Lipid Hydroperoxide Determination in Dark Chicken Meat through a Ferrous Oxidation−Xylenol Orange Method

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A ferrous oxidation−xylenol orange (FOX) method was adapted to measure lipid hydroperoxides (LHP) in raw and cooked dark chicken meat. Its applicability was evaluated using samples with different α-tocopherol contents or unsaturation degrees (both modulated by dietary supplementation). The FOX assay can work as an induced method because there is some oxidation of the sample extract during the incubation of the reaction. Consequently, it allows assessment of sample susceptibility to oxidation (response after some hours of incubation) and comparison of samples that are highly oxidized or readily susceptible to oxidation through their absorbance after 30 min of incubation. It is highly specific for LHP and showed a linear relationship between volume of meat extract and absorbance. However, the most suitable volume of extract and incubation time must be studied for each kind of sample. The use of butylated hydroxytoluene during this incubation is strongly discouraged because it attenuated the reaction by radical stabilization, thus diminishing Fe(III) formation and leading to a lower response.

Keywords: *Chicken meat; FOX; lipid hydroperoxide determination; xylenol orange*

INTRODUCTION

Lipid oxidative deterioration in meat is likely to occur during product processing, distribution, and storage. Primary products of lipid oxidation include lipid hydroperoxides (LHP). Their level rises as the food deteriorates, but as deterioration continues, this level reaches a plateau and then falls, as a consequence of their reaction with other food components (Gardner, 1979) or their evolution to secondary oxidation products (Moore and Roberts, 1998). Hence, lipid oxidation gives rise to a series of detrimental changes leading to the generation of potentially toxic compounds and undesirable flavors, as well as to the loss of nutritional value (Asghar et al., 1988; Chow, 1992; Brown and Jessup, 1999; Guardiola et al., 1996; Kubow, 1990; Smith and Johnson, 1989). It is then necessary to find accurate and reproducible methods to follow up lipid oxidation in muscle foods.

LHP value is determined in meat products to evaluate their oxidative stage and thus their quality. A great variety of methods have been proposed to assess LHP in biological samples and foods. Among them, the methods of the AOAC (1997) and the AOCS (1989), both through iodometric titrations, are frequently used, despite their lack of sensitivity and some important sources of interference (liberation of iodine by air oxidation of the potassium iodide and reaction of iodine with some lipid components, especially iodine addition to fatty acid double bonds). To improve the sensitivity of these methods, several iodine spectrophotometric and potentiometric methods have been developed (Hicks and Gebicki, 1979; Lezerovich, 1985; Oishi et al., 1992). In addition, some chromatographic (HPLC and GC) and spectroscopic (NMR and ESR) methods with higher reproducibility, sensitivity, and selectivity have been proposed (Frankel et al., 1990; van Kuijk et al., 1990; Yamamoto et al., 1990; Yang, 1992; Yang et al., 1991). However, these methods are not easily adapted to routine screening of large numbers of samples. Therefore, there is a need for simple, reproducible, sensitive, and selective methods, and several attempts have been made to develop them (Gray and Monahan, 1992; Miyazawa et al., 1993; Yang, 1992). Two of these attempts have attracted special attention: (i) determination of LHP by Fourier transform infrared (FTIR) spectroscopy, which has been successfully applied to edible oils (Ma et al., 1997; van de Voort et al., 1994); and (ii) the ferrous oxidation−xylenol orange (FOX) method, which, in addition to edible oils, has been successfully applied to food and biological samples (Burat and Bozkurt, 1996; Hermes-Lima et al., 1995; Jiang et al., 1991, 1992; Nourooz-Zadeh, 1998; Nourooz-Zadeh et al., 1994, 1995, 1997; Shantha and Decker, 1994; Södergren et al., 1998; Wolff, 1994). This method consists of the peroxide-mediated oxidation of ferrous ions in an acidic medium containing the dye xylenol orange (XO), which binds the resulting ferric ions to produce a blue-purple complex with a maximum of absorbance between 550 and 600 nm. According to Jiang et al. (1992), 3 mol of Fe (III) are formed per mole of LHP.

$$\text{Fe(II)} \xrightarrow{\text{LHP}} \text{Fe(III)} \xrightarrow{\text{XO}} \text{Fe(III)}-\text{XO (1:1 complex)}$$

The FOX method has been reported to have high sensitivity, being comparable to or even better than that

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of the iodometric assay through spectrophotometry (Jiang et al., 1991, 1992; Nourooz-Zadeh et al., 1995) and the official International Dairy Federation (IDF) method (Burat and Bozkurt, 1996; Shantha and Decker, 1994). With regard to the specificity of the method, it has been shown that acyl and alkylhydroperoxides and H_2O_2 have the same reactivity in the FOX assay, whereas endoperoxides are almost or totally unreactive (Jiang et al., 1992). However, in liposomes, low-density lipoproteins (LDL), and edible oils, reacting compounds were shown to be mainly LHP from the sample, because 80−85% of the color yield was due to non-H_2O_2 hydroperoxides (Jiang et al., 1992; Nourooz-Zadeh et al., 1994, 1995).

In addition, the FOX method has been reported to be precise and simple (Burat and Bozkurt, 1996; Jiang et al., 1991, 1992; Nourooz-Zadeh, 1998; Nourooz-Zadeh et al., 1995; Shantha and Decker, 1994; Södergren et al., 1998). However, there is a drawback: both (i) the amount of sample extract needed to obtain a quantifiable response and (ii) the incubation time for the reaction to be completed depend on the kind of sample (Hermes-Lima et al., 1995), so both have to be assayed for each new case.

Here we examine whether the FOX assay can be used to determine LHP values in raw and cooked dark chicken meat. We chose the method proposed by Hermes-Lima et al. (1995) as it has been applied to animal tissues and it does not require sample lipid extraction, thus minimizing the chance of LHP loss during solvent evaporation. This loss occurs even when solvent evaporation is carried out under a nitrogen stream (Nourooz-Zadeh et al., 1994). In addition, to assess the occurrence of lipid peroxidation during the assay itself, we also report data on the effect of nitrogen and butylated hydroxytoluene (BHT) on the colorimetric reaction.

MATERIALS AND METHODS

Reagents and Standards. BHT, 13(*S*)-hydroperoxy-(9*Z*,-11*E*)-octadecadienoic acid (HPODE), pyrogallol, *dl*-α-tocopherol, and xylenol orange (XO) (ACS grade) were from Sigma (St. Louis, MO). Cumene hydroperoxide (CHP) and triphenylphosphine (TPP) were purchased from Aldrich (Gillinham, Dorset, U.K.). Sulfuric acid (96%) and ammonium ferrous sulfate hexahydrate (both of analytical grade) were obtained from Panreac (Barcelona, Spain). Methanol used in the FOX method and α-tocopherol analysis was of HPLC grade (SDS, Peypin, France), whereas that used in fatty acid determination was of analytical grade (Panreac). Distilled deionized water was used throughout. XO, CHP, and ferrous ammonium sulfate solutions were prepared immediately before use.

Sample Preparation. Dark chicken meat samples were obtained from two factorial designs. The first experiment assessed the influence of dietary fat source (linseed, sunflower and oxidized sunflower oils, and beef tallow; 6% of fat supplementation), α-tocopherol [0 or 225 mg of *dl*-α-tocopheryl acetate (α-TA)/kg of feed], and ascorbic acid (0 or 110 mg/kg of feed) supplementation on lipid oxidation in raw and cooked dark chicken meat (16 dietary treatments, 240 animals). The second experiment examined the effect of the dietary α-TA supplementation according to dose (75, 150, and 225 mg/kg of feed) and days of supplementation prior to slaughter (0, 10, 21, 32, and 43 days) (15 dietary treatments, 225 animals). For raw samples, legs were hand-deboned, ground (meat and skin), and vacuum packaged in plastic bags. For cooked samples, legs were hand-deboned, vacuum packaged, cooked, ground, and vacuum re-packaged. In the first experiment, samples were cooked in a pressure cooker at 80 °C for 35 min, and in the second, samples were cooked in an oven at 90 °C (95% relative humidity), to an internal temperature of 80 °C. Samples were stored at −80 °C until analysis. Both experimental designs were conducted in triplicate. In addition, some raw chicken legs were also purchased from local supermarkets. They were hand-deboned, ground, and immediately analyzed or vacuum packaged and stored at −20 °C until analysis.

FOX Method. Three grams of meat was weighed into a 50 mL centrifuge tube with a screw cap. Fifteen milliliters of cold HPLC grade methanol (−20 °C) was added, and the content was homogenized for 30 s at 12000 rpm using a Polytron PT 3000 (Kinematica, Lucerne, Switzerland). Homogenate was then centrifuged (3 min at 1400*g*), and the supernatant was removed for assay. In a new glass tube with a screw cap (13 × 100 mm), the following reagents were sequentially added (conducted in triplicate): 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic XO, 1100 − *X* μL of methanol (volume of methanol necessary to make up the final volume to 2 mL), and *X* μL of tissue extract (*X* stands for the volume of tissue extract that results in an absorbance value of 0.7−0.8). A blank was prepared using methanol instead of tissue extract. Final concentrations of reagents in the reaction mixture were 0.25 mM ferrous ammonium sulfate, 25 mM H_2SO_4, and 0.1 mM XO, in water/methanol (25:75). Reaction mixtures were incubated at room temperature for the time needed for the reaction to reach a stable end-point, and the absorbance at 560 nm was then read against the blank, using a Shimadzu UV-160A spectrophotometer. The LHP value of samples is expressed as milligrams of CHP per kilogram of meat, and it was determined with reference to an experimental linear regression curve performed with CHP as standard (0.2−21.2 nmol/mL of reaction mixture) ($Y = 4.11 \times 10^{-3} + 5.74 \times 10^{-2}X$, $r^2 = 0.9987$, where *Y* stands for the concentration in nmol/mL of CHP in the reaction mixture and *X* for the absorbance of the reaction mixture). All of the glassware used had been previously rinsed with distilled deionized water to eliminate interfering ions. All procedures were performed under attenuated light. When time course assays were carried out, the reaction took place in special optical glass cuvettes with Teflon caps. For tests in which cuvettes were filled with N_2, cuvettes were flushed with N_2, reagents were added to the cuvette after they had been mixed in a glass tube (13 × 100 mm), N_2 was flushed again, and the cuvette was immediately tightly capped (thus, oxygen was only partially removed from the reaction medium). In tests performed to check the specificity of the method or the influence of BHT on the reaction, a FOX reagent was prepared daily by mixing all components (ferrous ammonium sulfate, H_2SO_4, XO, methanol, and water) in a volumetric flask (see below).

Furthermore, it was checked that an increase in absorbance at 560 nm always involved a decrease in absorbance at 440 nm, which is the absorption maximum corresponding to free XO.

Specificity of the Method. Eighty microliters of a methanolic sample extract (raw or cooked sample from diets supplemented only with sunflower oil or beef tallow, $n = 2$) was incubated with 20 μL of 10 mM methanolic TPP for 30 min at room temperature. Next, 1900 μL of FOX reagent was added and, after 4 h of reaction, absorbance at 560 nm was measured. Controls contained 20 μL of methanol instead of TPP solution. FOX reagent was prepared by mixing its components in appropriate concentrations (20.7 mg of ferrous ammonium sulfate, 52 mL of water, 290 μL of H_2SO_4, and 17.0 mg of XO, made up to 200 mL with methanol) so that the addition of 1900 μL of the reagent gave the final concentrations in the reaction mixtures mentioned above in the FOX method description.

Precision. Eight aliquots of a sample were weighed and simultaneously analyzed. Then the coefficient of variation (CV) was calculated. Precision was assessed using two samples: a raw one from the first experiment from broilers fed a diet supplemented only with sunflower oil (volume of tissue extract = 75 μL; incubation time = 4 h) and a cooked sample from the second experiment from broilers fed for 10 days a basal diet supplemented with 150 mg of α-TA/kg of feed (volume of tissue extract = 140 μL; incubation time = 80 h).

Determination of Fatty Acid Composition. One gram of sample was weighed into a 32 × 210 mm tube, 20 mL of chloroform/methanol (2:1, v/v) was added, and the mixture was homogenized for 40 s at 19800 rpm using a Polytron PT 2000. The extract was filtered through a Whatman No. 1 filter paper into a 50 mL screw-capped tube, and the residue was re-extracted twice: first with 7 mL (30 s at 19800 rpm) and then with 5 mL (10 s at 19800 rpm) of the same solvent. Ten milliliters of distilled water was added to the tube, which was agitated and then centrifuged at 400*g* for 20 min. The chloroform extract was transferred to a round-bottom flask through anhydrous sodium sulfate and was concentrated to ~1 mL in a vacuum rotatory evaporator at 35 °C. The remaining solvent was removed, first in a light nitrogen stream and then by leaving the flask in a vacuum desiccator at 10 mmHg overnight. Fatty acid methyl esters were prepared from the extracted lipid fraction and determined as described by Guardiola et al. (1994), with a few modifications.

α-Tocopherol Determination. α-Tocopherol was determined following a still unpublished method. Two grams of sample was homogenized in 5 mL of absolute ethanol containing 1% pyrogallol (w/v) and 0.012% BHT (w/v) with a Polytron PT 2000 (30 s at 20000 rpm). Next, 10 mL of 1.6 N methanolic KOH was added, and saponification was carried out at 70 °C for 30 min. Nonsaponifiables were then extracted with petroleum ether, filtered through a 0.45 μm Teflon membrane, and, after solvent evaporation under a nitrogen stream at 30 °C, the residue was redissolved with absolute ethanol. Twenty microliters of this solution was injected into a series 1100 Hewlett-Packard liquid chromatograph equipped with a C18 column (25 × 0.46 cm) packed with 5 μm-80 Å Extrasil ODS2 and a C18 precolumn packed with 5 μm-100 Å Kromasil ODS2. Sample compounds were isocratically eluted with methanol and detected through a 1046A Hewlett-Packard spectrofluorometric detector (excitation and emission wavelengths selected were 288 and 330 nm, respectively). Sample α-tocopherol content was determined by means of an experimental calibration curve, using α-tocopherol as external standard.

RESULTS AND DISCUSSION

Optimization of Reactants. In the method proposed by Hermes-Lima et al. (1995), methanol was used to prepare only the LHP extract and water was used in all other cases (for ferrous, XO, and H_2SO_4 solutions and to make up the volume). Unfortunately, this part of their procedure is not suitable for chicken meat samples because, when volumes of methanolic extract ≥50 μL were added to the reaction tube, some turbidity appeared. To achieve a clear reaction mixture, the methanol/water ratio in the medium had to be increased. Complete solubility of the methanolic extract (up to 150−200 μL, depending on the raw or cooked chicken meat sample being analyzed) was achieved when methanol, instead of water, was used to prepare XO and H_2SO_4 solutions and to make up the volume of the reaction mixture.

Because a few FOX methods using hydrochloric acid instead of sulfuric acid have been reported in the literature (Burat and Bozkurt, 1996; Shantha and Decker, 1994; Winterbourn et al., 1997), the influence of the two acids on the speed and intensity of the reaction was studied. For each acid (both 0.25 M in methanol) three aliquots of a meat extract were analyzed and their absorbance was read every 30 min for 6.5 h. As shown in Figure 1, although both acids showed the same stabilization time, the intensity of the reaction was higher with sulfuric acid. It is then recommended that sulfuric acid be used to obtain higher sensitivity.

Relationship between Volume of Sample Extract and Response. As reported by Hermes-Lima et al. (1995), we found a linear relationship between volume of tissue extract added in the reaction mixture and absorbance (Figure 2). Volumes assayed in separate experiments ranged from 50 to 150 μL (depending on the kind of sample), and linearity was checked, at different incubation times, in raw and cooked samples from chickens fed different diets, as well as in commercial fresh raw samples.

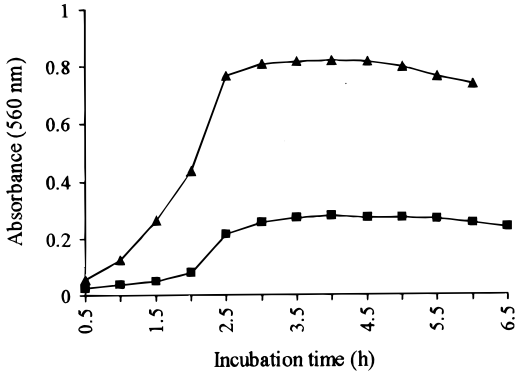

Figure 1. Influence of the acid on the speed and intensity of the reaction ($n = 3$). Reaction media (2 mL) contained (added in the following order) 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic sulfuric (triangles) or hydrochloric (squares) acid, 200 μL of 1 mM methanolic XO, 1000 μL of methanol, and aliquots (100 μL) of a methanolic extract from a raw chicken meat sample purchased from a local supermarket, which had been stored for 1.5 months at −20 °C.

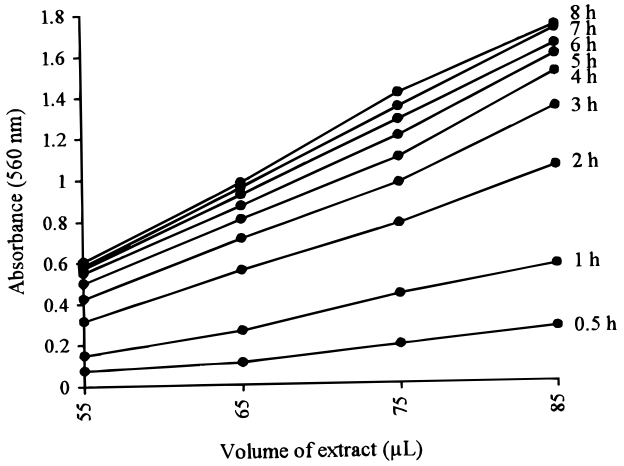

Figure 2. Absorbance values ($n = 2$) as affected by increasing the volume of meat extract added to the reaction mixture and the incubation time. Reaction media (2 mL) contained (added in the following order) 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic XO, methanol (to make up the volume), and aliquots of a methanolic extract from a raw meat sample from chickens fed a diet supplemented only with sunflower oil.

Factors Affecting the Reaction Time Course. The time required for the reaction to be completed depends on the kind of sample. To find it, Hermes-Lima et al. (1995) suggested incubating reaction mixtures for times ranging from 30 min to 24 h until the absorbance reaches a plateau and, at the same time, trying several volumes of extract to find the one that gives absorbance values of 0.7−0.8 at the reaction end-point. When this was assayed on chicken meat samples, higher absorbance values were obtained when reaction mixtures had been read every 30 min than when a single measurement was done at the end of a certain period. In fact,

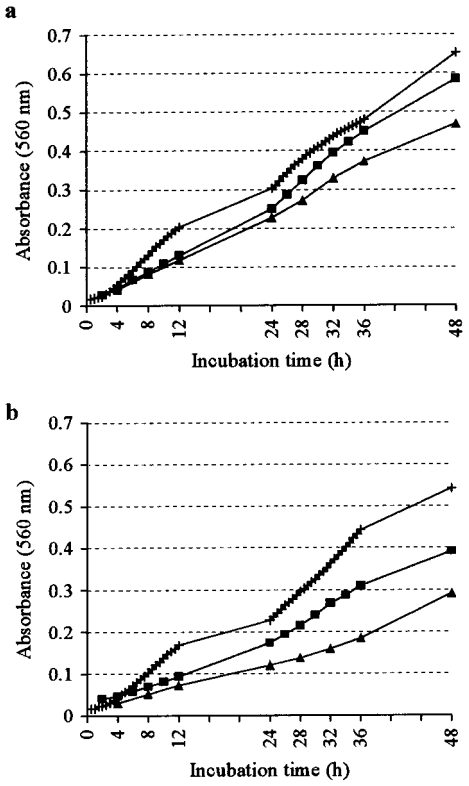

Figure 3. Influence of the frequency of the spectrophotometric measurements of the reaction mixtures on absorbance values ($n = 2$). Reaction mixtures containing aliquots (75 μL) of a raw chicken meat sample extract (from chickens fed a diet supplemented with 75 mg of α-TA/kg for 21 days) were incubated for 48 h, and their absorbance was measured every 30 min (crosses), 2 h (squares), or 4 h (triangles). Reaction media were as described in Figure 2. (a) results from an experiment carried out with air filling the empty space in the cuvettes, whereas in (b) N_2 was flushed in the cuvettes as described under Materials and Methods. Both experiments were conducted at the same time.

increasing the frequency of spectrophotometric measurements of a reaction mixture led to higher absorbance values (Figure 3a). This could be attributed to an oxygen- and light-related oxidation of peroxidable materials extracted from the sample (reaction mixtures were shaken before each reading, which facilitates contact of peroxidable material with oxygen in the presence of light). This is supported by the finding that the upper layer of the reaction mixture changed color more readily than the rest during incubation and, in addition, when the oxygen in the reaction medium was partially removed by an N_2 stream, absorbance values were lower, although they also tended to increase with higher frequency of reading (Figure 3b).

Influence of BHT Addition. To avoid LHP formation from nonperoxidized lipids from the sample, which may be present in the reaction medium, some authors strongly recommended the use of BHT (Jiang et al., 1991, 1992; Wolff, 1994). However, others (Hermes-Lima et al., 1995) discouraged BHT addition, claiming that it interferes in the reaction as it can react with alkoxyl radicals formed from the LHP, resulting in more stable species and thus diminishing Fe(III) generation. In the present study, when BHT was added to the reaction mixture (1.68 mg/2 mL), absorbance values were much lower than when N_2 was used, and they were much less affected by increasing the frequency of the measurements (Figure 4). Hence, there is some peroxidation of the lipids extracted from the sample during incubation. These results cast doubt on the theory of Hermes-Lima et al. (1995), according to which peroxidable lipids in the sample do not interfere in the reaction (at least during the first 12 h of incubation). However, that was checked in a model system with arachidonic acid, which is free from pro-oxidant components that may be present in a meat extract.

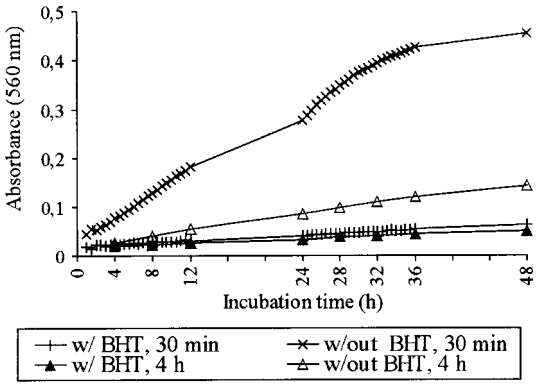

Figure 4. Influence of BHT addition in the reaction medium (0.84 mg/mL) and the frequency in the spectrophotometric measurements of the reaction mixture (every 30 min or every 4 h) on absorbance values ($n = 2$). Reaction media contained (added in the following order) 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic XO, 1025 μL of 7.45 mM methanolic BHT or methanol, and aliquots (75 μL) of a sample methanolic extract (same sample as in Figure 3).

The lower absorbance values obtained by means of BHT addition than by filling the cuvette with N_2 could be explained (i) by incomplete removal of the O_2 from the reaction medium in the N_2 stream and (ii) by the fact that, in addition to avoiding hydroperoxide formation from peroxidable material in the sample extract (Wolff, 1994), BHT leads to lower Fe(III) formation by turning alkoxyl radicals into more stable species (Hermes-Lima et al., 1995).

Moreover, when BHT (1.68 mg) was added to 2 mL of a reaction medium containing 4 μg of a linolenic hydroperoxide (HPODE) standard and the FOX reagent, absorbance (after 30 min of reaction) was 17.2% lower (Figure 5). However, when BHT was added before the FOX reagent, absorbance was much lower (43.6%). This result is consistent with the hypothesis that BHT leads to lower Fe(III) formation. Furthermore, this reaction is instantaneous, as the decrease in response was the same irrespective of whether HPODE and BHT had been in contact shortly or for 30 min before FOX addition. The lower decrease in response observed when FOX reagent was added before BHT could be attributed to the fact that the reaction between LHP and FOX reagent is instantaneous, and when BHT was added, the reaction had already started. However, when BHT was added before the FOX reagent, it attenuated the reaction from the first moment.

In addition, two cooked samples from the second experiment, one from chickens fed a nonsupplemented diet (8.6 mg of α-tocopherol/kg of meat) and the other from chickens fed a diet supplemented with α-TA (50.7 mg of α-tocopherol/kg of meat), were analyzed with and without BHT (1.68 mg of BHT added before the FOX reagent). Figure 6 shows that, as in the case of the HPODE standard, BHT addition also caused a clearly lower initial response (after 2 h of incubation) due to the attenuation of the reaction and, what is more,

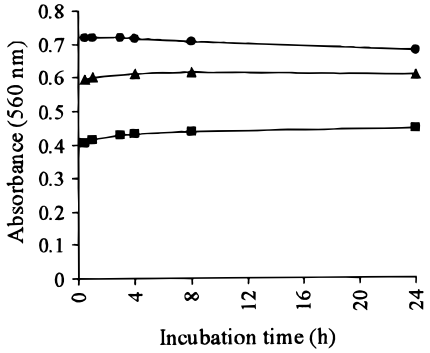

Figure 5. Effect of BHT addition (600 μL of 12.7 mM methanolic solution) on absorbance from HPODE standard (2 μg/mL) ($n = 2$). To 400 μL of 0.066 mM methanolic HPODE was added (a) 600 μL of methanol followed by 1 mL of FOX reagent (circles), (b) BHT solution followed by 1 mL of FOX reagent (squares), or (c) 1 mL of FOX reagent followed by BHT solution (triangles). FOX reagent was prepared in appropriate concentrations so that the addition of 1 mL of the reagent gave the desired final concentrations in the reaction mixtures (see descriptions of FOX method and specificity tests under Materials and Methods).

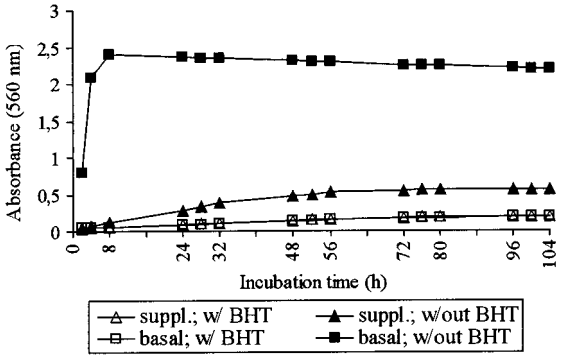

Figure 6. Time course analysis of cooked samples from chickens fed a nonsupplemented diet (basal) or a diet supplemented with α-TA (suppl.) depending on the BHT addition (0.84 mg/mL) in the reaction medium ($n = 3$). α-Tocopherol contents of samples from basal and supplemented diets were, respectively, 8.6 and 50.7 mg/kg. Reaction media contained (added in the following order) 860 μL of 8.9 mM methanolic BHT (open symbols) or methanol (solid symbols), 1 mL of FOX reagent, and aliquots (140 μL) of the sample methanolic extracts. Preparation of FOX reagent was as explained in Figure 5.

response remained lower because BHT protected the sample from oxidation during incubation. Thus, when BHT was added, the samples were not distinguished by this method (Figure 6).

Moreover, the method (without BHT) was also useful to distinguish between samples from the same experiment with more similar α-tocopherol levels (7.9, 13.6, and 18.2 mg/kg of meat) through their reaction curves (Figure 7). The lower the α-tocopherol content, the higher the susceptibility to oxidation and the higher the response after incubation.

The FOX Assay as an Induced Method. From the results of this study, it can be assumed that the FOX assay works as an induced method, because when it was applied to the HPODE standard, the reaction was complete after 30 min of incubation (Figure 5), whereas when it was carried out on chicken meat, a steady endpoint was reached only after several hours, depending on the oxidability of the sample (Figures 1 and 6−8). During this additional time, the extracted lipids from the sample undergo further oxidation due to oxygen and

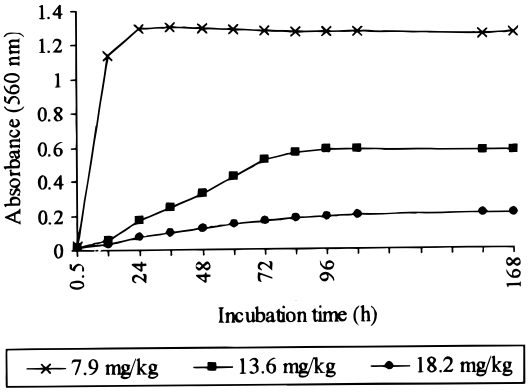

Figure 7. Time course analysis of raw chicken meat samples (65 μL extract aliquots) with different α-tocopherol contents ($n = 2$). Samples were obtained from chickens fed a diet supplemented with α-TA (75 mg/kg of feed) during different periods [0 (crosses), 21 (squares), and 43 (circles) days], which resulted in different α-tocopherol contents of the samples (7.9, 13.6, and 18.2 mg/kg of meat, respectively). Reaction media were as described in Figure 2.

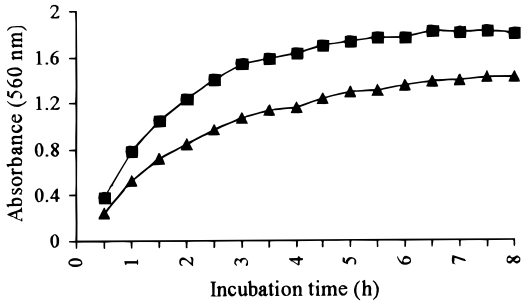

Figure 8. Time course analysis of raw chicken meat samples (100 μL extract aliquots) from chickens fed diets supplemented only with sunflower oil (squares) or beef tallow (triangles) ($n = 2$). Reaction media were as described in Figure 2.

the yet formed LHP, radicals, and other pro-oxidant substances extracted from the sample (reaction mixtures were incubated under attenuated light). Furthermore, in our conditions, Fe(II) from the sample and from the ferrous salt may be the main cause of lipid peroxidation by inducing the decomposition of LHP to alkoxyl radicals, which can attack other lipid molecules (Jadhav et al., 1996; Kanner et al., 1988). The extent of this induced peroxidation depends on the susceptibility of the sample to oxidation. In fact, dark chicken meat samples from chickens fed diets without α-TA supplementation showed higher initial absorbance values and needed shorter incubation times than samples from supplemented diets (Figures 6 and 7). This may be due to the fact that the latter had a higher α-tocopherol content and were thus more protected against oxidation during storage and processing (resulting in a lower initial response) and also during incubation (leading to a longer stabilization time and a lower absorbance value at the reaction end-point). On the other hand, raw and cooked samples from diets supplemented with beef tallow or sunflower oil (without α-TA addition) also yielded distinct curves (Figure 8), because the lipid fraction from animals fed sunflower oil-supplemented diets was more unsaturated (Table 1) and therefore more prone to oxidation. From these results, for samples with a low oxidation degree, this FOX method is more useful to measure sample susceptibility to LHP formation rather than its present level of LHP. However, in the case of highly oxidized or very susceptible to

Table 1. Fatty Acid Composition, Expressed as Compensated Area Normalization (Percent), of Raw Chicken Meat Samples from Animals Fed Diets Supplemented with Different Fat Sources

	dietary fat source	
	beef tallow	sunflower oil
total SFA[a]	**34.41**	**22.62**
total MUFA	**47.29**	**34.96**
C18:2 *n*−6	16.09	39.69
C18:3 *n*−6	0.16	0.32
C20:2 *n*−6	0.13	0.23
C20:3 *n*−6	0.15	0.25
C20:4 *n*−6	0.56	0.92
C22:4 *n*−6	0.14	0.27
C22:5 *n*−6	0.04	0.08
total *n*−6 PUFA	**17.26**	**41.75**
C18:3 *n*−3	0.88	0.63
C18:4 *n*−3	0.03	0.02
C20:4 *n*−3	0.01	traces
C20:5 *n*−3	0.02	0.01
C22:5 *n*−3	0.08	0.04
C22:6 *n*−3	0.05	0.02
total *n*−3 PUFA	**1.07**	**0.72**
total PUFA	**18.34**	**42.47**

[a] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

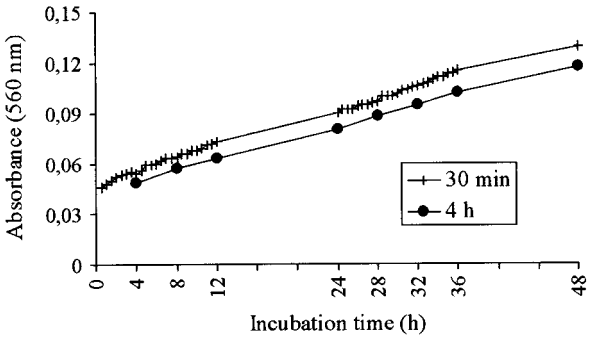

Figure 9. Absorbance of blanks (n = 2) as affected by the frequency of spectrophotometric measurements, which were performed every 30 min or 4 h. Blanks contained (added in the following order) 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic XO, and 1100 μL of methanol.

oxidation samples, the FOX method can be useful to compare these samples by measuring their LHP value after a short incubation time (30 min).

Stability of Ferrous Ions during Incubation. Ferrous ions undergo very little oxidation during incubation of blanks since their absorbance rose much less than the absorbance of samples (i.e., 0.03 AU versus 0.35−1.46 AU in 48 h) and was only slightly affected by increasing frequency of measurements (Figure 9) or N_2 medium (Figure 10). Moreover, BHT addition had no influence on it (data not shown). These observations are supported by the fact that Fe(II) is relatively stable to oxidation in dilute acids (Burriel et al., 1974) and by the findings of Hermes-Lima et al. (1995), who showed that ferrous ions in the acidic reaction media did not oxidize before addition of tissue extracts or peroxides.

Specificity of the Method. Pretreatment of sample aliquots with enzymes that reduce H_2O_2 (catalase) and hydroperoxides (glutathione plus glutathione peroxidase plus phospholipase A_2) or with triphenylphosphine (TPP), a specific hydroperoxide reductant that has no effect on H_2O_2 (Barnard and Wong, 1976; Nakumura

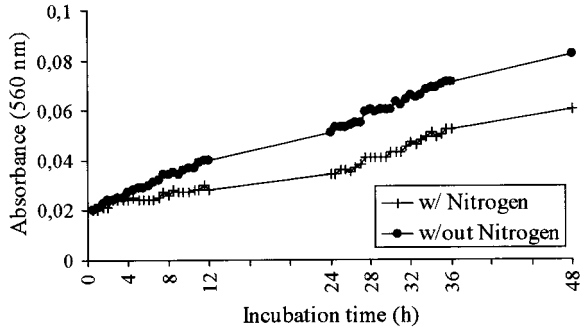

Figure 10. Absorbance of blanks (n = 2) depending on the presence of N_2 in the reaction medium. From 0 to 12 h and from 24 to 36 h, absorbance was measured every 30 min. Reaction media were as described in Figure 9, but for blanks under N_2 atmosphere, N_2 was flushed in the cuvettes as described under Materials and Methods.

and Maeda, 1991), has been used to discern color development due to authentic LHP from that due to H_2O_2 or other interfering components. In liposomes, LDL and edible oils, reacting compounds were shown to be mainly LHP from the sample because 80−85% of the color yield was due to non-H_2O_2 hydroperoxides (Jiang et al., 1992; Nourooz-Zadeh et al., 1994, 1995), whereas, in fresh plasma, a high proportion of the response was due to interfering components (Södergren et al., 1998). Nourooz-Zadeh et al. (1994) suggested that these interfering components were mainly ferric ions intrinsic to the sample. In our chicken meat samples, TPP reduced the color yield by 98.6 and 98.3% in raw and cooked samples, respectively. Furthermore, no differences were found between samples from sunflower oil and beef tallow supplemented diets. Therefore, these results indicate that, in dark chicken meat, this FOX assay is highly specific, as color development is due to authentic LHP rather than other sample components such as H_2O_2 or ferric ions from the meat. However, special attention must be paid to the presence of reducing/oxidizing compounds in the sample which, if they enter the reaction medium, may interfere in the reaction. Some of these have been described, such as glutathione, cysteine, uric acid, and ascorbic acid (Bleau et al., 1988; Hermes-Lima et al., 1995; Jiang et al., 1992; Wolff, 1994). Those studies, however, showed that the final effect of ascorbic acid (enhancing or lowering color development) depends on its concentration and reaction conditions. Moreover, compounds that bind ferric ions (e.g., desferrioxamine and ethylenediaminetetraacetic acid) may interfere through competition with XO for the ferric ions generated by the LHP-mediated oxidation of ferrous ions (Wolff, 1994).

Precision. Precision (CV) was 8.9 and 7.4% for the raw (LHP value = 275.8 mg CHP/kg) and cooked (LHP value = 42.5 mg CHP/kg) samples, respectively. These results are comparable to those reported in other works that applied methods using ferrous oxidation in xylenol orange to measure lipid hydroperoxides in plasma (CV = 7.6−12.1%; Södergren et al., 1998) and edible oils and fats extracted from foods (CV = 0.3−10%; Burat and Bozkurt, 1996; Nourooz-Zadeh, 1998; Nourooz-Zadeh et al., 1995; Shantha and Decker, 1994). In addition, studies in liposomes (Jiang et al., 1991) and edible oils (Nourooz-Zadeh, 1998; Nourooz-Zadeh et al., 1995) reported a similar or even better repeatability for the FOX methods than that of the iodometric method through spectrophotometry.

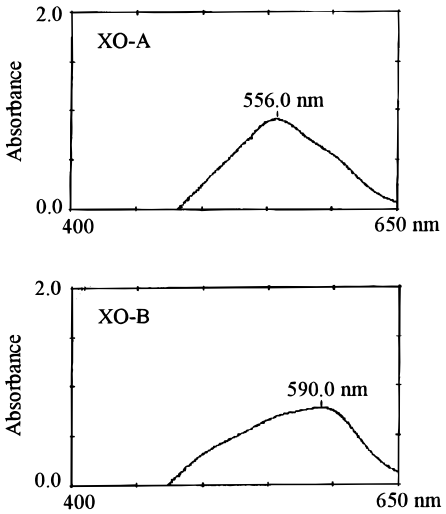

Figure 11. Absorbance spectra of the Fe(III)−XO complex formed from two different batches of XO after reaction with CHP for 30 min. Reaction media contained (added in the following order) 500 μL of 1 mM aqueous ferrous ammonium sulfate, 200 μL of 0.25 M methanolic H_2SO_4, 200 μL of 1 mM methanolic XO, 950 μL of methanol, and 150 μL of 0.21 mM methanolic CHP.

Table 2. Calibration Curves Depending on Xylenol Orange Batch and Wavelength of Reading[a]

wavelength, nm	xylenol orange batch	
	XO-A	XO-B
592	$Y = -0.0203 + 0.0388X$ ($r^2 = 0.9940$)	$Y = -0.0145 + 0.0475X$ ($r^2 = 0.9871$)
560	$Y = 0.0041 + 0.0575X$ ($r^2 = 0.9970$)	$Y = 0.0048 + 0.0440X$ ($r^2 = 0.9986$)

[a] Y = concentration of CHP in the reaction mixutre (nmol/mL); X = absorbance of the reaction mixture at the corresponding wavelenth.

Differences between XO Batches. Some unexpected differences were observed between two batches of XO from the same company (XO-A and XO-B). XO-A gave methanolic solutions (1 mM) with lighter color. Furthermore, reaction mixtures with high CHP concentrations (16 nmol/mL) were violet or blue when XO-A or XO-B were used, respectively. Consequently, the spectrum shape and the wavelength of the absorption maximum (λ_{max}) varied depending on the XO batch (Figure 11): λ_{max} = 556−561 nm for XO-A and λ_{max} = 590−594 nm for XO-B (depending on the hydroperoxide concentration). Calibration curves obtained (Table 2) indicate that, at 560 nm, XO-A was more sensitive to changes in CHP concentration, whereas at 592 nm XO-B was more sensitive. In fact, most of the authors who work with FOX methods (Bleau et al., 1998; Burat and Bozkurt, 1996; Jiang et al., 1991, 1992; Nourooz-Zadeh et al., 1994; 1995; Shantha and Decker, 1994; Södergren et al., 1998; Wolff, 1994) use 560 nm to measure the absorbance. It is thus concluded that when a series of samples are to be analyzed, it is highly recommended that the same lot of XO be used for all of them.

CONCLUSION

This highly specific FOX assay can be used in dark chicken meat to assess sample susceptibility to oxidation (FOX value after some hours of incubation, as an induced method) and to differentiate between highly oxidized or readily susceptible to oxidation samples (by means of their absorbance after 30 min of incubation). This may also be valid for other food and biological samples. However, the volume of sample extract and reaction time must be assayed for each kind of sample. In all cases, the use of BHT is inadvisable.

ABBREVIATIONS USED

BHT, butylated hydroxytoluene; CHP, cumene hydroperoxide; FOX, ferrous oxidation−xylenol orange; HPODE, 13(*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid; LDL, low-density lipoprotein; LHP, lipid hydroperoxides; α-TA, α-tocopheryl acetate; TPP, triphenylphosphine; XO, xylenol orange.

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