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Lipid-Oxidation-Induced Carboxymyoglobin Oxidation

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We evaluated the usefulness of the ratio A_{503}/A_{581} as a browning index (BI) for estimating brown color formation in solutions containing oxymyoglobin (OxyMb) and carboxymyoglobin (COMb). In split-chamber cuvette analyses with different proportions of metmyoglobin (MetMb), COMb and OxyMb, BI was highly correlated ($r = 0.93-0.94$) with direct estimation of MetMb. Moreover, A_{503}/A_{581} was not influenced by different COMb−OxyMb proportions. Second, we investigated 4-hydroxy-2-nonenal (HNE)-induced spectral changes in OxyMb and COMb solutions. At pH 7.4 and 37 °C, BI was greater in HNE-treated OxyMb and COMb samples than in aldehyde-free controls ($P < 0.05$). However, at pH 5.6 and 4 °C, HNE-induced browning was more pronounced in COMb than in OxyMb. These results indicated that COMb is susceptible to lipid-oxidation-induced browning in a pH- and temperature-dependent manner.

KEYWORDS: Myoglobin; carbon monoxide; carboxymyoglobin; lipid oxidation; 4-hydroxy-2-nonenal

INTRODUCTION

The consumer desirable red color of fresh meat may be stabilized by employing modified atmosphere packaging (MAP) that includes carbon monoxide (CO). Since 1985, the Norwegian meat industry has been using a gas mixture containing 0.3−0.5% CO (balance 60% CO_2 and 40% N_2) in retail-ready packages of beef, pork, and lamb (*1*). However, after 2 decades of successful application, Norway discontinued the use of CO MAP for red meat in July 2004 due to their entry into the European Union, which currently does not approve the use of CO in meat packaging (*2*). At the same time, CO MAP became more relevant for the United States meat industry (*3*) and was approved by the FDA (*4*) for use at a level of 0.4% in MAP systems for red meat.

CO binds strongly to myoglobin (Mb) to form a bright cherry-red pigment, carboxymyoglobin (COMb). From a biochemical aspect, it appears that deoxymyoglobin (DeoxyMb) is more readily converted to COMb than is oxymyoglobin (OxyMb) or metmyoglobin (MetMb). The absence of ligands in the sixth coordinate of heme iron in DeoxyMb is widely considered as the most favorable reason for its reaction with CO (*5*). Wolfe et al. (*6*) reported that 1% CO MAP enhanced MetMb reducing activity by preventing heme-catalyzed lipid oxidation, the latter of which produces free radicals known to inactivate enzymes (e.g., those associated with MetMb reduction) and catalyze heme oxidation. While investigating the influence of gaseous environments on MetMb reduction in ground beef systems, Lanier et al. (*7*) observed that MetMb reducing activity was enhanced in a CO-containing environment, possibly due to a stabilizing effect of CO on heme and by counteracting the inhibitory action of oxygen on MetMb reduction. Nevertheless, the reaction characteristics of CO with OxyMb and MetMb and the interconversion(s) between COMb and other Mb forms are poorly understood.

Previous research revealed that CO MAP decreased lipid oxidation and increased color stability in different meat systems (*8*−*15*). Furthermore, Hunt et al. (*16*) concluded that the use of 0.4% CO MAP improved beef color without masking spoilage; following the removal of product from CO MAP, meat color deteriorated during display in a manner similar to that of OxyMb. CO-treated porcine blood was reported to maintain a stable, attractive red color and was proposed for potential use as a colorant in meat products (*17*).

The absorption spectrum of COMb is very similar to that of OxyMb (*18*, *19*). Traditional equations used to estimate the relative proportions of Mb redox forms in aqueous solutions/meat extracts (*20*−*22*) have not included COMb and are based on the wavelength maxima for OxyMb, DeoxyMb, and MetMb. Therefore, the application of these equations to COMb-containing solutions would be inappropriate, because they do not account for the wavelength maxima and extinction coefficient(s) of COMb. Furthermore, the nearly identical spectra of COMb and OxyMb make it very difficult to determine the relative proportions of these two species and complicate the estimation of brown color formation in solutions containing both COMb and OxyMb.

Mancini and Hunt (*5*) noted that many fundamental concepts of COMb redox chemistry remain unresolved and COMb dynamics in the presence of lipid oxidation products has not been considered. It is well-documented that CO can act as an antioxidant in meat (*9*−*13*), but the redox stability of COMb

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when challenged with reactive lipid oxidation products has not been addressed previously.

4-Hydroxy-2-nonenal (HNE) is an α,β- unsaturated aldehyde formed by oxidation of ω-6 polyunsaturated fatty acids (*23*), and is very reactive toward proteins and enzymes (*24*). Free, unbound HNE has been detected in fresh beef, pork, and fish (*25*−*28*) and proposed as a marker to assess the quality of muscle foods containing significant amounts of polyunsaturated fatty acids (*26*). HNE has been used as a model aldehyde to investigate lipid-oxidation-induced OxyMb (*29*−*33*) oxidation.

The objectives of this research were to determine a suitable method to estimate brown color formation in COMb solutions and to compare lipid-oxidation-induced redox instability in COMb and OxyMb solutions.

MATERIALS AND METHODS

Materials and Chemicals. Equine heart metmyoglobin, sodium hydrosulfite, sodium citrate, and sodium phosphate were obtained from Sigma Chemical Co. (St. Louis, MO). HNE was obtained from Cayman Chemical Co. (Ann Arbor, MI), and PD-10 columns were obtained from GE Healthcare (Piscataway, NJ). All chemicals were of reagent grade or greater purity.

OxyMb Preparation. OxyMb was prepared by sodium hydrosulfite-mediated reduction of Mb (*34*). The residual hydrosulfite was removed by passing over a PD-10 column. The pH of Mb solution was adjusted with 50 mM sodium citrate (pH 5.6) or 50 mM sodium phosphate (pH 7.4) buffer.

COMb Preparation. To ensure the stability of the heme protein, all preparations were carried out in ice. In order to prepare COMb, equine MetMb was reduced with hydrosulfite (0.1 mg sodium hydrosulfite to 1 mg Mb), and the resulting DeoxyMb was bubbled with a gas mixture of 0.4% CO, 69.6% CO_2, and 30% N_2. Conversion of DeoxyMb to COMb was monitored spectrophotometrically (Shimadzu UV-2101PC spectrophotometer, Shimadzu Inc., Columbia, MD) by observing the shift in absorbance spectra between 500 and 600 nm. COMb spectra are very similar to those of OxyMb, and both myoglobin forms shared common peaks at 543 nm and 581 nm, albeit with different magnitudes of absorbance. Similar spectra were reported previously for COMb from tuna (*18*) and turkey (*19*). For COMb spectra, the magnitudes of the absorbance values at 543 nm were consistently greater than at 581 nm, whereas in OxyMb the reverse was true. The ratio of A_{543}/A_{581} for a solution of 100% COMb was always greater than one, whereas for 100% OxyMb it was less than one. The ratio decreased as the proportion of OxyMb, within the split-chamber cuvette, increased and was used to differentiate COMb from OxyMb.

The bubbling time needed to convert DeoxyMb to COMb was determined to be 40 min, the time point after which no further change in spectra was observed (**Figure 1**). Residual hydrosulfite was removed by passing the COMb preparation over a PD-10 column, equilibrated with buffer that was bubbled with the gas mixture containing 0.4% CO, 69.6% CO_2, and 30% N_2. Preliminary observations revealed that when non-CO-bubbled buffer was used to equilibrate the PD-10 columns, there was significant conversion of COMb to OxyMb, indicated by a decrease in A_{543}/A_{581}. This could be attributed to the potential oxygenation of COMb due to mass action by oxygen dissolved in the buffer. Hence, CO-bubbled buffer was utilized to equilibrate the PD-10 desalting columns and minimize conversion of COMb to OxyMb. In order to minimize any OxyMb formation from COMb following PD-10 treatment, desalted COMb samples were rebubbled with 0.4% CO for an additional 10 min.

Reaction with HNE. OxyMb and COMb were incubated with HNE (0.15 mM of Mb + 1.0 mM of HNE) at 4 °C, pH 5.6 (typical meat storage condition) in 50 mM sodium citrate buffer, or 37 °C, pH 7.4 (physiological condition) in 50 mM sodium phosphate buffer. Controls consisted of Mb plus a volume of ethanol equivalent to that used to deliver the aldehyde to treatments. Samples were scanned spectrophotometrically at specific incubation times from 700 to 450 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD).

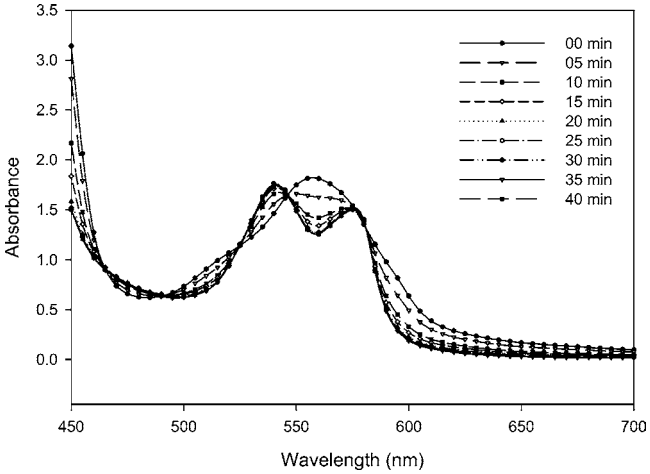

Figure 1. The effect of gas bubbling duration on the absorbance spectra of DeoxyMb. A gas mixture containing 0.4% CO, 69.6% CO_2, and 30% N_2 was bubbled through DeoxyMb (0.15 mM) in 50 mM sodium phosphate buffer, at pH 7.4.

Calculation of MetMb Formation. MetMb formation in samples containing only OxyMb was calculated according to Tang et al. (*21*). During incubation, OxyMb solutions were scanned spectrophotometrically from 650 to 500 nm using a Shimadzu UV-2101PC spectrophotometer (Shimadzu Inc., Columbia, MD) and MetMb formation was calculated using wavelength maxima at 503, 557, and 582 nm, representative for MetMb, DeoxyMb, and OxyMb, respectively.

Brown Color Formation in COMb Solutions. At present, direct estimation of brown color formation is not possible in COMb solutions. Hence, a browning index (BI) was derived to estimate brown color formation in COMb solutions and is addressed in Results and Discussion.

Statistical Analysis. The experimental design was a completely randomized design where each experiment was replicated three times (experiment 1 = pH 7.4 and 37 °C and experiment 2 = pH 5.6 and 4 °C). The four treatments had a one-way structure consisting of Mb derivatives with and without HNE (treatment 1 = COMb positive control with ethanol, treatment 2 = OxyMb positive control with ethanol, treatment 3 = COMb with HNE, and treatment 4 = OxyMb with HNE). Because pH and incubation temperature influence the rate of Mb oxidation, spectral observations were made at different time points for the two experiments. Time intervals were in minutes for trials evaluating pH 7.4, 37 °C and in hours for trials evaluating pH 5.6, 4 °C. Therefore, data within each of the two experiments were analyzed separately.

Within each trial, triplicate subsamples were analyzed, resulting in a total of 36 observations ($N = 36$; 3 trials × 3 subsamples × 4 treatments) for each of the two experiments. Subsamples within each trial were averaged for statistical analysis, resulting in $n = 3$. Type-3 tests of fixed effects for changes in browning index during incubation of OxyMb and COMb were evaluated using the general linear model (GLM) procedure of SAS (*35*). Means were generated for *F*-tests resulting from a significant ($P < 0.05$) effect of HNE on the redox stability of COMb and OxyMb. Standard errors of the mean were used to determine treatment differences and are reported as error bars in the figures.

Trials for determining the effect of gas bubbling on the absorbance spectra of DeoxyMb were replicated three times. For experiments using split-chamber cuvettes, triplicate subsamples were used for each combination of myoglobin derivatives (COMb and OxyMb, COMb and MetMb, and OxyMb and MetMb). Absorbance values at 525 nm (isobestic point for Mb) were used to verify results from split-chamber cuvette analyses.

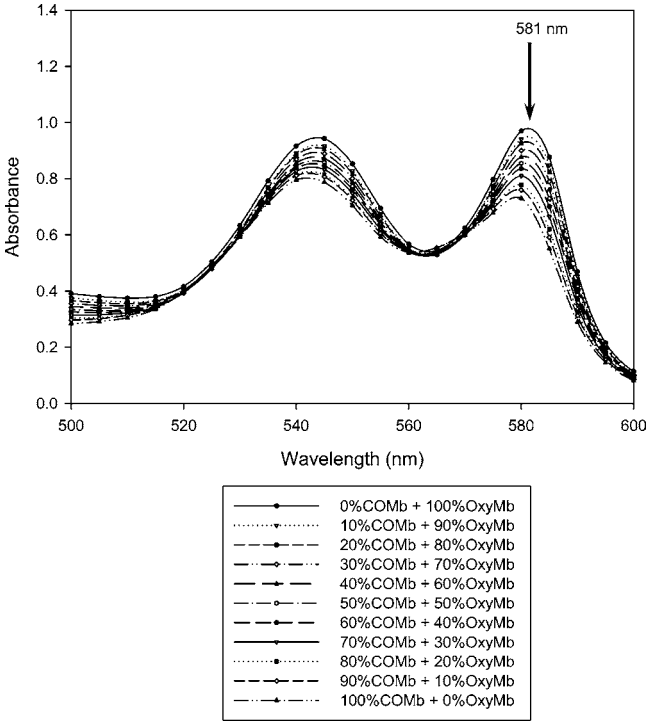

Figure 2. Absorbance spectra for Mb solutions containing different proportions of COMb and OxyMb at pH 7.4, in 50 mM sodium phosphate buffer. A split-chambered cuvette was used to avoid mixing of the two Mb forms. The absorbance maxima for COMb and OxyMb samples was close to 581 nm (between 580 and 582 nm, but not exactly identical).

RESULTS AND DISCUSSION

Determination of Brown Color Formation in COMb Solutions. During preliminary investigations, we observed the formation of a brown color in COMb solutions, similar in appearance to MetMb that forms during autoxidation of OxyMb solutions and concomitant with loss of red color during incubation at pH 7.4, 37 °C. However, the application of traditional equations for determining (*20*−*22*) MetMb in CO-treated Mb solutions is irrelevant, because they do not include extinction coefficient(s) for COMb at specific wavelengths. The similarity of COMb and OxyMb spectra further complicates matters. This necessitated deriving an index to calculate the formation of brown color in COMb solutions. We observed that equine COMb and equine OxyMb spectra shared a common peak at 581 nm, albeit with different absolute intensity at the same Mb concentration. Peak intensity at 581 nm was lower for COMb spectra than for OxyMb spectra. Our preliminary experiments with split-chamber cuvettes using variable proportions of OxyMb and COMb (0−100%, with increments of 10%) showed a prominent peak at 581 nm for all combinations (**Figure 2**). The absorbance spectra for Mb solutions with different proportions of COMb and OxyMb are presented in **Figure 2**. The absorbance peak at 581 nm was prominent, irrespective of different COMb−OxyMb proportions and was adopted as a reference wavelength maximum for both COMb and OxyMb.

Usefulness of A_{503} To Estimate Browning. During incubation of COMb and OxyMb solutions at pH 7.4, 37 °C, we observed that the magnitude of the peak at 581 nm decreased, whereas the peak magnitude at 503 nm corresponding to the wavelength of maximal absorption for MetMb (*21*) increased with an observed visual increase in browning and loss of red color. However, the absolute intensity of the peak at 503 was lower for COMb spectra, when compared with OxyMb, at the same Mb concentration. Interestingly, this finding was similar to the peak intensity at 581 nm observed in COMb and OxyMb solutions. This suggested that the relative proportion of MetMb increased with a concomitant decrease in the proportion(s) of COMb and OxyMb in the solutions due to OxyMb oxidation and presumed heme oxidation in COMb. Hence, the ratio of A_{503}/A_{581} was used as an index to measure the relative proportions of brown color (MetMb) and red color (COMb and/or OxyMb) in Mb solutions. Additionally, split-chamber cuvette analyses with variable amounts of COMb + OxyMb revealed that A_{503}/A_{581} was constant ($A_{503}/A_{581} = 0.39$) for all combinations of these myoglobins, suggesting that this ratio would not be influenced by COMb−OxyMb interconversions. The absorbance spectra for Mb solutions containing different proportions of OxyMb and MetMb are shown in **Figure 3**. The estimation of MetMb, using the equation of Tang et al. (*21*), was highly correlated with A_{503}/A_{581} ($r = 0.93$).

A similar experiment, using split-chamber cuvettes, was performed using variable proportions of COMb and MetMb to validate the usefulness of A_{503}/A_{581} for brown color formation in COMb solutions. The absorbance spectra for Mb solutions containing variable proportions of COMb and MetMb are shown in **Figure 4**. Although there is no specific method to calculate the proportion of MetMb in the presence of COMb, DeoxyMb, and/or OxyMb, we were able to estimate the relative proportion of MetMb based on the actual amount used in the different chambers of the split-chamber cuvette. Here also, proportion of MetMb was strongly correlated with A_{503}/A_{581} ($r = 0.94$), as we observed in the OxyMb + MetMb split-chamber cuvette experiments. This result further validated our assumption that A_{503}/A_{581} could estimate the proportion of brown color in COMb solutions. On the basis of the actual amounts of COMb and OxyMb delivered to the split-chamber cuvettes, the proportion of red colored non-MetMb pigments decreased with an increased A_{503}/A_{581}. These observations led us to adopt A_{503}/A_{581} as a BI to estimate the formation of brown color in Mb solutions. Therefore, browning index is expressed as $BI = A_{503} \div A_{581}$.

Incubation of HNE with COMb and OxyMb. The effect of HNE on BI in COMb and OxyMb solutions during incubation at pH 7.4 and 37 °C is presented in **Figure 5**. BI was greater in HNE-treated OxyMb and COMb samples compared to controls ($P < 0.05$). Moreover, changes in BI followed the same trend in HNE-treated COMb and OxyMb samples. This observation is significant because it is widely believed that COMb is more stable to autoxidation than OxyMb. However, our observations suggested that, under physiological conditions and when challenged with HNE, brown color formation proceeded in COMb similar to that in OxyMb. This could be attributed partly to the well-documented photodissociation phenomena in COMb (*36*) in which the CO molecule initially bound to iron escapes from the heme crevice when exposed to light. Moreover, it is well-known that the mass action of oxygen, at relatively high concentrations, accelerates dissociation of CO from heme proteins; hyperbaric oxygen is used as a treatment for CO poisoning in human beings (*37*). Similarly, investigations to determine the fate of CO in ground beef using ^{14}C-labeled CO observed that ^{14}CO escaped from beef systems to the atmosphere during storage (*38*). Once CO is released from COMb, it is logical to expect that the resulting Mb (DeoxyMb) molecule would be converted to OxyMb by atmospheric oxygen and/or subsequently oxidized to brown MetMb in the presence of reactive aldehydes in aqueous solutions. It is possible that nucleophilic attack and subsequent adduction by HNE might occur in COMb in a manner similar to that observed in OxyMb

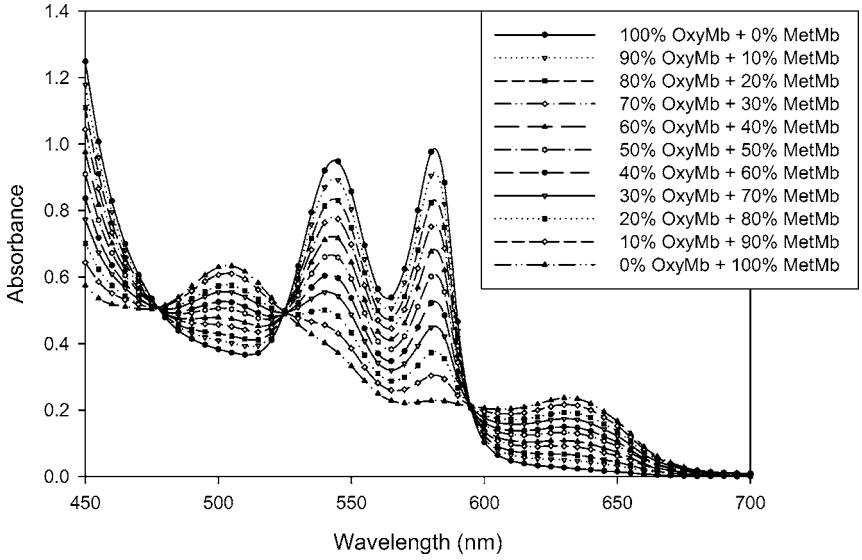

Figure 3. Absorbance spectra for Mb solutions containing different proportions of OxyMb and MetMb at pH 7.4, in 50 mM sodium phosphate buffer. A split-chambered cuvette was used to avoid mixing of the two Mb forms.

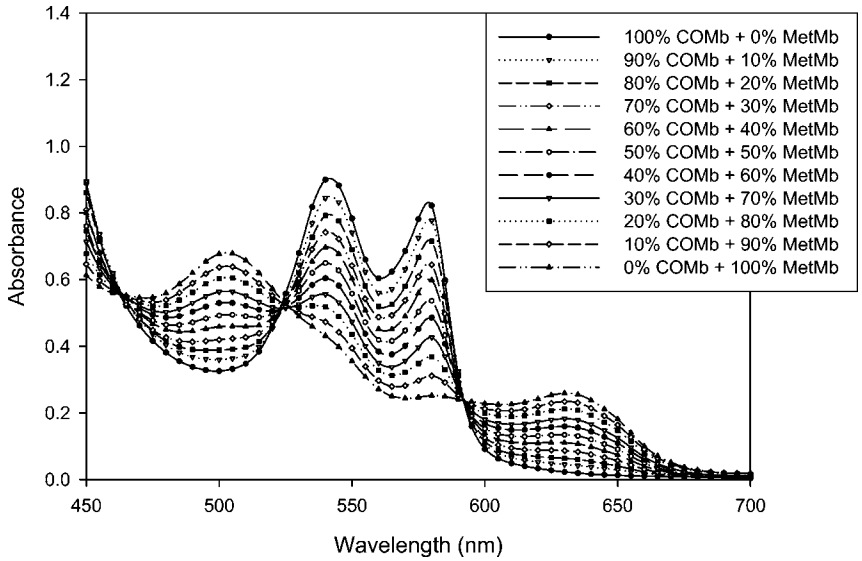

Figure 4. Absorbance spectra for Mb solutions containing different proportions of COMb and MetMb at pH 7.4, in 50 mM sodium phosphate buffer. A split-chambered cuvette was used to avoid mixing of the two Mb forms.

(*29*−*32*) and compromise the redox stability of COMb. Further investigations using mass spectrometry are essential to validate potential aldehyde adduction and covalent modifications in COMb.

The changes in BI of COMb and OxyMb samples during incubation with HNE were also investigated at pH 5.6, 4 °C and are shown in **Figure 6**. BI values were significantly lower in COMb than OxyMb samples ($P < 0.05$). This observation was in agreement with the widely accepted fact that the cherry-red color of CO-treated meat is more stable than the red color of conventionally bloomed meat (*1*). However, HNE increased BI values in COMb ($P < 0.05$), but not OxyMb solutions (**Figure 6**). Autoxidation of OxyMb would be expected to progress rapidly at pH 5.6 (*39*), potentially fast enough to mask the redox destabilizing effect of HNE. Similar results were observed during the incubation of HNE with equine OxyMb (*32*), bovine OxyMb (*30*), and porcine OxyMb (*31*). Our findings suggested that when exposed to HNE, COMb undergoes browning more rapidly than OxyMb at pH 5.6 and 4 °C. Nevertheless, BI was significantly lower ($P < 0.05$) in HNE-treated COMb than in HNE-treated OxyMb and indicated lower brown color formation in COMb compared with OxyMb when challenged with lipid oxidation products under typical meat storage conditions. However, one should be aware that discoloration and myoglobin oxidation in complex muscle food systems are governed by a variety of other factors, including species, pH, temperature, packaging type, partial pressure of oxygen, light, and microbial growth. These factors, along with the presence/absence of antioxidants/prooxidants, also affect lipid oxidation, which contributes to meat discoloration.

The A_{503}/A_{581} ratio, BI, was established to estimate the relative proportion of brown color in aqueous solutions containing COMb and/or OxyMb. Estimation of MetMb is not practical in the presence of COMb, DeoxyMb, and/or OxyMb, and hence, BI could represent an appropriate index to measure brown color formation in CO-treated meat. HNE, a reactive product of lipid oxidation, induced browning in COMb and OxyMb solutions. The results of the present study suggested that COMb is susceptible to lipid-oxidation-induced browning in a pH- and temperature-dependent manner. Further investigations are underway to elucidate the molecular interaction between COMb and lipid oxidation products.

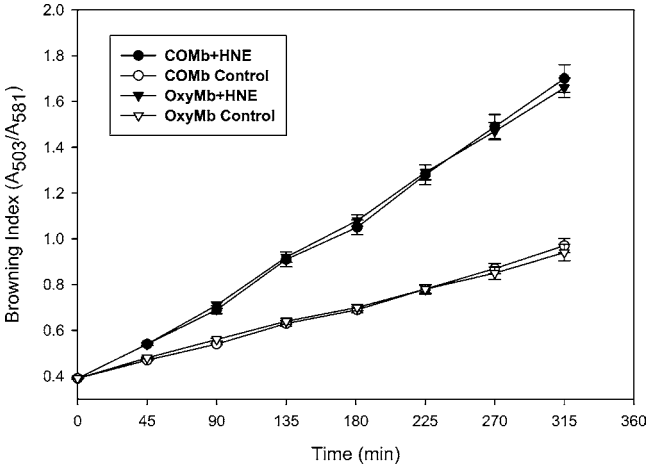

Figure 5. The changes in browning index (A_{503}/A_{581}) during incubation of COMb or OxyMb (0.15 mM) with HNE (1.0 mM) at pH 7.4 and 37 °C, in 50 mM sodium phosphate buffer. Means were generated for *F*-tests resulting from a significant ($P < 0.05$) effect of HNE on the redox stability of COMb and OxyMb. Standard errors that determine treatment differences are reported as error bars in the figures. Higher BI values indicate increased browning.

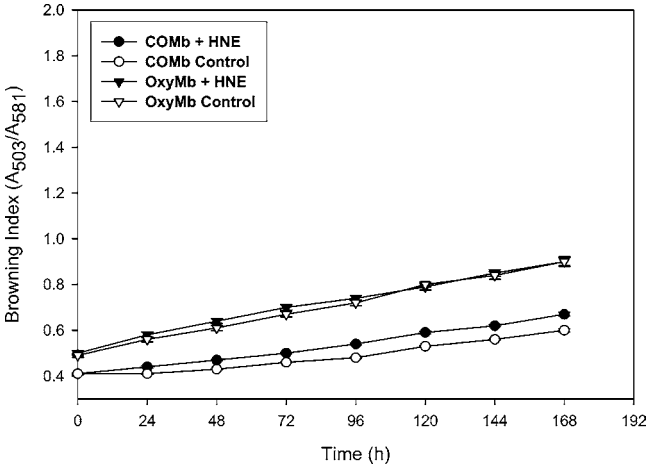

Figure 6. The changes in browning index (A_{503}/A_{581}) during incubation of COMb or OxyMb (0.15 mM) with HNE (1.0 mM) at pH 5.6 and 4 °C, in 50 mM sodium citrate buffer. Means were generated for *F*-tests resulting from a significant ($P < 0.05$) effect of HNE on the redox stability of COMb and OxyMb. Standard errors that determine treatment differences are reported as error bars in the figures. Higher BI values indicate increased browning.

ABBREVIATIONS USED

BI, browning index; Mb, myoglobin; COMb, carboxymyoglobin; OxyMb, oxymyoglobin; DeoxyMb, deoxymyoglobin; MetMb, metmyoglobin; HNE, 4-hydroxy-2-nonenal; MAP, modified atmosphere packaging; CO, carbon monoxide.

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