soluble sugars (principally sucrose, fructose, and glucose) were subtracted from the 532 nm signal by measurements at 600 and 440 nm, respectively. This TBARS procedure (*34*) eliminates any 532 nm artifacts contributed by the presence of interfering compounds according to the following equations:

$$[(Abs532_{+TBA} - Abs600_{+TBA}) -$$

$$(Abs532_{-TBA} - Abs600_{-TBA})] = A \quad (1)$$

$$[(Abs440_{+TBA} - Abs600_{+TBA}) \times 0.0571] = B \quad (2)$$

$$MDA \; equiv \; (nmol \cdot mL^{-1}) = (A - B/157000) \times 10^6 \quad (3)$$

**Generation and Phase Separation of LOOHs.** Lipid−OOHs were generated via LOX or autoxidation. The LOX samples contained boric acid buffer (200 mM; pH 9.0), linoleic acid (9.7 mM), Tween-20 (2.2 mM), and 11250 units·mL$^{-1}$ of LOX protein and were moderately stirred in the dark for ~24 h. The autoxidation samples contained the same constituents minus the LOX and were moderately stirred in the dark at room temperature for 48 h. The controls in both systems did not contain linoleic acid. One hundred microliters from each of the LOX and autoxidation solutions was added to 10 mL of 80:20 (v/v) ethanol/water grinding medium and vigorously stirred for a few seconds on a Vortex-Genie. One and a half milliliters from each solution was then added to 1.5 mL of heptane, followed by mixing and centrifugation at 3000*g* for 10 min, which yielded an upper organic phase and a lower aqueous phase (*37*). The phases were separated, with 500 $\mu$L from each being evaporated with $N_2$ gas, and were then reconstituted to 1 mL with 100% methanol. One hundred microliters from the reconstituted organic and aqueous phases was used to determine the presence of LOOHs via the FOX assay.

Linoleic acid−OOHs were also generated according to a Sigma-Aldrich protocol (*38*), which were then quantified using LC-MS. Five hundred microliters of 95% ethanol was added to 500 $\mu$L of linoleic acid, which was then combined with 50 mL of 200 mM boric acid buffer (pH 9.0). One to three hundred microliters of Tween-20 was also added to solubilize the fatty acid. Five milliliters of this solution was then combined with 20 mL of boric acid buffer into which ~9500 units·mL$^{-1}$ of LOX was added and moderately stirred at room temperature for ~18 h. Controls did not contain the fatty acid substrate but did have LOX. Following the reaction period, the −OOH solution was diluted 30-fold with methanol and had an absorbance of 1.5−2.0 units at 234 nm. The calculation of LOOH concentration was based on the formation of conjugated dienes in which 1 absorbance unit = 0.12 $\mu$mol of linoleic acid oxidized (= 0.12 $\mu$mol of −OOH formed). The LOOH solution was kept at −20 °C and was stable for several weeks.

**Protein Oxidation.** A 500 $\mu$M aqueous stock solution of BSA was continuously agitated on a magnetic stirring plate for ~20 h at room temperature to induce protein oxidation and was then held at room temperature for an additional 20 h. A fatty acid solution (10 mM) consisting of 25 mL of boric acid (200 mM; pH 9.0), 65 $\mu$L of Tween-20, and 80 $\mu$L of linoleic acid was continuously stirred on a magnetic plate for 17−18 h to induce fatty acid oxidation. Twenty-five milliliters of the fatty acid solution was added to the same volume of the BSA mixture, which was then stirred for 1−1.5 h. Three concentrations of BSA [0.08 (~1.2 $\mu$M), 0.32 (~4.7 $\mu$M), and 3.0 (~44 $\mu$M) mg·mL$^{-1}$] were tested separately and in combination with three concentrations of linoleic acid [3.38 (12.1 $\mu$M), 13.5 (48.2 $\mu$M), and 124 (444 $\mu$M) $\mu$g·mL$^{-1}$] for FOX reactivity.
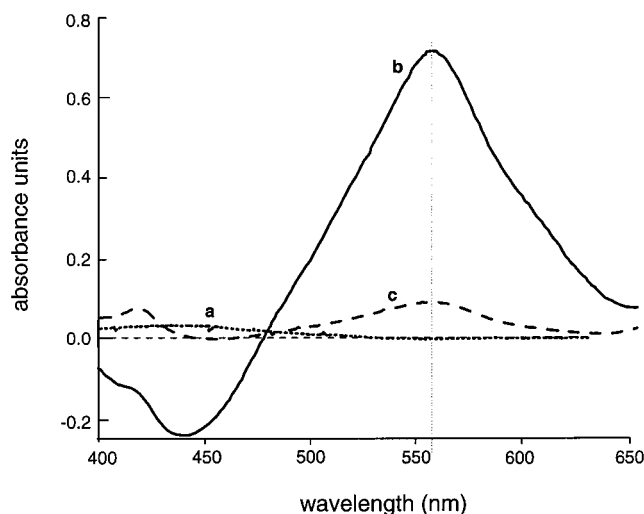


**Figure 2.** Spectrophotometric scan from 400 to 650 nm of 200 $\mu$L of methanol (blank) (a), 100 $\mu$L of spinach extract aliquot and 100 $\mu$L of methanol (no TPP) (b), or 100 $\mu$L of spinach extract aliquot and 100 $\mu$L of 10.0 mM TPP (c). Following a 30-min incubation, 2000 $\mu$L of FOX reagent was added to samples a–c with the absorbances being measured 2.5 h later.

**UV-B Treatment.** To induce lipid peroxidation, plant tissue segments were continuously exposed for 10−12 days to UV-B radiation generated by unfiltered UV-B lamps (Philips FS-40 UV-B lamps, Blacklock Medical Products, Delta, BC), which generated radiation from approximately 280 to 400 nm with 86% of the irradiance in the UV-B (280−320 nm) band. The levels of UV-B irradiance were monitored daily by a UV-Biometer, model 501A (Solar Light Co., Philadelphia, PA), which was weighted with the erythmal action spectra. Biologically effective UV-B (UV-B$_{BE}$) radiation was then determined by converting the units of the biometer into spectroradiometric readings weighted with the generalized plant damage action spectra, normalized to unity at 300 nm (*39, 40*).

**Liquid Chromatography—Mass Spectrometry (LC-MS).** LC-MS analyses were performed to quantify linoleic acid-OOHs using a Hewlett-Packard (Palo Alto, CA) HP1100 LC system connected with a Perkin-Elmer SCIEX (Concord, ON) API-165 single-quadrupole MS system equipped with an ion-spray source. Analyses were conducted in negative ion mode using selected ion monitoring of [M − H]$^-$ ions. Separations were performed on a 50 × 2 mm i.d. column packed with 3 $\mu$M BDS-Hypersil C8 silica with 0.20 mL·min$^{-1}$ of pH 2.4 mobile phase comprising acetonitrile/water (75:25, v/v) with 2 mM ammonium formate and 50 mM formic acid.

**Statistical Analysis.** Data from the plant tissue experiment were subjected to analysis of variance with mean separation performed by the Fisher LSD method (*41*). Regression equations for the standard curves were determined with Sigma Plot version 5. Data expressed as means ± standard errors were calculated with SAS software (*42*).

## RESULTS AND DISCUSSION

The spectrophotometric absorption profile indicates that the spinach extracts treated with and without TPP have maximum absorbances at 560 nm (Figure 2). Lipid hydroperoxides were differentiated from interfering compounds by incubating some of the samples with the −OOH reductant TPP for 30 min prior to the addition of the FOX reagent, whereupon the LOOH content is determined by the simple equation

$$[-TPP] - [+TPP] =$$

$$[Abs560 \; LOOH + Abs560 \; interfering \; compounds] -$$

$$[Abs560 \; interfering \; compounds] = Abs560 \; LOOH$$

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Although the precise identification of the interfering compounds is not known, phenolic (e.g., flavonoid) compounds likely represented a large portion of these substances as they have aborption maxima in the 500−600 nm range (*43*, *44*). Hodges et al. (*34*) demonstrated that anthocyanins and other compounds strongly interfere with the assessment of the lipid peroxidation product MDA as determined by the conventional TBARS assay. In the present study, the contribution of non-LOOH absorbances to the 560 nm signal is accounted for via the difference between the −TPP and +TPP samples.

A potential contribution to the FOX assay in plant tissue extracts is $H_2O_2$, as this molecule does not react with TPP and, if present, may inflate the 560 nm signal. The possible $H_2O_2$ interferences were eliminated in this study by treatment of samples with catalase prior to reagent addition (data not shown). Hence, incubation of samples with 500−1000 units of catalase $mL^{-1}$ for 15−30 min is highly recommended to test for the presence of $H_2O_2$ and, if necessary, to include catalase routinely (*35*, *45*).

Lipid−OOH content was quantified by calibration using $H_2O_2$ for both the FOX and the iodometric assays. In animal and artificial system applications, $H_2O_2$ is often used as the reference unit for LOOH measurements due to its high FOX reagent reactivity and the ease and simplicity in establishing standard curves from known stock concentrations (*27*, *32*, *35*, *46*). A calculated molar extinction coefficient ($\epsilon$) for $H_2O_2$ of 60342 $M^{-1}$ $cm^{-1}$ (data not shown) at 560 nm in FOX reagent is nearly identical to the $\epsilon$ of 60000 $M^{-1}$ $cm^{-1}$ for linoleic acid−OOHs reported by Gay et al. (*45*), indicating that $H_2O_2$ is a suitable standard against which LOOH concentrations in biological tissue can be measured. In addition, the $H_2O_2$-generated stoichiometric yield of 2.0−3.0 $Fe^{3+}$ formed per −OOH reacted is similar to more complex biological −OOHs than either cumene− or *tert*-butyl−OOHs (*45*).

In the early development of this assay, maximal color development consistently occurred during a 2.5-h incubation period for pure chemical hydroperoxides species (e.g., $H_2O_2$, cumene−OOHs, and linoleic acid−OOHs). When the LOOH content of various plant extracts was being determined, the time required for maximal color development was inconsistent for different tissue, sometimes occurring between 120 and 180 min and often requiring much longer time periods (data not shown). Hence, the designation of a benchmark time period (e.g., 30 min; *31*) for maximal color measurement is not reliable as this plateau may occur hours after initial incubation of the sample and reagents. In addition, the development of color in plant tissue extracts is often curvilinear the longer the incubation period, with the degree of curvilinearity being affected by the endogenous tissue levels of polyunsaturated fatty acids (data not shown). To circumvent these problems and to standardize the method of determining LOOH concentration regardless of tissue type and condition, the color development occurring exactly 10 min following sample introduction into the FOX reagent was recorded. During this 10-min period, the 560 nm absorbance developed linearly in all systems tested.

An important assumption underlying the use of the FOX assay is that separation of the lipid from the aqueous fraction is not necessary as the methanolic−$H_2SO_4$ FOX reagent denatures lipoproteins sufficiently to allow interaction of $Fe^{2+}$ ions with LOOHs (*27*, *47*). Intimated in this assumption is that the change in polarity conferred by the formation of the LOOH group(s) changes the fundamental hydrophobicity of the fatty acid, resulting in an increased degree of hydrophilicity and thus permitting chemical reactions within the methanol-based solvent.

**Table 1.**  $H_2O_2$ Equivalents (Micromolar) in the Polar and Nonpolar Phases for LOOHs Derived via LOX and Autoxidation[a]

| phase | mode of oxidation | |
|---|---|---|
|  | LOX | autoxidized |
| polar | 45 ± 3 | 29 ± 4 |
| nonpolar | 0 | 0 |

[a] Each mean ± standard error is based upon four replicate samples.

**Table 2.**  $H_2O_2$ Equivalent Values for Nonspiked and Spiked (+11 $\mu$M Linoleic Acid−OOH) Avocado and Spinach Samples[a]

| plant material | protocol | nonspiked ($\mu$M) | spiked ($\mu$M) | difference |
|---|---|---|---|---|
| avocado | LC-MS | 0 | 5.8 | 5.8 |
|  | FOX | 5.7 | 12.9 | 7.2 |
| spinach | LC-MS | 0 | 7.7 | 7.7 |
|  | FOX | 7.4 | 12.5 | 5.1 |

[a] Each mean comprises four replicate observations.

To determine whether a separate lipid extraction is required for the FOX method, the nonpolar and polar phases of spinach leaf extracts were separated (*37*) and then each was assayed for LOOHs with the modified FOX protocol. The results clearly show that the 560 nm signal resides in the polar phase (Table 1); hence, a separate lipid extraction is not necessary if the intent is to use the FOX assay as a general indicator of the presence of LOOHs. The omission of a lipid extraction procedure is a marked benefit as it not only reduces the time and labor involved in sample processing but also reduces the degree of time-dependent degradation of the metastable −OOHs. If the experimental intent is to detect the presence of LOOHs in purified lipid systems or among different classes of lipids, then a lipid phase extraction would be required.

The presence of authentic linoleic acid−OOHs in avocado and spinach samples was verified by LC-MS techniques, which validated corresponding FOX results following the addition of ∼11 $\mu$M linoleic acid−OOHs to avocado and spinach samples (Table 2). The similar difference values between the nonspiked and spiked samples indicates good agreement between the two methods for detection of the added linoleic acid−OOHs. However, a small residual FOX signal of unknown origin was present in the nonspiked samples (Table 2). The possibility that excess, sample-derived $Fe^{3+}$ caused the artifact was tested by incubating nonspiked spinach extracts with 100 $\mu$L of the reducing agent mercaptoethanol (10 mM), 100 $\mu$L of sample, and 100 $\mu$L of TPP for 30 min followed by the addition of the FOX reagent. The data indicated that ferric ions were not the source of the residual signal as absorbances were identical to the TPP-treated samples (data not shown). It may be that the residual absorbance was caused by hydroperoxide species that the LC-MS analysis did not detect as only linoleic acid−OOHs were quantified, although TPP should have reduced all −OOH groups present. Hence, an important objective for future work with plant systems will be identification and elimination of all potential non-OOH artifactual absorbances.

To determine if the FOX assay differentiates between lipid− and protein−OOHs, BSA was autoxidized at room temperature for ∼20 h. The data indicate that the FOX assay did not detect protein−OOHs due to their absence or the inaccessibility of the −OOH group to $Fe^{2+}$-induced reduction (Table 3). If −OOH moieties formed on the BSA protein, it may be that they were largely unavailable for reaction with $Fe^{2+}$ due to the complex secondary, tertiary, and quaternary structure of protein molecules, possibly compounded with the high concentration of

**Table 3.** Absorbance at 560 nm for (i) BSA Protein, (ii) BSA and Tween-20, and (iii) BSA, Tween-20, and Linoleic Acid Solutions[a]

| treatment | BSA[b] (mg mL$^{-1}$) | linoleic acid[c] ($\mu$g mL$^{-1}$) | Abs560 ($-$TPP $-$ +TPP) |
|---|---|---|---|
| BSA | 0.08 | 0 | 0 |
| | 0.32 | 0 | 0 |
| | 3.0 | 0 | 0 |
| BSA + Tween-20 | 0.08 | 0 | 0 |
| | 0.32 | 0 | 0 |
| | 3.0 | 0 | 0 |
| BSA + Tween-20 + linoleic acid | 0.08 | 3.38 | 0.090 |
| | 0.32 | 13.5 | 0.17 ± 0.02 |
| | 3.0 | 124 | 0 |

[a] Each Abs560 value is a mean ± standard error of three observations. [b] BSA concentrations: 0.08 mg mL$^{-1}$ = ~1.2 $\mu$M, 0.32 mg mL$^{-1}$ = ~4.7 $\mu$M, and 3.0 mg mL$^{-1}$ = ~44 $\mu$M. [c] Linoleic acid concentrations: 3.38 $\mu$g mL$^{-1}$ = 12.1 $\mu$M, 13.5 $\mu$g mL$^{-1}$ = 48.2 $\mu$M, and 124 $\mu$g mL$^{-1}$ = 444 $\mu$M.

**Table 4.** LOOHs and TBARS in Various Plant Tissues Prior to and Following 10–12 Days of Continuous UV-B$_{BE}$ Exposure[a]

| tissue | UV-B exposure (days) | FOX[b] | iodometric[b] | TBARS[c] |
|---|---|---|---|---|
| avocado | 0 | 20 ± 1 a[d] | 17 ± 3 a | 7 ± 1 |
| | 11 | 268 ± 55 a | 241 ± 57 b | 33 ± 3 |
| pear | 0 | 21 ± 4 a | 47 ± 26 a | 0 |
| | 12 | 190 ± 14 a | 189 ± 26 a | 120 ± 7 |
| potato | 0 | 0 a | 0 a | 0.7 ± 0.3 |
| | 10 | 9 ± 1 a | 17 ± 3 a | 10 ± 1 |
| red cabbage | 0 | 0 a | 0 a | 6 ± 3 |
| | 12 | 5.2 ± 0.9 a | 0 b | 29 ± 3 |
| red pepper | 0 | 0 a | 0 a | 45 ± 3 |
| | 12 | 28 ± 6 a | 11 ± 10 b | 74 ± 5 |
| spinach | 0 | 199 ± 5 a | 110 ± 12 b | 27 ± 2 |
| | 10 | 384 ± 12 a | 341 ± 51 a | 234 ± 5 |

[a] Each value is a mean of four sample replicate measurements ± standard error. Tissues were exposed to a continuous daily UV-B$_{BE}$ irradiance of ~83 kJ m$^{-2}$. [b] FOX and iodometric assay units: $\mu$M H$_2$O$_2$ equiv (g FW)$^{-1}$ mL$^{-1}$. [c] TBARS assay units: nmol MDA equiv (g FW)$^{-1}$ mL$^{-1}$. [d] FOX and iodometric assay means for the same UV-B exposure day having different letters are significantly different at the 5% $\alpha$ level.

BSA used. When autoxidized linoleic acid was added to a similar BSA solution, an absorbance signal at 560 nm was observed at the low and medium fatty acid concentrations, indicating the presence of $-$OOHs. Interestingly, at the highest concentration of BSA and fatty acid, no Abs560nm signal was detectable (Table 3). Albumin has antioxidant properties (*56*), can bind fatty acids (*48*), and can protect them from oxidation by inhibiting $-$OOH formation (*49*). At the highest level of BSA tested (~44 $\mu$M), the data suggest that any $-$OOH groups formed on the free fatty acids were either chemically altered or bound by the BSA, resulting in no Fe$^{2+}$-induced reduction and no subsequent binding of Fe$^{3+}$ to xylenol orange (Table 3). Recent work on protein$-$OOH detection has shown that BSA$-$OOHs generated with $^{60}$CO irradiation are measurable via the ferric$-$xylenol orange complex (*45*). However, the oxidizing environment created with $\gamma$-radiation in that study would be much more extreme than that induced in the present work by moderate stirring of the BSA mixture for 20 h in the presence of ambient O$_2$ levels. Nonetheless, when protein concentrations in the aqueous$-$base mixture were in the range commonly found in plant tissue [micrograms to milligrams·(g FW)$^{-1}$], protein$-$OOHs were not detected with the FOX assay, whereas LOOHs were.

Following 10$-$12 days of UV-B$_{BE}$ exposure, marked increases in TBARS and LOOHs for all tissues were observed, with the exception of the iodometric measurements of red cabbage for the latter (Table 4). Past work demonstrates that UV-B$_{BE}$ radiation oxidizes membrane fatty acids directly or indirectly in tissue as diverse as *Arabidopsis thaliana* LER (*50*), cucumber (*Cucumis sativus* L.) (*51*, *52*), the marine phytoplankton *Tetraselmis* sp. (*53*), several species of microalgae (*54*), isolated thylakoid membranes (*40*), and some of the same plant material used in this study (*34*). Recently, a less intense hormic dose of germicidal UV-C on tomato (*Lycopersicon esculentum* L.) fruit prior to storage caused a significant induction of TBARS and other lipid peroxidation markers within the first 5 days of storage, suggesting that membranes are direct targets of UV damage (*55*).

Comparison of the LOOH measurements by the FOX and iodometric assays show that these methods generated fairly similar readings at day 0 and following the UV exposure period as judged by statistical nonsignificance or numeric closeness of means (Table 4). In some cases, as with pear at day 0, the LOOH averages for the two methods were 2-fold different but were statistically similar due to the large variation in the iodometric measurements. For avocado and red cabbage tissue following the UV exposure, the LOOH means, although

statistically different, were considered similar from a practical standpoint due to the relatively small numeric range between them.

The plant extract LOOH values generated by the FOX assay tended to be higher than those produced with the iodometric method (Table 4); Nourooz-Zadeh et al. (*32*) reported similar results when LOOHs in various vegetable oils were assayed, with some of the FOX-derived averages being >5-fold higher than those obtained with the iodometric assay. Gay et al. (*45*) recently found that iodometric measurements of absolute LOOH concentrations in human serum were not reliable as the liberated iodine reacted with serum protein. This protein$-$iodine reaction may explain why the iodometric means were often numerically less than the FOX-derived values in the present study (Table 4).

The iodometric assay did not indicate the presence of LOOHs in plant extracts when the concentrations were below 11 $\mu$M H$_2$O$_2$ equiv, whereas the FOX assay measured concentrations as low as 5 $\mu$M (Table 4). Nourooz-Zadeh et al. (*32*) also found that lower $-$OOH levels in edible oils were not measurable with the iodometric method as there appeared to be a certain $-$OOH threshold below which the assay could not quantify. This lower measurement limit may be due to or be exacerbated by the rapid increase in the signal once the sample is introduced into the reagent, the high mobile baselines, the high O$_2$ sensitivity, and the high intra-assay sample variation (*32*). The smaller standard errors associated with the FOX assay in the present work indicate that the FOX method is more reproducible than the iodometric assay, generating less sample-to-sample variation in plant extracts (Table 4).

For plant tissue extracts, the FOX assay is valuable as a relative measure of incipient lipid peroxidation. At this point, the assay does not distinguish between different types of LOOH molecules and is therefore a general indicator of the presence of LOOH. Nonetheless, the assay is relatively simple, requires inexpensive chemicals, produces results rapidly, is not sensitive to ambient O$_2$ or light levels, and does not require heating or special reaction conditions, and the lipid components do not need to be separated from the rest of the cellular homogenate. To distinguish LOOHs from the contribution of pigments and other compounds to the 560 nm signal, it is necessary that some samples are treated with TPP. Modifications to the FOX assay

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originating from this work include the sample preparation protocol and quantifying LOOH concentrations in samples during the linear phase of color development, i.e., exactly 10 min following reagent introduction into the sample. The value of the FOX assay from a physiological perspective lies in its ability to detect initial rather than more advanced fatty acid oxidation; hence, early membrane-associated stress events can be detected. Studies are presently being pursued to more precisely identify the nature of the LOOHs responsible for generating the FOX assay signal in plant extracts.

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