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Particulate Metmyoglobin Reducing Activity and Its Relationship with Meat Color

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There is controversy about the effect of storage time on metmyoglobin (MetMb) reducing activity in the sarcoplasmic fraction of meat and the role of this reducing activity in maintaining the color of red meat. The presence of metmyoglobin reducing activity in the myofibrillar fraction of muscle extracts was investigated as a possible reason for this controversy. NADH-dependent MetMb reducing activity was found in the particulate fraction of meat following sedimentation of a meat homogenate at 35 000*g*. There was 5.8 times more MetMb reducing activity in the particulate fraction compared with that of the sarcoplasmic (supernatant) fraction. The presence of metmyoglobin reducing activity in the myofibrils (MMRA), defined as the activity in the sediment after centrifugation of a meat homogenate at 2 000*g*, was confirmed. The myofibrillar fraction contained 63% of the total MetMb reducing activity available in the meat. The relationship between muscle MetMb reducing activities and meat color parameters was investigated in a beef patties system. The particulate MetMb reducing activity (PMRA) in beef patties was found to be a good indicator of the total metmyoglobin reducing activity in meat and was positively correlated with the color parameters of the patties, suggesting that PMRA may be associated with red meat color of meat patties.

KEYWORDS: Metmyoglobin reducing activity; myofibrillar; sarcoplasmic; color; color stability

INTRODUCTION

Many investigators have emphasized the importance of meat color because of its association with the market value of meat (*1*, *2*). The amount and state of myoglobin (Mb) in meat are the most important determinants of meat color. In living animals, there is equilibrium between the reduced form of Mb, which is purple in color, and the oxygenated form, oxymyoglobin (OxyMb), which is bright red. In both forms the heme iron is reduced (ferrous state). Upon exposure of freshly cut meat to air, the conversion of Mb to OxyMb is due to the covalent binding of molecular oxygen to the free binding site of heme. Metmyoglobin (MetMb), which is tan-brown in color, is formed by the oxidation of heme iron from the ferrous to the ferric state. During storage, accumulation of MetMb at the meat surface is the major factor leading to fresh meat discoloration (*3*). This redox reaction can be reversible under specific conditions, depending on MetMb reducing activity, cofactors, and oxygen availability. MetMb reducing enzymes can reduce MetMb to Mb, which can then be oxygenated to bright red OxyMb. However, the role of MetMb reducing activity in the maintenance of fresh meat color is debatable. Some investigators (*4*−*6*) have reported that MetMb reducing activity is the controlling factor retarding the accumulation of MetMb. Other researchers (*7*−*10*) have found no evidence to support this theory.

There are conflicting reports on the effect of storing meat on MetMb reducing activity. Some researchers have found no change or an increase in MetMb reducing activity throughout the storage time (*7*, *9*, *10*), whereas others have reported up to a 50% decrease (*11*, *6*).

The standard method to determine MetMb reducing activity is to use the supernatant of muscle homogenates after centrifugation at either 35 000*g* (*9*, *12*, *13*) or 2 000*g* (*7*). There have been no reports, to our knowledge, on whether MetMb reducing activity resides in the myofibrillar fraction in the pellet. Given the controversy on the effect of storage time on MetMb reducing activity, it is reasonable to speculate that MetMb reducing activity may be present in the myofibrillar fraction of meat.

Bekhit et al. (*14*) observed an apparent loss of MetMb reducing activity in beef patties during storage at 2 °C when measured in the presence of a standard amount of NADH in the assay (0.1 mL of 1 mM NADH). The activity was recovered, however, by using 0.1 mL of 2 mM NADH in the assay. Indeed, ensuring the availability of sufficient amount of NADH is crucial for the expression of the full activity of MetMb reductase (*9*, *13*). Therefore, in the present study, we measured the MetMb

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reducing activity at different concentrations of NADH during storage to evaluate the requirement for NADH.

The objectives of the present study were to investigate (i) the presence of MetMb reducing activity in the myofibrillar fraction of muscle as a possible reason for the reported increase of MetMb reducing activity found in aged meat (*7*, *9*, *10*) and (ii) to study the relationship between color and MetMb reducing activities during cold storage of meat.

MATERIALS AND METHODS

Reagents. Potassium ferrocyanide and equine myoglobin (purity 90%) were obtained from Sigma (St. Louis, USA). Potassium ferricyanide was from Laboratory Chemicals (Dagenham, England). NADH (disodium salt grade1, purity 97%), sodium dihydrogen orthophosphate anhydrous (purity 98%), and disodium dihydrogen orthophosphate anhydrous (purity 99%) were from BDH Laboratory Supplies (Poole, England).

Sample Preparation. *Experiment 1. Fractionation of MetMb Reducing Activity in Beef Longissimus Dorsi (LD).* Meat of normal pH (pH = 5.68, 48 h postmortem) was obtained from a facility processing beef for export. The meat samples were frozen and stored at −80 °C until analyzed.

Experiment 2. Effect of Time Postmortem and Storage Time on Color, Color Stability, Assay NADH Concentration, and Metmyoglobin Reducing Activity in Beef Patties. Fresh meat was obtained from carcasses of Angus heifers (612 ± 18 kg live weight, $n = 11$) reared on pasture and slaughtered in processing facilities licensed to export. The animals were mechanically stunned with a captive bolt and processed, and the carcasses were hung at 4 °C. The meat from the carcasses had a normal pH (range 5.54−5.62) 24 h postmortem. The carcasses were hung in the chiller for 5 days during which time steaks 20 mm thick were cut from the longissimus et lumborum muscles at 1, 2, and 4 days postmortem (1, 2, and 4 PM). The sampling was started at the 13th rib for the 1 PM sample and then toward the lumbar part of the muscle for the subsequent samples as outlined in **Figure 1**. Samples from the 11 animals corresponding to each postmortem day were assigned randomly to three batches. Thus, each batch was meat from 3−4 animals. The visible fat and connective tissue were removed from the meat prior to chopping into small pieces. Each batch of meat was then ground using a food processor (Braun multipractic MC100, Braun Co.). Meat patties (3 patties/batch) were formed by pressing the samples into Petri dishes and patties were placed in polystyrene trays covered with an oxygen permeable poly(vinyl chloride) film (oxygen permeability > 2000 mL m^{-2} atm^{-1} 24 h^{-1} at 25 °C, AEP FilmPac(Ltd), Auckland, New Zealand) and stored at 4 °C in a fluorescent illuminated display cabinet. Samples were taken from each pattie after 0, 1, 3, and 6 days of storage, frozen and stored at −80 °C until analyzed. Biochemical and color measurements on each sample were determined in triplicate.

pH Measurements. The pH of the steaks was determined at 24 h postmortem using an InLab 427 pH combination puncture electrode (Mettler-Toledo Process Analytical Inc., Wilmington, MA) attached to a Hanna HI 9025 pH meter.

Color and Color Stability Measurements. The color of meat patties was measured using a Minolta chromameter CR-210 {Observer 2°, illuminant D_{65}}, which had been standardized with a white tile [$L^* = 98.14$, $a^* = -0.23$, and $b^* = 1.89$]. Color was described as coordinates, i.e., L^*, a^*, and b^* (where L^* measures relative lightness, a^* relative redness, and b^* relative yellowness). Three replicate measurements were taken from each pattie 1 h after producing the patties and, subsequently, after 1, 3, and 6 days of storage at 4 °C. The human perception of color is based on (i) the hue angle (H), which represents the relative position of the color between redness and yellowness, (ii) chroma (C), which assesses the color intensity, and (iii) lightness (Eagerman et al., 1977). The a^* and b^* measurements were used to calculate the hue angle ($H = \tan^{-1} b^*/a^*$), chroma ($C = (a^{*2} + b^{*2})^{1/2}$). Color stability was expressed as the rate of change (derived from the models of fitted data in **Figure** 6) in L^*, H, and C.

Preparation of Equine Metmyoglobin for MetMb Reducing Activity Measurements. Equine myoglobin was dissolved in 2.0 mM phosphate buffer (pH 7.0), oxidized with a slight excess of $K_3Fe(CN)_6$, dialyzed (10000 MW cutoff membrane) against water, and further dialyzed against 2.0 mM phosphate buffer (pH 7.0) at 4 °C. The metmyoglobin solution was concentrated in Centriprep 10 concentrators (Amicon Inc., USA) to 0.75 mM based on spectrophotometric measurement at 525 nm ($\epsilon_{525} = 7700$ L mol^{-1} cm^{-1}) (Anderson and Skibsted, 1992). Metmyoglobin was stored in 5-mL aliquots at −100 °C until used.

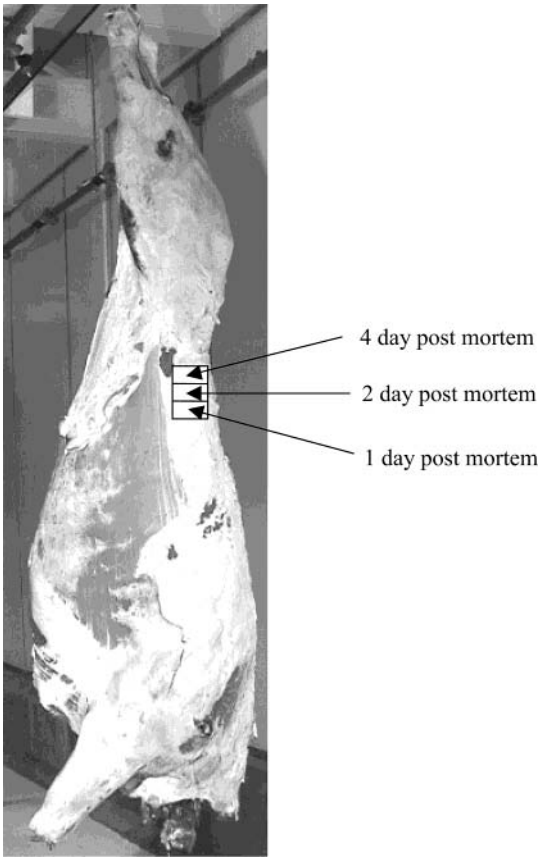


Figure 1. The sampling procedure showing the location for each postmortem day, which remained constant among all the carcasses.

Determination of Metmyoglobin Reducing Activity in Sarcoplasmic and Myofibrillar Fractions. *Sample Fractionation.* MetMb reductase extracts were obtained using a modification of standard techniques (*11*, *13*) as shown in **Figure 2A**. A 6-g sample was homogenized for 30 s in 20 mL of cold extraction buffer (2.0 mM phosphate buffer, pH 7.0 containing 1.0 mM EDTA and 0.1 mM dithiothreitol) with a Polytron PT 3100 (Polytron, Littau, Switzerland) set at 13 500 rpm. The homogenate was centrifuged at 35 000*g* for 30 min at 4 °C, and the supernatant was filtered through Whatman No.1 filter paper. The pellet was extracted twice more as described above. The three supernatant extracts were pooled and used as the source for sarcoplasmic metmyoglobin reducing activity (SMRA). The resultant pellet, after three extractions, was suspended in 100 mL of the extraction buffer and used as the source for particulate metmyoglobin reducing activity (PMRA) ($n = 8$).

Another fractionation protocol (*7*) was used to verify that the sediment reducing activity resided in the myofibrillar fraction (**Figure 2B**). In this protocol, a 2-g sample of minced meat was homogenized in 20 mL of cold extraction buffer as described above, and the resultant homogenate centrifuged at 2 000*g* for 20 min at 4 °C. The supernatant was filtered as before, and the pellet was extracted twice more with 20 mL of extraction buffer as described above. The three supernatant extracts were pooled, the final pellet was suspended in 100 mL extraction buffer, and the supernatant and the suspension were used, respectively, as sources for sarcoplasmic (SMRA) and myofibrillar (MMRA) MetMb reducing activity ($n = 8$).

Metmyoglobin Reducing Activity Measurements. Metmyoglobin reductase activity was determined as previously described (*13*) using

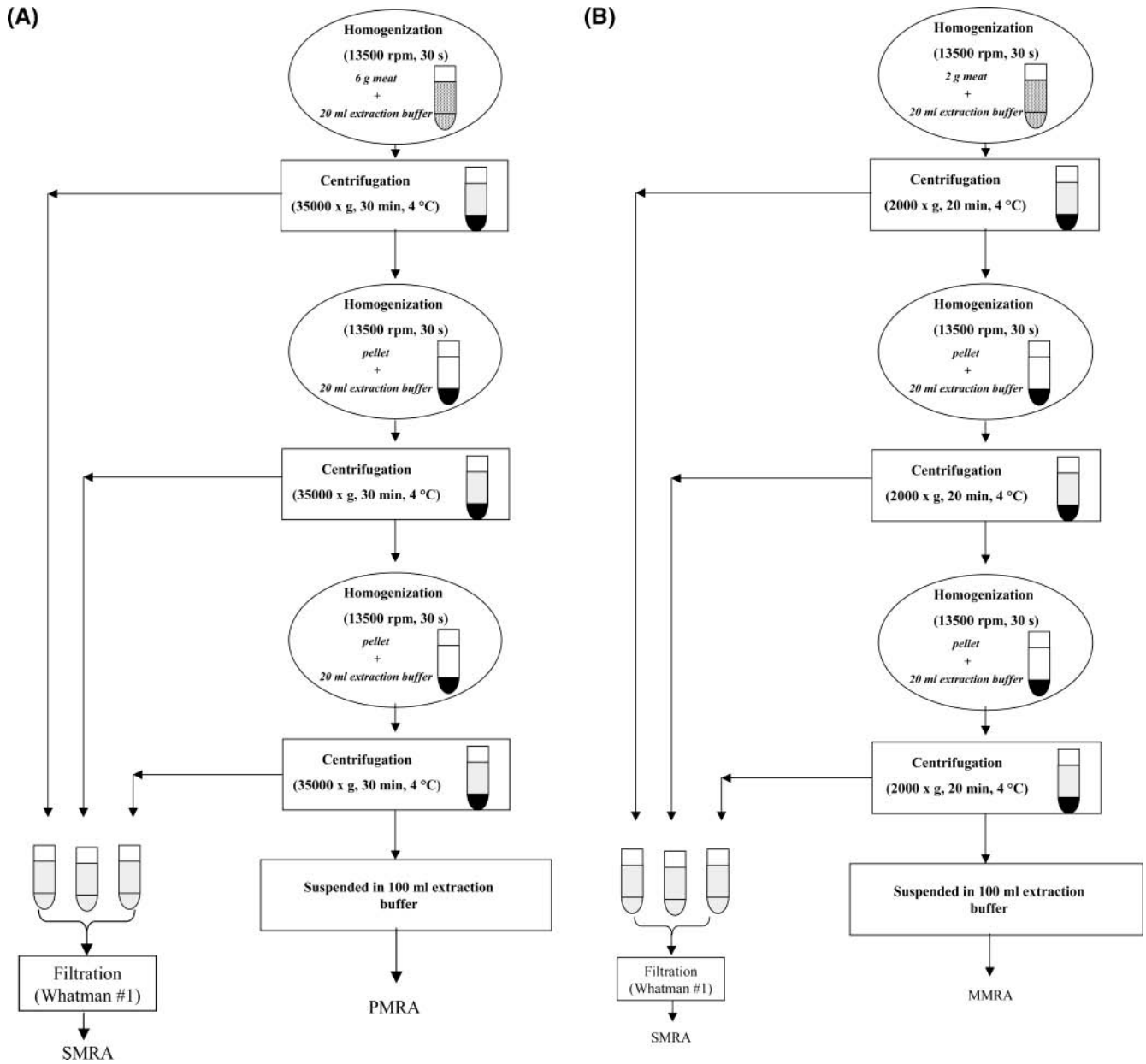


Figure 2. (A) Schematic of the extraction process of MetMb reducing activity in various fraction of meat utilizing the procedure of Reddy and Carpenter (*12*) and Mikkelsen et al. (*13*). (B) Schematic of the extraction process of MetMb reducing activity in various fraction of meat utilizing the procedure of Echevarne et al. (*7*).

a Unicam UV4 spectrometer (Unicam Ltd., UK). The standard assay mixture contained 0.1 mL of 5 mM EDTA; 0.1 mL of 50 mM phosphate buffer (pH 7.0); 0.1 mL of 3.0 mM $K_4Fe(CN)_6$; 0.2 mL of 0.75 mM MbFe(III) in 2.0 mM phosphate buffer (pH7.0); 0.1 mL of 2.0 mM NADH; muscle extract (0.3 mL for SMRA and 50 μL for PMRA), and water to a final volume of 1 mL assay mixture. The assay mixture pH was 6.4, and the assay was carried out at 25 °C. The reaction was initiated by adding NADH and was monitored by the change in absorbance at 580 nm. Blanks contained all of the additions except NADH, which was replaced by water. The activity was calculated as the mean of three determinations on each pattie and expressed as nanomoles of MetMb reduced per minute per gram of meat. Total metmyoglobin reducing activity (TMRA) was calculated as the sum of PMRA or MMRA and SMRA.

The metmyoglobin reducing activity measurements were repeated using different concentrations of NADH (1, 2, 3, and 4 mM) to examine the effect of storage time on the amount of NADH required for the expression of full activity.

Statistical Analysis. The analysis was performed using Genstat software (5th ed., release 4.21). Data from the color parameters and MetMb reducing activities were meaned over the three patties, resulting in 36 values per variable (3 postmortem time (PM) × 3 batches × 4 storage time (ST)). These values were then subjected to a split plot analysis of variance, with the nine batches treated as main plots. PM was the main plot treatment factor, and ST was the subplot treatment factor. The statistical model was $Y_{ijk} = \mu + (PM)_i + \epsilon_{ij} + (ST)_k + (PM{\cdot}ST)_{ik} + \epsilon_{ijk}$, where $\epsilon_{ij} = batch_j + (PM{\cdot}batch)_{ij}$. Orthogonal polynomial contrasts were calculated using times of 1, 2, and 4 days for PM and 0, 1, 3, and 6 days for ST and used to decide appropriate terms to be included in the chosen model. The resulting regression equations were used to plot 3-D plots using Maple V software (release 5.1.).

The fully nested ANOVA design of Minitab (release 13.1, Minitab inc.) was used to test for NADH concentration effects with SMRA, PMRA, and TMRA as the responses and NADH concentrations (1, 2, 3, and 4 mM), PM, and ST as the factors. Means of main effects were plotted and considered significantly different at $p < 0.05$ by the least significance difference test.

Table 1. Postmortem Time and Storage Effects[a]

	postmortem time			storage time			interaction		residual	
variable	df	ms	S	df	ms	S	ms	S	df	ms
SMRA	2	10870.5	***	3	3912.2	***	26031.4	***	18	108.3
PMRA	2	20764	***	3	293260	***	202841	***	18	13016
TMRA	2	126441	**	3	359246	***	239816	***	18	13292
*L**	2	0.4218	ns	3	9.5167	***	0.8046	**	18	0.1734
C	2	12.538	*	3	528.41	***	2.82	***	18	0.3455
H	2	22.88	***	3	102.33	***	17.8	***	18	0.3469

[a] Values are degrees of freedom (df), mean squares (ms), and significance (S) from variance analysis. ns = nonsignificant. * = significant at ($p < 0.05$). ** = significant at ($p < 0.01$). *** = significant at ($p \leq 0.001$).

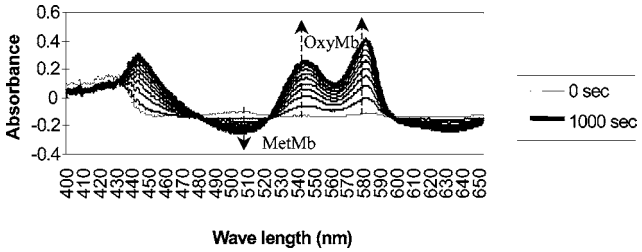


Figure 3. Spectral changes during metmyoglobin reduction using the myofibrillar muscle fraction.

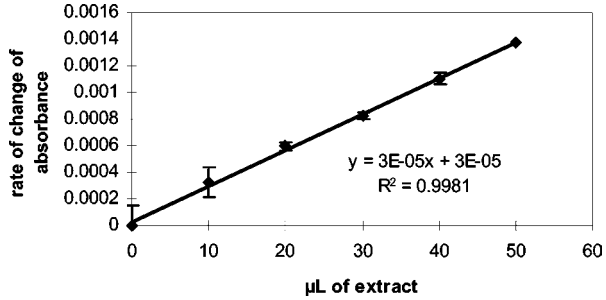


Figure 4. Effect of various amounts of myofibrillar extract on the changes in absorbance. Errors bars represent ± SD.

RESULTS

Experiment 1. Fractionation of MetMb reducing activity in beef Longissimus Dorsi (LD). The spectral scan of the assay mixture for the particulate fraction is shown in **Figure 3**. A decrease in peaks at 505 and 630 nm, which characterize metmyoglobin, and an increase in 540 and 580 nm peaks, which characterize oxymyoglobin, was observed during the reaction period. No reduction of MetMb was found if the particulate fraction or NADH was omitted from the assay.

The rate of change in absorbance was proportional to the amount of added particulate fraction (**Figure 4**). The sarcoplasmic MetMb reducing activity was 367 nmol min^{-1} g^{-1}. The 35 000*g* particulate MetMb reducing activity was 5.8 times greater than the sarcoplasmic MetMb reducing activity (**Figure 5**). Using the 2 000*g* fractionation protocol of Echevarne et al. (*7*), the myofibrillar fraction (the sediment of 2000 g of meat homogenate), after three washes with extraction buffer as described in Material and Methods, contained 63% of the total MetMb reducing activity. It should be noted that the 35 000*g* particulate fractions contains myofibrils, mitochondria, and other parts of the muscle cells, whereas the 2 000*g* pellet contains mainly myofibrils. The 2 000*g* myofibril pellet contained 74% of the MetMb reducing activity found in the 35 000*g* pellet. The MetMb reducing activities for sarcoplasmic and particulate fractions of LD muscle using the two fractionations protocols are in **Figure 5**.

Figure 5. Metmyoglobin reducing activity in sarcoplasmic and myofibrillar fraction of LD muscle. Errors bars represent ± SD

Preliminary work on the particulate fraction showed that following the change in absorbance at 580 nm (the initial linear part of the increase in absorbance), directly after mixing the assay mixture, gave consistent results. The alternative approach of measuring the reaction product, after a fixed period of incubation followed by centrifugation, gave variable results. This was due to the lag time between the addition of the reactants and the start of the reduction reaction, which varied from one sample to another. In this study, the enzyme activity was calculated from the initial linear part of the increase in absorbance.

Experiment 2. Effect of Time Postmortem and Storage Time on Color and Color Stability of Beef Patties. The color parameters, lightness (*L**), chroma (*C*), and hue (*H*) of the beef patties were affected by storage time as shown in **Figure 6** and **Table 1**. The postmortem time only had a significant ($p < 0.001$) impact on *C* and *H* after the patties were stored for 6 days. The interactions between time postmortem and storage time were significant ($p < 0.01$) for all color parameters (**Table 1**). Although the initial lightness of 4 PM patties tended to be lower than the lightness of 1 and 2 PM patties, there were no significant differences found between the treatments. The 3-D plot showed that *L** has polytonic properties with variable changes occurring on storage.

At the end of the storage period the 4 PM patties exhibited *C*-values significantly higher ($p < 0.05$) and *H*-values significantly lower ($p < 0.05$) than those of the 2 PM patties. The orthogonal polynomials analysis showed a significant effect for the linear and quadratic components of storage time for the regression with *L**, *C*, and *H*, which indicates that the quadratic models are appropriate. The linear and quadratic components of time postmortem has a significant effect for the regression with *H*, but only the linear component had a significant effect on *C*. Together, storage time and time postmortem accounted for 73.2%, 98.8%, and 96.9% of changes in *L**, *C*, and *H*, respectively.

The main effects of time postmortem and storage time are shown in **Figure 7**. *L**-values had a tendency to decrease as the time postmortem and the storage time increased. *C*-values tended to increase in the 2 PM samples but decreased with

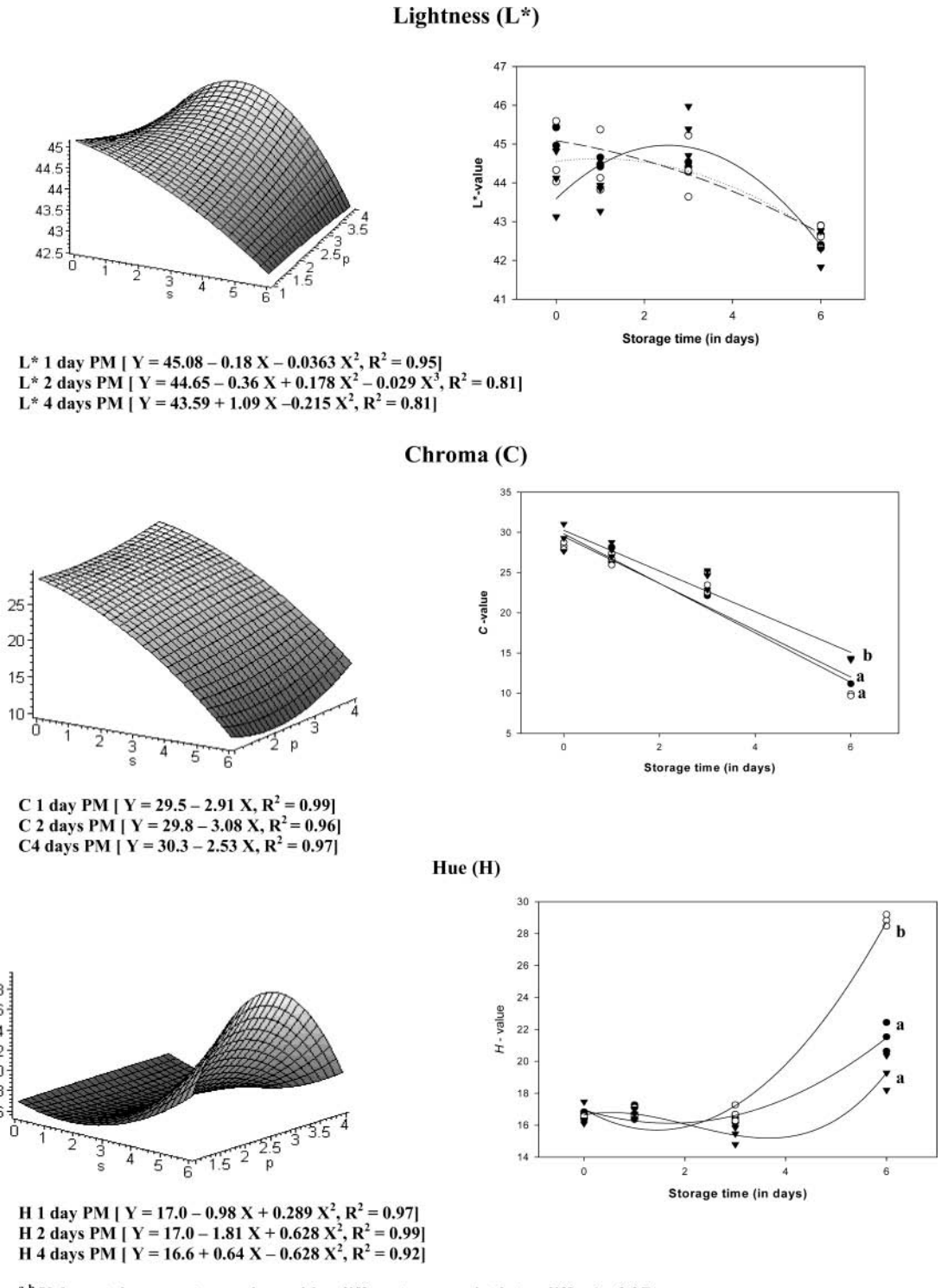
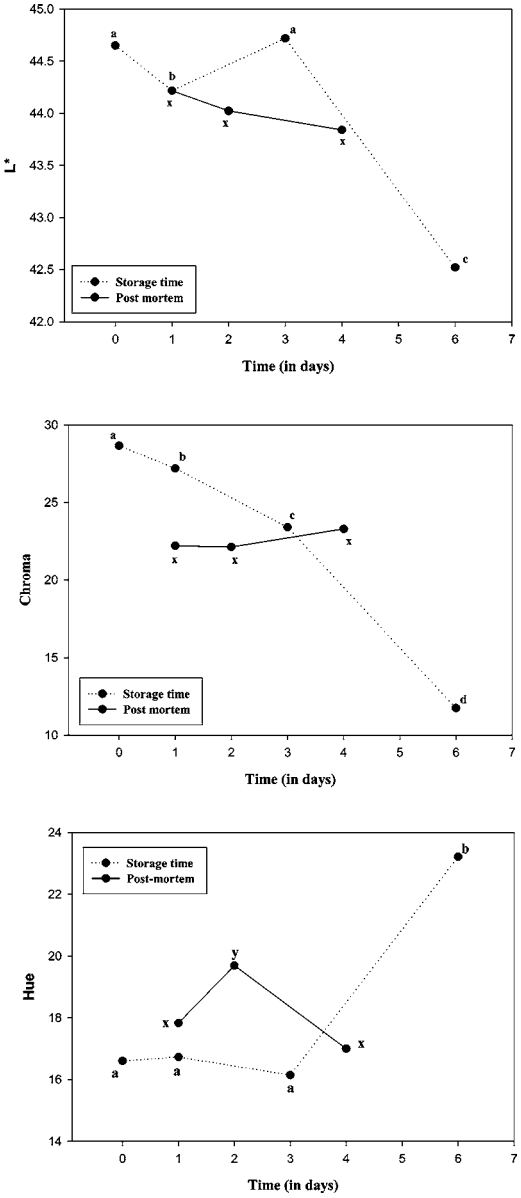


Figure 6. Changes in color parameters of beef patties as affected by time postmortem during storage for 6 days at 2 °C.

storage. The *H*-values of 1 PM patties were the same as 4 PM patties. However, both 1 and 4 PM patties had values lower than those of the 2 PM patties. The *H*-values were relatively stable during the first 3 days of storage but subsequently showed a substantial increase.

The color parameter data were used to develop appropriate descriptive models to calculate color stability (**Figure 6**). Color stability was calculated as the rate of change in the color parameters. The rates of change in *C* and *H* were lower for 4 PM patties than for the 1 and 2 PM patties. This result suggests that 4 PM patties exhibited better color stability than any other treatment.

Effect of Time Postmortem, Storage Time, and Assay NADH Concentration on Metmyoglobin Reducing Activity in Beef Patties. *Sarcoplasmic Metmyoglobin Reducing Activity (SMRA).* SMRA was significantly ($p < 0.001$) affected by time postmortem, storage time, and their interactions (**Table 1**). After 24 h of storage at 4 °C, there was no change in SMRA. The average SMRA was significantly ($p < 0.001$) increased by 26% after 3 and 6 days of storage (**Figure 8**). An apparent increase



a-d, x-y Values at the same storage time with a different superscript letter differ ($p<0.05$)

Figure 7. Main effects of time postmortem and storage time on the color parameters of beef patties during storage at 2 °C.

(45%) in SMRA was observed in 2 PM meat patties at 0 storage time compared with the 1 PM patties at 0 storage time. However, SMRA for the 2 PM patties tended to decrease as storage time increased, and after 6 days of storage only 70% of the initial activity was found. No differences were observed in SMRA in the 2 PM patties at 1 and 3 days of storage.

The SMRA for 4 PM patties displayed a relative increase at 1 day but decreased after 3 days of storage. However, after 6 days of storage the SMRA was similar to that at 0 time. The 4 PM patties had SMRA slightly lower than that of 2 PM patties at 0 storage time. However, there was a significant increase in the SMRA of the 4 PM patties after 1 day of storage compared with that of the 2 PM. Furthermore, 4 PM patties had SMRA higher than that of the 1 PM patties at 0 and 1 day of storage.

The main effects of time postmortem, storage time, and NADH level on SMRA are shown in **Figure 9**A. SMRA increased as the time postmortem increased and decreased as the storage time increased. The concentration of NADH used in the assay did not exhibit any consistent trends or major effects on SMRA of the 1, 2, or 4 PM patties (**Figure 9A**). However,

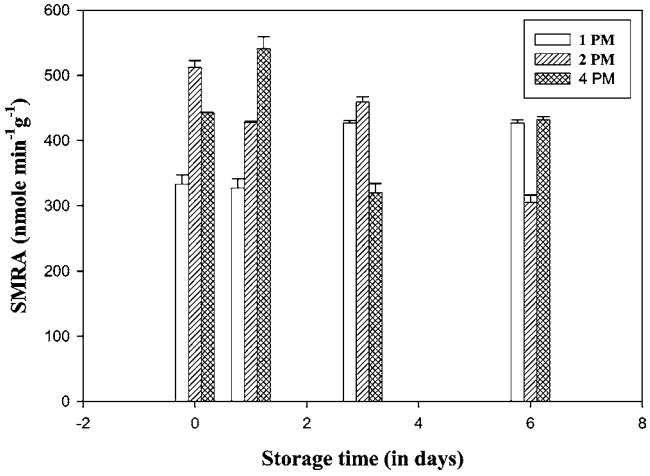


Figure 8. Effect of time postmortem and storage time on SMRA of beef patties during storage at 2 °C. Errors bars represent ± SD

lower SMRA values were associated with 1 mM NADH compared with those for 2, 3, and 4 mM NADH.

Particulate Metmyoglobin Reducing Activity (PMRA). PMRA was significantly affected by time postmortem and storage time and their interactions ($p < 0.001$, **Table 1**). However, PMRA was not affected by batches and its interactions. PMRA in 1 PM patties decreased during the first 3 days storage at 4 °C (**Figure 10**). After 6 days of storage, PMRA was similar to that found at 1 day of storage. A significant reduction in PMRA was observed in the 2 and 4 PM (**Figure 10**) patties after 1 and 3 days of storage, respectively. The main effects plot of time postmortem and storage time on PMRA is shown in **Figure 9B**. PMRA decreased after 2 days postmortem and remained the same thereafter. PMRA decreased as the storage time increased.

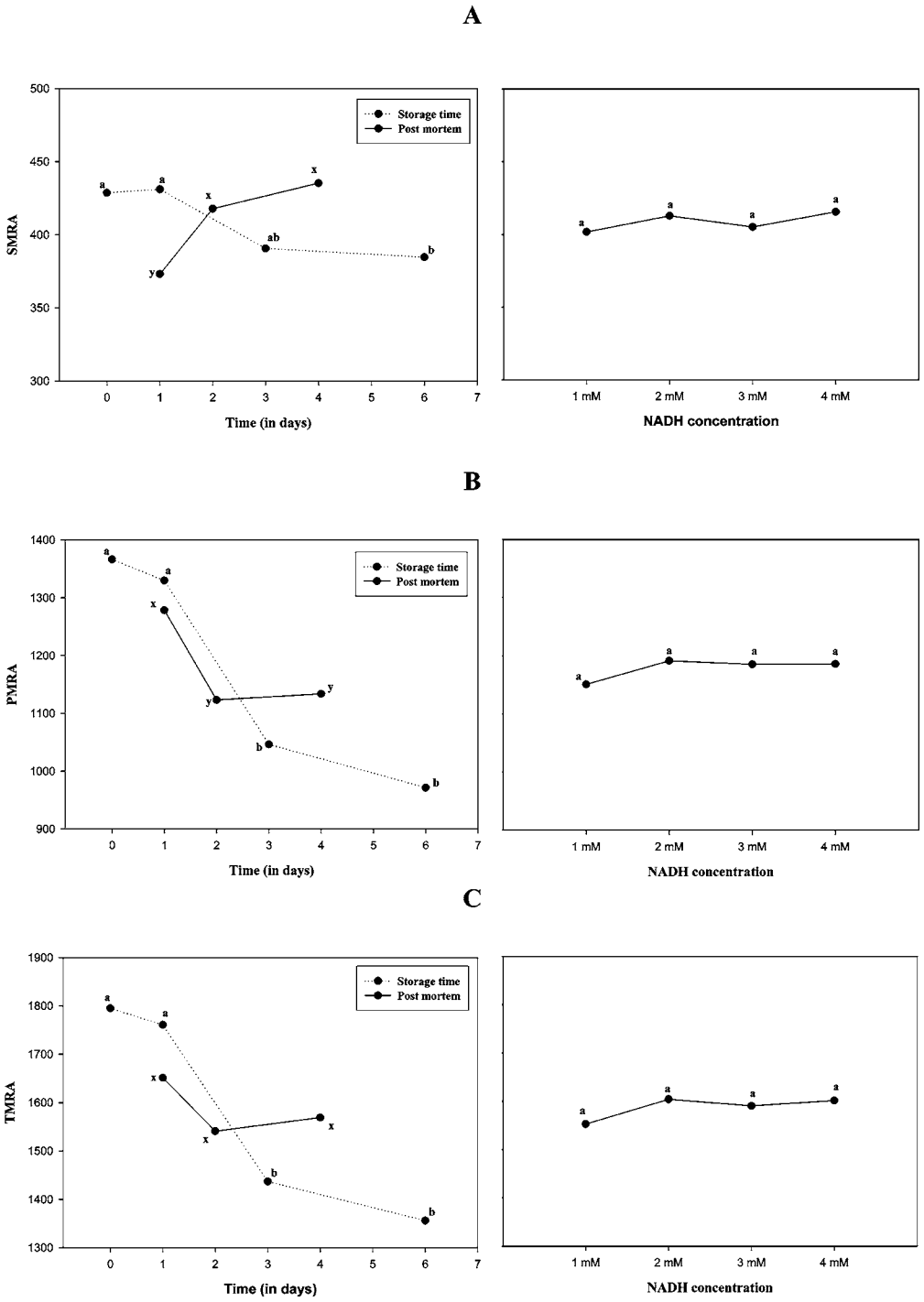
The NADH concentration in the assay did not have any effect on PMRA. However, the main effect plot of NADH level on PMRA shows that 1 mM NADH produced slightly lower but insignificant PMRA values compared with those for 2, 3, and 4 mM NADH (**Figure 9B**).

Total Metmyoglobin Reducing Activity (TMRA). The effects of time postmortem, storage time, and NADH level on TMRA are shown in **Figure 11**. TMRA for 1 PM patties remained relatively constant over the 6 days of storage. TMRA was stable at 0 and 1 day storage in the 2 and 4 PM patties and decreased after 3 days of storage.

The main effects of time postmortem, storage time, and NADH level on TMRA were similar to those observed with PMRA (**Figure 9C**).

DISCUSSION

Fractionation of MetMb Reducing Activity in Beef Longissimus Dorsi (LD). The presence of MetMb reducing activity in the soluble fraction of muscle extracts is well-documented for bovine (*11*), ovine (*9*), and porcine (*13*) muscles. Previous published studies (*7*, *9*, *10*) indicated that an increase in muscle MetMb reducing activity occurred during storage. One possible explanation for the increase in measured activity is the relocalization of MetMb reducing activity from the myofibrillar fraction to the soluble fraction of the muscle. To investigate this hypothesis, LD muscle samples were fractionated into sarcoplasmic and particulate fractions, and the level of MetMb reducing activity was determined in these two fractions. MetMb reducing activity was present in the particulate fraction, as shown by the decrease in 505 and 630 nm peaks, which characterize



[a-b, x-y] Values at the same storage time with a different superscript letter differ ($p<0.05$)

Figure 9. Main effects of time postmortem, storage time, and assay NADH concentration on (A) SMRA, (B) PMRA, and (C) TMRA.

metmyoglobin, and an increase in 540 and 580 nm peaks, which characterize oxymyoglobin. No reduction of MetMb occurred in the absence of the particulate fraction or NADH, indicating that the reduction is enzymatic and requires NADH as a source of electrons (reducing equivalents).

The sarcoplasmic MetMb reducing activity of 367 nmol min^{-1} g^{-1} was similar to the results previously reported (*9*, *17*). Particulate and sarcoplasmic MetMb reducing activities had a ratio of 5.8:1. From the results it is apparent that 85% of available muscle MetMb reducing activity was unaccounted for in the previous studies (*9*−*13*). An earlier report (*7*) employed a different fractionation protocol and obtained a 2 000*g* supernatant from a beef homogenate, which after centrifugation at 100 000*g* yielded an insoluble fraction. This insoluble fraction had 4−7 times more MetMb reducing specific activity than the original 2 000*g* supernatant. Given the differences in the fractionation protocol it is possible that the activity in the 35 000*g* particulate fraction in the present study corresponds to the same activity in the 100 000*g* sediment (*7*). If this is correct then the 2 000*g* sediment should contain little or no MetMb reducing activity. To test this, we prepared a washed 2 000*g* sediment and measured the MetMb reducing activity. The 2 000*g* sediment (which contains mainly the myofibrillar fraction) contained 63% of the total MetMb reducing activity.

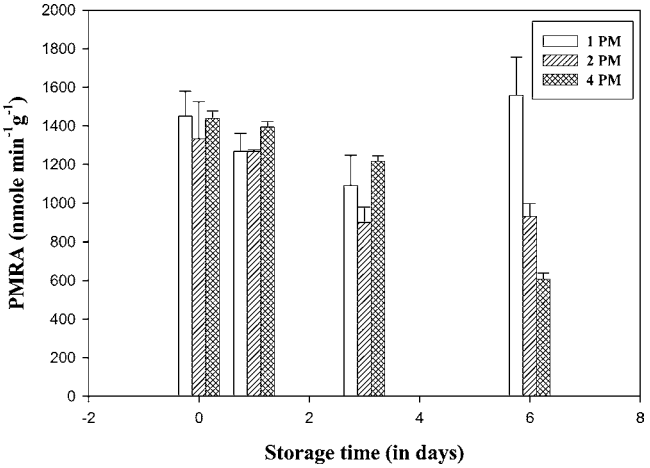


Figure 10. Effect of time postmortem and storage time on PMRA of beef patties during storage at 2 °C. Errors bars represent ± SD

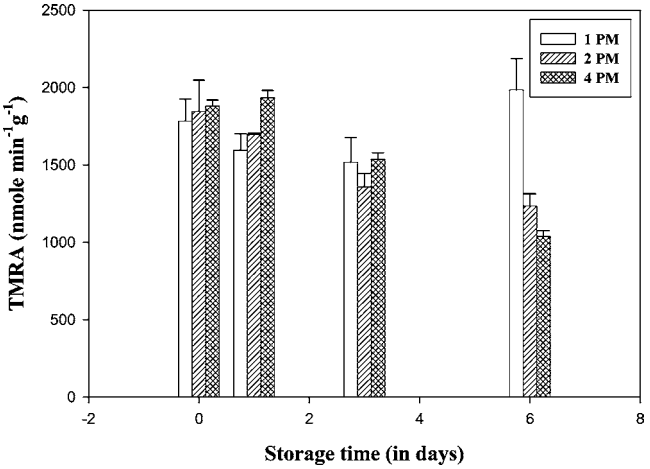


Figure 11. Effect of time postmortem and storage time on TMRA of beef patties during storage at 2 °C. Errors bars represent ± SD

This result indicates that PMRA is different from that previously reported (*7*). The results also confirm that the myofibrils contained MetMb reducing activity. This myofibrillar MetMb reducing activity is 63% of the total reducing activity in meat. The difference in MetMb reducing activity between the 2 000*g* and 35 000*g* fractionation protocols used in our study reflects the MetMb reducing activity in the mitochondrial fraction, which are the cellular components that precipitate on applying 35 000*g* to 2 000*g* supernatant.

Effect of Time Postmortem and Storage Time on Color and Color Stability in Beef Patties. Meat color stability has been reported to improve by aging meat for several days (*18*, *11*). The effect of time postmortem on the color stability in our study is consistent with these reports. The aging effect may be due to a decrease in oxygen consumption rate (OCR) over the aging period (*18*, *11*). Another factor revealed by the main effect analysis is that the intensity of the red color (*C*) in 4 PM treatment was higher than that in the 1 and 2 PM treatments. This is probably due to the known evaporative weight loss effect of chilling during aging (*19*), which results in a relative increase in the concentration of pigments. This possible explanation is supported by the decrease in *L**-values with the increase in time postmortem (**Figure 7**). The decrease in nonbound moisture would decrease the reflected light from the meat and produce lower *L**-values (*20*).

Effect of Time Postmortem, Storage Time, and Assay NADH Concentration on Metmyoglobin Reducing Activity in Beef Patties. There are contradictory reports on the effect of storage time on SMRA, which is the previously reported metmyoglobin reducing activity in meat muscles by investigators. Lanari and Cassens (*8*) observed no significant changes in MRA over 7 days of storage in beef muscles. However, some investigators have found muscle MRA decreased during storage of beef (*11*) and pork muscles (*6*), while others have observed an increase in this activity during the storage of beef (*7*, *10*) and lamb muscles (*9*). In the present study, the statistical analysis revealed that SMRA in beef patties was affected by time postmortem, storage time, and NADH concentrations. The main effects plots for the means revealed that SMRA increased with the increase in time postmortem, with values of 375, 420, and 435 nmol min^{-1} g^{-1} at 1, 2, and 4 days time postmortem, respectively. SMRA also increased slightly after 1 day of storage time but decreased to 92% and 88% of its initial value after 3 and 6 days of storage, respectively. The leakage of cell matrix material into the sarcoplasm due to aging seems to maintain or increase the SMRA (*9*). That was evident from the increase in the SMRA during the storage period for patties made at 1, 2, and 4 days time postmortem. The increase in sarcoplasmic MetMb reducing activity in aged meat has been shown to have in a negative correlation with color stability (*7*, *9*). This may be due to the presence of PMRA in the myofibrillar fraction of meat.

One might assume that the MRA measured at any time during storage will be influenced by the actions of protease enzymes on MRA during storage. The proteases break down the muscle cells and release the MRA enzymes from the tightly bound membranes. These proteases also catabolise the MRA enzymes and, consequently, reduce their activity.

From previous MRA characterization studies (*21*, *13*, *9*), it was noted that 0.1 mL of 1 mM NADH was sufficient to express the maximum activity of MRA. However, the meat used in the characterization studies were 48 h postmortem at the most. In previous studies (*14*, *22*) it was observed that after 9 days of storage of meat patties, MRA was lost in some samples but the activity was recovered by doubling the amount of standard NADH (0.1 mL 1 mM NADH). We did not observe a similar effect in the current study, probably because of the shorter storage period. However, 0.1 mL of 1 mM NADH produced numerically lower MRA. The usage of 0.1 mL of 2 mM NADH in the assay seems to be optimal for maximum activities and is recommended for the MRA assay.

The color parameters−MetMb reducing activities and storage time relationships are shown in **Tables 2** and **3** on the basis of patties and batches, respectively. The overall correlation coefficients indicate that storage time was negatively associated with PMRA and TMRA and to a lesser extent with SMRA. Positive correlations were found between PMRA, TMRA, *L**, and *C*. Weaker correlations were found between PMRA, TMRA and H. In general, there was no significant correlations between SMRA, *L**, and *C*.

Metmyoglobin reducing activity in meat has been measured by several different assays. Initially, MetMb reducing activity (MRA) was measured by oxidizing meat with potassium ferricyanide, with pigment reduction being followed spectrophotometrically (*23*). Ledward (*24*) developed the aerobic reducing activity (ARA) in which the meat was oxidized by storage for 24 h in 1% O_2. The MetMb reduction was then monitored for 24 h aerobically using a reflectance spectrophotometer. Another method widely used is the extraction method

Table 2. Overall Correlation Coefficients between MetMb Reducing Activities, Lightness (*L**), Chroma (*C*), and Hue (*H*) in Raw Beef Patties during Storage at 2 °C[a]

	storage (days)	*L**	C	H
METMb reducing activity				
sarcoplasmic				
1 mM NADH	–0.22	–0.11	0.26	–0.34
2 mM NADH	–0.23	–0.14	0.26	–0.33
3 mM NADH	–0.31	–0.09	0.33	–0.34
4 mM NADH	–0.27	–0.11	0.30	–0.37
myofibrillar				
1 mM NADH	–0.59**	0.43*	0.48**	–0.22
2 mM NADH	–0.52***	0.31	0.41*	–0.26
3 mM NADH	–0.65***	0.45*	0.55**	–0.33
4 mM NADH	–0.65***	0.43*	0.55**	–0.25
total				–0.30
1 mM NADH	–0.63***	0.39*	0.54**	
2 mM NADH	–0.55**	0.26	0.45*	–0.34
3 mM NADH	–0.70***	0.40*	0.61***	–0.41*
4 mM NADH	–0.67***	0.36	0.58**	–0.32
H	–0.69***	–0.68***	–0.81***	
C	–0.96***	0.69***		
*L**	0.69***			

[a] Correlation: * = significant at $p < 0.05$, ** = significant at $p < 0.01$, *** = significant at $p \leq 0.001$.

Table 3. Overall Correlation Coefficients between MetMb Reducing Activities, Lightness (*L**), Chroma (*C*), and Hue (*H*) between Batches during Storage at 2 °C[a]

	*L**	C	H
MetMb reducing activity			
SMRA	–0.15	0.26	–0.33
PMRA	0.41	0.43	–0.29
TMRA	0.35	0.48	–0.36
H	–0.73**	–0.83***	
C	0.81***		
*L**			

[a] Correlation: * = significant at $p < 0.05$, ** = significant at $p < 0.01$. *** = significant at $p \leq 0.001$.

of Hagler et al. (*21*) as modified by Reddy and Carpenter (*12*). In this method, the supernatant of an extracted sample is used in a mixture (containing EDTA, potassium ferricyanide, MetMb, and phosphate buffer), and the reduction of MetMb is followed spectrophotometrically at 580 nm. This variation in the methods used to assay MetMb reducing activity has resulted in a number of studies that report the results differently and thus are difficult to compare.

Faustman and Cassens (*25*) found that differences in color stability between longissimus (good color stability muscle) and gluteus medius (poor color stability muscle) do not appear to be due to the aerobic MetMb reducing capacity as measured by the ARA method. Echevarne et al. (*7*) also found no correlation between MetMb reductase activity, measured under aerobic as well as anaerobic conditions, and the color stability of different bovine muscles. On the other hand, Ledward (*24*) reported a highly significant correlation between the equilibrium concentration of MetMb (% MetMb) and ARA. He suggested that the lack of correlation between MRA and MetMb formation (color stability) could be because either the technique for MRA estimation does not accurately measure the activity of the enzymatic reduction or the effective aerobic reduction does not occur by this mechanism.

The result in our study suggests that PMRA is a good indicator of TMRA. Also, the results suggest that PMRA is associated with meat color parameters. However, since the intermuscular variability in color stability has been found to be a good model for meat color studies (*26*), the PMRA−color stability relationship in various muscles is currently under investigation.

In conclusion, it has been shown that a large proportion of the MetMb reducing activity is present in the myofibrillar muscle fraction. This activity may explain some of the contradictory results on the relationship between MetMb reducing activity and color stability of fresh meat. Also, the myofibrillar metmyoglobin reducing activity was found to be a good indicator for the total metmyoglobin reducing activity in meat and may be associated with meat color.

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