Optimization of Enzymatic Assay for the Measurement of Lipoxygenase Activity in Organic Solvent Media

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ABSTRACT: Lipoxygenases (LOX; EC 1.13.11.12) are an important class of non-heme iron enzymes that catalyze the di-oxidation of PUFA to hydroperoxy FA, which can be measured by the xylenol orange method (FOX). To determine the enzymatic production of these FA in organic solvent media, the FOX assay was optimized using the standard cumene hydroperoxide. An increase in the proportion of methanol from 0 to 75% in the FOX reagent resulted in a 93% increase in the molar absorption coefficients at 560 nm. In addition, the presence of linoleic acid in the cumene hydroperoxide sample enhanced the formation of the FOX complex, resulting in a 50% increase in the sensitivity of the method. Moreover, when perchloric acid was used, the source of ferrous ions and presence of denatured LOX had little effect on the sensitivity of the FOX assay whereas sensitivity decreased by 40–46% with sulfuric acid. The overall results demonstrated that the modified FOX assay may be used for the precise and accurate measurement of hydroperoxy FA obtained by LOX activity in organic solvent media.

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KEY WORDS: FOX assay, lipid hydroperoxides, lipoxygenase, organic solvent media, xylenol orange.

Lipoxygenases (LOX; EC 1.13.11.12) are an important class of non-heme iron enzymes that catalyze the regio- and stereospecific di-oxidation of PUFA containing a *cis,cis*-1,4-pentadiene moiety to hydroperoxy-FA, considered as precursors of flavor compounds. In addition, LOX are ubiquitously found in plants, microorganisms, and various animal tissues, where they are implicated in physiological activities (1).

Most spectrophotometric methods reported for the determination of LOX activity were developed for an aqueous medium, with the most common being based on the absorption of hydroperoxy-FA containing a *cis,cis*-1,4-pentadiene moiety at 234 nm (2); however, this method remains limited by its inability to detect hydroperoxides that lack a conjugated diene chromophore (3). The ferrous oxidation assay, using xylenol orange (FOX) or ferrous thiocyanate, was reported as an alternative spectrophotometric method for the determination of lipid hydroperoxides, with the latter assay showing lower sensitivity compared with the FOX assay (4) and used for the quantification of high amounts of lipid hydroperoxides.

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The FOX assay is based on the oxidation of ferrous ions (Fe^{2+}) by hydroperoxides into their ferric counterparts (Fe^{3+}), which, in turn complex with the xylenol orange salt to form a chromophore that absorbs at 560 nm (5), with a molar absorption coefficient (ε_{560}) of 4.5×10^5 M^{-1} cm^{-1} for lipid hydroperoxides in a methanol-based reagent (6). To measure hydroperoxides in liposome or lipoprotein suspensions, Jiang *et al.* (7) also used methanol in the preparation of the FOX reagent. In addition, the specificity of the FOX assay to measure lipid hydroperoxides in an extract can be improved by the addition of triphenylphosphine (TPP) (8,9), and its sensitivity can be increased by the use of sucrose (10) or the replacement of sulfuric acid by perchloric acid (11).

Over the last 10 yr, LOX biocatalysis in organic solvent media has gained great interest (12–14). Numerous advantages are associated with biocatalysis in nonconventional media, including a decrease in the rate-limiting depletion of oxygen, changes in enzyme specificities, and increases in the thermostability of enzymes and the solubility of hydrophobic substrates (15). Nonpolar solvents are generally considered as appropriate reaction environments for biocatalysis in organic solvent media (12–14,16); however, when such solvents are mixed with the polar FOX reagent, the reaction assays can become turbid and hence limit the accuracy of the spectrophotometric measurements. In addition, the FOX method can have a certain degree of interference due to the presence of LOX, a non-heme iron protein that could potentially complex with xylenol orange.

The overall objective of the present study was to optimize the FOX assay to rapidly and accurately quantify hydroperoxides, obtained by LOX activity, in organic solvent media, and to evaluate its performance as an enzymatic assay. The specific objective was to optimize the FOX assay in terms of various parameters including the methanol/water ratios; degree of solvent degassing; hexane/FOX reagent ratios; the presence of different reagent components including BHT, sulfuric acid, or perchloric acid in combination with ferrous sulfate or ammonium ferrous sulfate; and its specificity and substrate selectivity.

MATERIALS AND METHODS

Materials. Commercial soybean LOX type I-B (131,000 U/mg solid, with a specific activity of 15.72 μmol oxidized linoleic acid/min/mg solid), xylenol orange salt [3,3′-bis(*N,N*-di(carboxymethyl)aminomethyl)-*o*-cresol-sulfonephthalein sodium salt], BHT (2,6-di-*tert*-butyl-*p*-cresol), and cumene

[(1-methylethyl)benzene] hydroperoxide were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic (*cis*-9,*cis*-12-octadecadienoic), α-linolenic (*cis*-6,*cis*-9,*cis*-12-octadecatrienoic), and arachidonic (*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic) acids were purchased from Nu-Chek-Prep Inc. (Elysian, MN). Ammonium thiocyanate, ammonium ferrous sulfate hexahydrate, and ferrous sulfate of ACS grade as well as tris(hydroxymethyl)aminomethane, HCl, TPP, and all the solvents used were purchased from Fisher Scientific (Pittsburgh, PA). Meso-tetraphenyl-porphine copper(II) and perchloric acid were purchased from Aldrich Chemical Co. (Milwaukee, WI) whereas sulfuric acid was obtained from LabChem Inc. (Pittsburgh, PA). Sodium phosphate (Na_2HPO_4) was purchased from ACP Chemicals (Montréal, Canada). The LC-Si cartridges were obtained from Supelco (Bellefonte, PA).

FOX assay. The FOX assay was carried out according to a modification of the procedure described by Jiang *et al*. (7). To prepare the FOX reagent, methanol and deionized water were degassed independently for 10 min using a Bransonic ultrasonic cleaner (Model 3510; Bransonic, Danbury, CT). The FOX reagent was composed of 250 μM Fe^{2+} ions, 25 mM sulfuric acid, and 100 μM xylenol orange in a methanol/water mixture (90:10, vol/vol) and used within 4 h after preparation. FOX assays were performed in triplicate. FOX assays were initiated by the addition of 25 μL of hydroperoxide sample in hexane to 2.0 mL of the FOX reagent. Ethanol (96%) was then added to the mixture to complete the volume to 2.1 mL. The mixture was stirred with a Vortex (Genie 2; Fisher Scientific) for 10 s and stirred again every 5 min for a total reaction time of 15 min. Absorbance was measured after 20 min of reaction time at 556 nm against a blank assay containing all the components in the FOX assay except the hydroperoxide, using a Beckman spectrophotometer (Model 650; Beckman Instruments, Inc., Fullerton, CA). A calibration curve was used based on a wide range of concentrations of cumene hydroperoxide, ranging from 0.025 to 0.4 mM (7).

Optimization of FOX assay. (i) Methanol/water ratio and degassing. FOX assays were optimized by varying the methanol/water mixture ratios, including 0:100, 45:55, 60:40, 75:35, and 90:10 (vol/vol), of the FOX reagent. The stability of the FOX reagent was also assessed by degassing the water and methanol for different time periods ranging from 0 to 15 min.

(ii) Effect of BHT and linoleic acid. The effect of 4 mM BHT on the FOX assay was also investigated in the absence and presence of 7 mM linoleic acid. BHT as well as linoleic acid was prepared in methanol and added to the hydroperoxide samples before the FOX assay. To ensure that an increase in the sensitivity of the FOX assay was due to the presence of linoleic acid in the samples and not its potential auto-oxidation, all samples were blanked against a constant concentration of linoleic acid.

(iii) Effect of acid and Fe^{2+} source. The effects on the FOX assay of perchloric acid as well as sulfuric acid at a final concentration of 25 and 85 mM, respectively, and either ammonium ferrous sulfate or ferrous sulfate as a Fe^{2+} source at a final concentration of 250 μM were also investigated in the presence and absence of denatured LOX (3.2 μg). Denaturation was achieved by incubating LOX in Tris-HCl buffer (0.1 M, pH 9.0) for 5 min at 100°C. The denatured enzyme was added to the hydroperoxide sample before the FOX assay.

Effect of TPP reduction on the specificity of FOX assay. The specificity of the FOX assay was assessed, using TPP, according to the procedure described by Nourooz-Zadeh (5). Calibration curves of cumene hydroperoxide, in the presence and absence of TPP, were obtained as follows. A 25-μL methanol solution, with and without 10 mM TPP, was added to an equal amount of the sample. The mixture was stirred for 30 min before adding 2 mL of FOX reagent. Ethanol was added to complete the volume to 2.1 mL. Samples were analyzed spectrophotometrically at 560 nm, and the difference in absorbance between the TPP-treated and the untreated samples was used to determine the measurements of lipid hydroperoxides. The TPP reduction effect was also investigated for hydroperoxide samples containing 7 mM linoleic acid.

Selectivity of FOX assay. To investigate the substrate specificity of the assay, standard curves of different FA hydroperoxides were prepared using the modified FOX method with perchloric acid and ferrous sulfate in the reagent preparation. To prepare the standard curves, 14 mM linoleic acid was also added to all samples.

Preparation of FA hydroperoxide standards. Following the procedure described by Schieberle *et al*. (17), photooxidation of linoleic, linolenic, and arachidonic acids yielded their corresponding hydroperoxides, hydroperoxyoctadecadienoic (HPOD), hydroperoxyoctadecatrienoic (HPOT), and hydroperoxyeicosatetraenoic (HPETE) acids, respectively. The reaction medium was composed of 1 g linoleic, linolenic, or arachidonic acid, 22 mL of benzene, and 2 mg of meso-tetraphenyl porphine as a sensitizer. Photooxidation was performed at 15°C by bubbling a stream of O_2 into the reaction medium, which was stirred for 70 min while being subjected to a 500 W halogen light, through a 1 cm layer of deionized water used to filter IR radiation. The separation of hydroperoxides from the residual FA was performed following the method of Toschi *et al*. (18), using LC-Si cartridges. Photooxidized and cumene hydroperoxides were quantified with the ferrous thiocyanate assay as described by Wurzenberger and Grosch (4).

LOX assay in organic solvent media. The enzymatic reaction was initiated by the addition of 20 μL of LOX suspension (8 to 24 μg protein in 0.1 M Tris-HCl buffer; pH 9.0) to 0.98 mL of the hexane reaction mixture, containing 7 mM linoleic acid. The mixture was gently stirred at room temperature. At different time intervals (0 to 5 min), an aliquot was withdrawn and the concentration of HPOD was quantified, according to the modified FOX assay using perchloric acid and ferrous sulfate in the reagent preparation. Aliquots of 25 to 200 μL were taken from the 1-mL reaction medium and analyzed for the presence of HPOD. All experiments were performed in triplicate. After mixing and transferring the FOX assay mixture to the spectrophotometric cells, a sufficient period of time (5 min) was allowed for the dissipation of the formed emulsion. In addition, freshly prepared

solutions of linoleic acid and the respective HPOD were used to obtain the standard curves.

RESULTS AND DISCUSSION

Preparation of reaction medium for FOX assay. Depending on the nature of the sample, different ratios of methanol/water in the FOX reagent have been used to enhance hydroperoxide solubility and avoid turbidity in the assay (19,20). The results (Fig. 1) show that an increase in the proportion of methanol from 0 to 75% (vol/vol) in the FOX reagent produced a 93% increase in the molar absorption coefficients (ε_{560}) for cumene hydroperoxide; however, further increases in the proportion of methanol to 90% (vol/vol) slightly decreased the ε_{560} value from 197,250 to 181,780 M^{-1} cm^{-1}. These experimental findings clearly indicate that the presence of a high proportion of methanol enhanced the sensitivity of the FOX assay, which could be attributed to an increase in the solvation of hydroperoxides (21).

Since the occurrence of turbidity at lower methanol proportions (≤60%) may have affected the validity of the assay, as indicated by the lower correlation coefficients (r^2) for the linear regression of the calibration curves (≤0.95), the volume of the hydroperoxide sample prepared in hexane was increased from 25 to 200 μL. Although an increase in the ratio of non-polar hexane to polar xylenol reagent could have potentially produced turbidity, the experimental results (data not shown) demonstrated the absence of turbidity with the 200-μL sample volume, as indicated by the corresponding r^2 value of 0.997; this high value validated the selection of using a methanol/water ratio of 90:10 (vol/vol) in the reagent for the FOX assay. Similarly, Eymard and Genot (20) used a methanol/water mixture at a ratio of 91:9 (vol/vol) for quantifying fish lipid hydroperoxides. However, the experimental data (Fig. 1) do not show a relationship between cumene hydroperoxide and absorbance for methanol content below 60%. The absence of such linear relationship may be due to a lower solubility of cumene hydroperoxide in low concentrations of methanol. Nevertheless, Hermes-Lima *et al.* (22) used a lower methanol concentration (10%) for the determination of extracted lipid hydroperoxides from animal tissues.

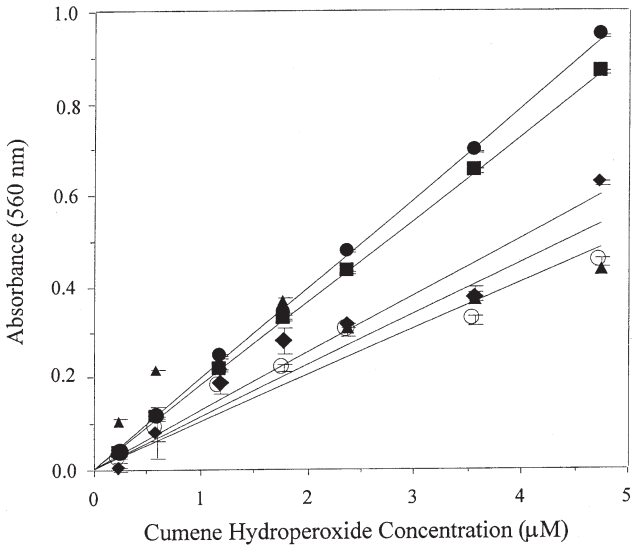

FIG. 1. Effect of methanol content of the xylenol reagent on the sensitivity and reproducibility of the calibration curve for hydroperoxide quantification in hexane. Cumene hydroperoxide calibration curves were performed in xylenol orange (FOX) reagent composed of 100% deionized water (○), and in 45 (▲), 60 (◆), 75 (●), and 90% methanol (■) in deionized water. Error bars represent mean ± SD (n = 3).

In using the 90:10 methanol/water ratio for FOX assay, the absorbance of the ferric ion-xylenol orange (Fe-XO) complex was found to be relatively unstable, with more than 20% variation within a 2 h incubation (data not shown), which may have been due to the oxidation of lipids by oxygen. To improve the stability of the Fe-XO complex, the FOX reagent was degassed for periods ranging from 0 to 15 min. The experimental findings (data not shown) indicated that the stability of the FOX reagent increased with the degree of degassing. The color developed by the reagent, after being degassed for 10 min, was stable upon reaction at room temperature over a period of 2 h. However, this increase in stability was associated with a concomitant decrease in the sensitivity of the FOX assay by 20 and 29%, corresponding to 2 and 15 min of degassing, respectively. This concomitant decrease in FOX sensitivity upon degassing may have been due to the removal of dissolved oxygen in the reagent solution, which would otherwise have played a role in the oxidation complex of the chain radical of the xylenol reagent (5). Since a compromise between the two parallel phenomena, i.e., a decrease in sensitivity with a concomitant increase in stability, was necessary, the FOX reagent was degassed for 10 min prior to use throughout the study.

Effect of BHT addition on FOX assay. Wolff (6) reported that the addition of BHT, a chain-breaking antioxidant, to the FOX reagent prevented the oxidation of lipids during analysis. However, the use of BHT in the FOX assay still remains ambiguous: Some authors recommend the addition of BHT (5,7,23), whereas others discourage it (19,20,22). The effect of 4 mM BHT in the reagent on the FOX assay was investigated in the absence and presence of 14 mM linoleic acid.

Figure 2 shows that in the absence of linoleic acid, BHT has no effect on the ε_{560} value of cumene hydroperoxide. The results also show that in the presence of 14 mM linoleic acid, BHT produced a 32% decrease in the ε value of cumene hydroperoxide, whereas in the absence of BHT, a 50% increase in the ε value was observed. These findings suggest that in the absence of BHT, nonoxidized lipids, such as linoleic acid, can promote an oxidation chain reaction of the Fe^{2+} ions and their subsequent complexation with the xylenol orange compound thereby increasing the ε value of the FOX assay whereas in the presence of BHT, the opposite effect occurs, preventing the overall oxidation of Fe^{2+} ions by both linoleic acid and hydroperoxides.

Although it is difficult to propose a mechanism to explain the effect of linoleic acid on the formation of Fe^{3+} ions, the experimental findings (Fig. 2) are in agreement with those reported in the literature, where lower ε values were found in the

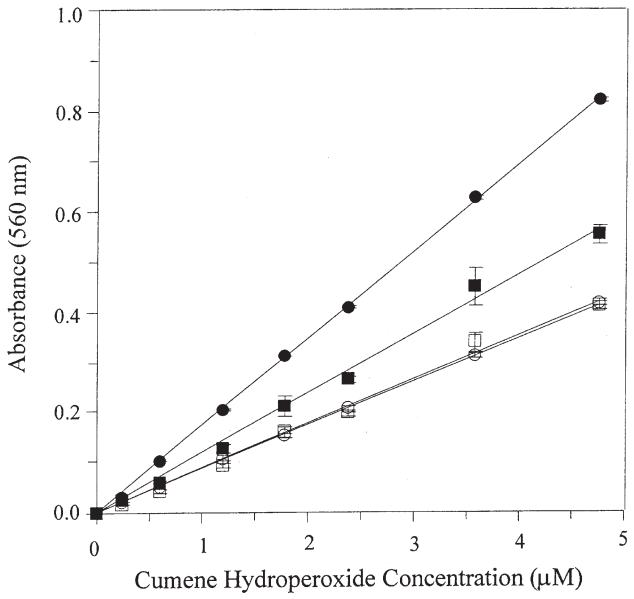

FIG. 2. Effect of BHT and linoleic acid on the sensitivity and reproducibility of the calibration curve. Calibration curves were performed using the FOX reagent mixture in the presence of BHT (□ and ■) and absence of BHT (○ and ●). Linoleic acid was added to the hydroperoxide sample for the calibration curves with the filled symbols (● and ■). For abbreviation see Figure 1. Error bars represent mean ± SD (n = 3).

presence of BHT, which could be attributed to limited oxidation of lipids and stabilization of the intermediate alkoxyl radicals of the FOX colorimetric reaction (19,22). The overall results suggest that the presence of linoleic acid in the cumene hydroperoxide samples produced more accurate measurements for the construction of a calibration curve whereas the addition of BHT to the FOX reagent led to a decrease in the sensitivity of the assay in the presence of linoleic acid and was therefore omitted for all subsequent trials.

Optimization of reactants for FOX assay. Several studies have indicated that the absorbance of the ferric ion-xylenol orange (Fe-XO) complex shows a strong dependence on the pH of the assay, with a maximal absorbance obtained in the pH range of 1.7–1.8 (9). The effect of sulfuric acid and perchloric acid at a final concentration of 25 and 85 mM, respectively, on the FOX assay was investigated, using either ferrous sulfate or ammonium ferrous sulfate as a source of Fe^{2+} ions. The experimental results (data not shown) indicated that the overall absorbance profiles of the spectrophotometric scans of several different FOX colorimetric reaction mixtures consisted mainly of two peaks, the first peak with a maximum absorbance (λ_{max}) between 440 and 453 nm, corresponding to the xylenol orange reactant, and the second peak with a λ_{max} between 553 and 558 nm for the Fe-XO complex. The results also showed that when using ammonium ferrous sulfate as a source of Fe^{2+} ions, the λ_{max} of the Fe-XO complex was at 553 and 558 nm with sulfuric acid and perchloric acid, respectively while with ferrous sulfate as a source of Fe^{2+} ions, the λ_{max} was at 556 nm with both acids.

Table 1 shows that, in the presence of perchloric acid, the molar absorption coefficients (ε_{560} values) of the Fe-XO complex were 105,110 and 110,380 M^{-1} cm^{-1} with ferrous sulfate and ammonium ferrous sulfate, respectively. However, in the presence of sulfuric acid, the ε_{560} value increased by a factor

TABLE 1
Molar Absorption Coefficients of the Ferric Ion-Xylenol Orange Complex for Cumene Hydroperoxide Using Different Xylenol Orange Reagents

Reagent conditions[a]	ε_{560} (M^{-1} cm^{-1})[b]	pH Δ[c]	pH[d]	
			Before	After
Perchloric/fs	105,110 (±1,030)[e]	0.47	1.68	2.15
Perchloric/ammonium fs	110,380 (±890)[e]	0.29	1.6	1.89
Perchloric/adjusted ammonium fs	118,880 (±120)[e]	0.32	1.75	2.07
Perchloric/fs/LOX[f]	109,430 (±3,320)[e]	0.27	1.69	1.96
Sulfuric/fs	166,570 (±1,410)[e]	0.23	1.63	1.82
Sulfuric/ammonium fs	76,570 (±250)[e]	0.04	1.66	1.70
Sulfuric/adjusted ammonium fs	88,480 (±130)[e]	0.08	1.75	1.83
Sulfuric/fs/LOX[f]	101,150 (±370)[e]	0.61	1.73	2.34
Sulfuric/fs/TPP/with LA[g]	66,740 (±2,470)[e]		Not applicable[h]	
Sulfuric/fs/TPP/without LA[g]	103,250 (±2,540)[e]		Not applicable[h]	

[a]The FOX (xylenol orange) reagent was prepared using either perchloric or sulfuric acid as acid and ferrous sulfate (fs) or ammonium fs as a source of ferrous ions. The term "adjusted" indicates that the pH of the reagent was adjusted before the assay.
[b]The molar absorption coefficient (ε) was calculated using cumene hydroperoxides. Linoleic acid was present in all samples at a 7 mM concentration. All curves were performed in triplicate.
[c]The pH variation was defined as the difference between the pH values of the reagent mixture before and after reaction.
[d]The pH was measured before the addition of the hydroperoxide samples and after the colorimetric assay.
[e]The SE of the curve was calculated from the average of triplicate samples using SigmaPlot (SPSS, Chicago, IL).
[f]Lipoxygenase (LOX) denatured protein (3.2 μg) was added to the standard to evaluate the effect of the ferric ions of the enzyme.
[g]Linoleic acid (LA).
[h]The differences in the absorbance of the samples treated and nontreated with triphenylphosphine (TPP) were used for the construction of calibration curves and to determine the molar absorption coefficient.

of 2.2 with ferrous sulfate compared with that obtained with ammonium ferrous sulfate. These results indicate that in the presence of perchloric acid, the source of the Fe^{2+} ions had no effect on the sensitivity of FOX assay whereas with sulfuric acid, the ε_{560} values were dramatically affected, suggesting that the low ε_{560} value obtained with ammonium ferrous sulfate could have been related to a lack of compatibility between these two compounds. The results also show that when the initial pH of the FOX reagent mixture was adjusted to 1.75 with sodium phosphate powder in the presence of ammonium ferrous sulfate, the ε_{560} values slightly increased by a factor of 1.1 and 1.2 with perchloric acid and sulfuric acid, respectively. However, since ammonium ferrous sulfate showed a certain degree of interference in the FOX assay, ferrous sulfate was used throughout the study. In the presence of denatured LOX possessing Fe^{2+}/Fe^{3+} ions at its active site, the ε_{560} value of the FOX assay slightly increased by 4% in the presence of perchloric acid and dramatically decreased by 40% with sulfuric acid compared with the ε_{560} values obtained in absence of the denaturated enzyme. The experimental results also showed that in the presence of denatured LOX, variations in the pH of the reaction were more important with sulfuric acid than with perchloric acid. The high degree of interference of the LOX protein in the FOX assay with sulfuric acid may be explained by the lower capacity of the medium to adjust itself to the dramatic pH changes occurring during the reaction compared with perchloric acid (11).

In contrast to the literature (9,11), the overall experimental findings (Table 1) indicate that there was no correlation between the sensitivity of the assay and the pH of the reagent mixture. Although variations in pH before and after the reaction were important with both perchloric acid and sulfuric acid, smaller changes in the ε_{560} values were observed in the presence of perchloric acid. Gay and Gebicki (11) also reported the use of perchloric acid for the FOX assay at a lower pH optimum of 1.1 and with limited sensitivity to minor changes in pH. Perchloric acid was hence used in the optimized FOX assay.

Effect of TPP on the specificity of the FOX assay. Nourooz-Zadeh *et al.* (24) reported that the use of TPP could increase the specificity of the FOX assay by reducing lipid hydroperoxides into alcohols so that only the potentially interfering substances, such as ferric ions or phenolic compounds, are measured (5,8). Differences in the absorbances, obtained using the FOX assay, between TPP-treated and untreated samples would therefore provide a specific quantification of lipid hydroperoxides (9). Figure 3 shows the calibration curves of TPP-treated and untreated cumene hydroperoxide samples in the presence and absence of 14 mM linoleic acid. The results indicate that in the absence of linoleic acid, the TPP-treated cumene hydroperoxide samples did not absorb at 560 nm; however, in the presence of linoleic acid, a relative ε_{560} of 98,680 M^{-1} cm^{-1} was observed, suggesting that the presence of linoleic acid greatly affected the reduction of cumene hydroperoxide by TPP. Nourooz-Zadeh *et al.* (24) indicated that H_2O_2, used as a reference in the FOX assay, was not reduced by the TPP.

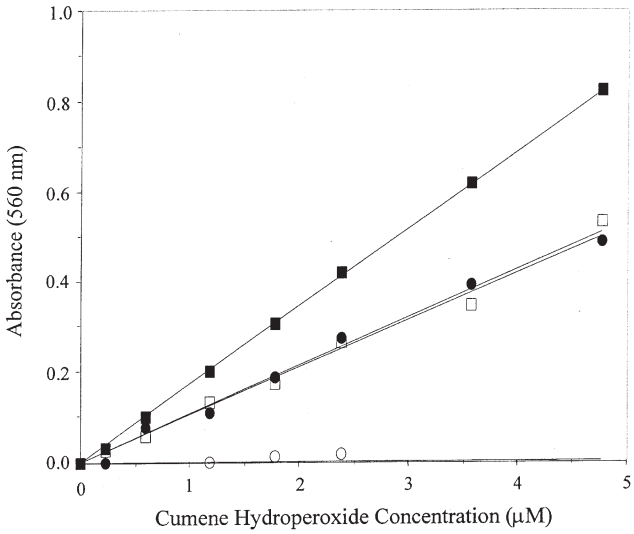

FIG. 3. Effect of triphenylphosphine (TPP) on the specificity of the FOX assay. The absolute absorbance of the colorimetric reaction calibration curve was measured with samples treated with TPP (○ and ●) and without TPP (□ and ■). Linoleic acid (14 mM) was added to the hydroperoxide sample for the calibration curves with the full symbols (● and ■). All other parameters of the FOX reagent remained unchanged. For abbreviation see Figure 1. Error bars represent mean ± SD (n = 3).

Selectivity of the FOX assay. To investigate the specificity of the FOX assay, standard curves of different FA hydroperoxides were prepared using perchloric acid and ferrous sulfate in the FOX reagent. The results (Table 2) indicate a high dependence of the FOX assay on the nature of the hydroperoxides. The highest ε_{556} value, 172,540 M^{-1} cm^{-1}, was obtained for linoleic acid hydroperoxides while those of highly oxidizable lipids, including linolenic and arachidonic acids, showed lower ε_{556} values of 93,060 and 81,510 M^{-1} cm^{-1}, respectively. The ε_{556} values (Table 2) for cumene hydroperoxide were within the range of those reported by Gay and Gebicki (11) and were much higher than those reported by Michaels and Hunt (25) and Gay *et al.* (21). In addition, the overall ε values, estimated for the lipid hydroperoxides, were higher than those of 47,000 and 51,200 M^{-1} cm^{-1} previously reported in the literature by Jiang *et al.* (7) and Gay and Gebicki (11), respectively. The discrepancies between the ε values obtained in the present study and those reported in literature (7,11,21) could be related to the nature of the solvent, the reference source, and the assay conditions as well as the presence of a linoleic acid chain radical-promoting compound.

The results (Table 2) suggest that an increase in the degree of unsaturation of the FA hydroperoxides affected the sensitivity of the FOX response. To interpret this phenomenon, the number of Fe^{3+} ions, generated by each –OOH group of the different hydroperoxides, was estimated using the ε_{560} of 20,100 M^{-1} cm^{-1} obtained for the ferric ions of the Fe-XO complex reported by Gay *et al.* (26). Table 2 shows that linoleic acid hydroperoxides produced twice the amount of Fe^{3+} ions per molecule as the amounts produced by linolenic or arachidonic acid hydroperoxides. Overall, the ratios of Fe^{3+} ions per cumene and

TABLE 2
Molar Absorption Coefficients of the Xylenol Orange Assay[a] and the Relative Proportion of Yielded Fe^{3+} Ions

Reference[b]	Molar absorption coefficient (M^{-1} cm^{-1})	(Fe^{3+}:HP)[c] ratio
Cumene HP	111,800 (±7,590)[d]	5.6
HPOD	172,540 (±9,700)[d]	8.6
HPOT	93,060 (±2,680)[d]	4.6
HPETE	81,510 (±2,440)[d]	4.1

[a]The FOX (xylenol orange) assay conditions are described in the Materials and Methods section.
[b]The references, used for the construction of the calibration curve, were cumene hydroperoxide (cumene HP) and hydroperoxides (HP) of linoleic (HPOD; hydroperoxyoctadecadienoic), linolenic (HPOT; hydroperoxyoctadecatrienoic), and arachidonic (HPETE; hydroperoxyeicosatetraenoic) acids.
[c]The ratio of Fe^{3+}:HP was defined as the number of Fe^{3+} ions generated by the reference and calculated as the extinction coefficient of the complex over that of the ferric ions, according to Gay *et al.* (26), in the same reagent.
[d]The SE was calculated using SigmaPlot software (SPSS, Chicago, IL).

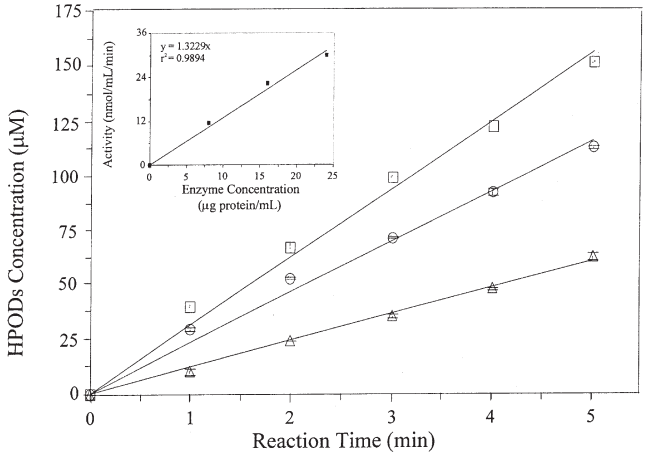

FIG. 4. Kinetics of the formation of hydroperoxides of linoleic acid (hydroperoxyoctadecadienoic acids, HPOD) obtained by lipoxygenase activity, using 8 (△), 16 (●), and 24 (■) mg protein/mL reaction, determined using the FOX assay. Inset: v_i is the initial velocity of end product production, expressed in nmol of HPOD per mL reaction volume per min. Error bars represent mean ± SD (n = 3).

lipid hydroperoxides were found to be higher than those reported in literature (7,11,21,26), which may be due to the different methods used for quantification of hydroperoxides. Using various hydroperoxide references, Gay *et al.* (26) attempted to explain the different ratios of Fe^{3+} ions per hydroperoxide molecule on the basis of the mechanism for the production of radicals, but these authors did not provide a definite mechanism for the hydroperoxide references that produced higher amounts of Fe^{3+} ions, such as the cumene hydroperoxide. However, it has been reported (5,11,21) that alkoxyl radicals (Eq. 1) formed during the colorimetric assay produce methyl free radicals by β-elimination, which could promote, in the presence of oxygen, the formation of different radical species capable of oxidizing Fe^{2+} ions.

$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO^{\bullet} + OH^{-} \quad [1]$$

Measurement of LOX activity. LOX activity has been shown to be relatively higher in hexane media compared with other organic solvents (13), which can be explained by its lower polarity (16) that prevents it from stripping the essential water layer surrounding the enzyme molecule (27).

The kinetics of HPOD production by LOX were investigated, using a range of enzyme concentrations varying from 8 to 24 μg/mL, in the hexane reaction medium. The HPOD, produced at defined time intervals, were quantified by the modified FOX assay as described previously. The results (Fig. 4) show that a linear increase in HPOD concentration occurred with reaction time, demonstrating the high sensitivity of the assay even at the initial velocity stage. In addition, the initial velocity stage of the enzymatic reaction implied that the substrate was partially converted (<10%) into its respective end product, this condition being crucial as the presence of linoleic acid as substrate was shown to increase the ε_{556} of the FOX assay. Hence, a defined amount of linoleic acid (7 to 14 mM) was added to all samples used for the construction of calibration curve.

The initial velocity of HPOD production increased proportionally with enzyme concentration (insert of Fig. 4), indicating that the reaction was kinetically catalyzed by LOX and not by auto-oxidation. Although the determination of LOX activity in the nonpolar organic solvent medium was reported previously (12–14), the overall experimental results reported suggest that the modified FOX assay may be a convenient, rapid, and reproducible method for LOX assay in organic solvent media.

The experimental data from this study showed that the modified FOX assay could be used with acceptable precision and sensitivity to quantify LOX activity in hexane. However, a proper calibration curve, based on using the same components that were in the reaction medium, is mandatory since the presence of nonperoxidized lipids could dramatically influence the sensitivity of the assay.

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