Development of a Model System to Mimic Beef Bone Discoloration

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ABSTRACT: Some current practices used in the meat industry (blast chilling, enhancement, modified atmosphere packaging (MAP)) appear to result in darkening of the bone in fresh meat. The objective of this study was to develop a model system that could be used to evaluate intervention strategies to prevent this discoloration. Beef rib bones were removed from carcasses, split along the transverse plane from the proximal to the distal end of the rib, and then frozen (-20 °C) or held at 4 °C for 24 h. Half were exposed to a phosphate/salt enhancement solution while half served as the control. Samples were packaged in air or modified atmosphere packaged (MAP: 80% O2/20% CO2) and displayed in a retail case (4 °C, 24 h). Visual discoloration occurred during the 1st 10 h of display. More darkening (brown, green/black) was observed in previously frozen samples, whereas samples held at refrigeration temperature were redder. CIE L*, a*, and b* values determined after 24 h indicated that samples held in refrigeration before packaging were lighter and redder. After 24 h at 4 °C, previously frozen samples contained more methemoglobin/g protein in the bone marrow than did bone samples that had never been frozen.

Keywords: bone discoloration, freezing, enhancement, packaging

Introduction

Visual color is a primary quality trait that consumers use when purchasing retail beef cuts (Robbins and others 2003). Consumers will make a no-purchase decision when metmyoglobin (brown) concentration reaches 30% to 40% of the total pigments on the surface of fresh beef (Carpenter 2001). Up to 20% of all beef cuts sold in U.S. retail stores are discounted or even discarded due to losses associated with freshness (Morgan and others 1991). Undesirable color change, from red to brown, has been observed in bone marrow from beef. This discoloration has been noted by the industry as problematic with respect to display life of retail cuts (Mancini and others 2004).

The mechanism of bone darkening has been widely accepted to be similar to that of muscle discoloration (Faustman and Cassens 1990). Hemoglobin undergoes oxidation-reduction reactions similar to those described for myoglobin.

Rib bones contain primarily red marrow in the medullary cavity. Red marrow is found in the spongy bone of the epiphysis of long bones, and in the main bodies of the sternum, ribs, and vertebrae (Forest and others 1975; Field and others 1978), all of which can be contained in bone-in retail cuts. Red marrow is the primary source of red blood cells and, as such, contains high levels of hemoglobin.

Under low oxygen tension, myoglobin (Mb) and hemoglobin (Hb) iron exists in the Fe2+ state. The electron deficiency of iron allows it to interact ionically with water in the absence of other stronger electron pair–donating ligands, which could form covalent linkages. Visually these pigments are dark red-purple, exhibiting diffuse absorption in the green area of the spectrum (approximately 555 nm; deMan 1999). Exposed to O2 for a short period of time, Mb and Hb reversibly bind O2 producing oxymyoglobin (MbO2) or oxyhemoglobin (HbO2), both of which are bright red, absorb less light in the 600 to 700 nm (red) region, and have higher CIE a* values (CIE 1978) than the reduced forms.

At high pH, heme iron exists predominantly in the Fe2+ state; low pH accelerates Fe2+ conversion to Fe3+. While O2 can bind to Fe3+ only, many other ligands (CN, NO, CO) can bind to either Fe2+ or Fe3+ producing different colors. The central iron atom oxidizes (Fe3+) if it loses an electron producing metmyoglobin (MbFe3+1) or methemoglobin (methB), which exhibits increased absorption at about 505 nm (blue region of spectrum), a slightly increased absorbance in the 630 nm (red) region and decreased absorbance in the yellow region (about 550 nm). It is visually brown or gray-brown and is often associated with higher CIE b* values. MbFe3+3 and HbFe3+3 can degrade through a number of pathways producing green, yellow, and dark brown pigments. In the presence of reducing agents, such as ascorbate, the porphyrin ring can oxidize, producing cholemyoglobin, which has an absorption maximum at 628 nm and is visually green (deMan 1999). Clearly, a variety of endogenous and exogenous conditions have the capacity to alter, either temporarily or permanently, these pigments.

To prevent the growth of spoilage bacteria and maintain meat quality, meat temperature must be brought down to 4 °C within 16 h. Rapid cooling techniques are widely used for these purposes (Romans 2001; Obene and others 2004). The lower the temperature, the greater the effect. However, formation of ice crystals during rapid chilling and/or cold storage can damage the pigment molecules, encouraging discoloration. In addition, rapid chilling may alter oxygen solubility into tissues.

Phosphates and NaCl solutions are generally used in enhancement solutions to increase palatability (Higgings and others 1999; Rhee 2001; Robbins and others 2003). While developed in the poultry and pork industries, the beef industry has been rapidly adopting the enhancement process. Salt is a known prooxidant and phosphates alter pH. During enhancement, they are introduced in high concentrations (because of ultimate dilution with meat) to achieve the desired final concentration in the product. Lean meat color can...
be preserved by controlling oxygen in the package atmosphere; oxygen concentration is critical in maintaining meat in a “fresh like” condition (Bremer and others 1992). Thus, vacuum packaging or modified atmosphere packaging (MAP), with 1 or more gases, can be used to design conditions to maximize the microbiological shelf life of lean meat; however, the absence of oxygen or very high oxygen concentrations (80% in high oxygen MAP) can alter color in the short term (purple deoxy- forms) and in the long term (red to brown as the oxy- form converts to the met- form). Various packaging gas- ses may encourage or discourage these changes (Warren and others 1992; Gill 1996; Mancini and others 2005).

These industrial practice, such as enhancement, MAP and blast chilling, may have negative effects on color. To evaluate the effectiveness of potential intervention strategies, a model system was needed that would consistently produce the darkening effect in bone tissue. Beef rib bones were chosen as the bone source material because of their high hemoglobin content. Using various pre- packaging temperature treatment, exposure to enhancement so- lution, and gas atmosphere in the package combinations, the objective of this study was to develop a model system that would produce the dark bone phenotype consistently enough that we could evaluate treatments that would prevent it.

Materials and Methods

Determination of ideal hemoglobin extraction conditions

Beef rib bones were obtained within 24 h postmortem from the Univ. of Illinois Meat Science Laboratory (Urbana, Ill., U.S.A.). To establish the ideal extraction conditions for use in the remainder of the study, fresh hemoglobin was extracted from rib bones. Ribs were split along the transverse plane from the proximal to the distal end of the rib using a band saw (Hobart Corp., Troy, Ohio, U.S.A.) to expose the marrow portion. Hemoglobin was extracted for 20 h, with shaking, from fresh 12- to 15-cm beef rib bone sections (n = 8/treat- ment combination) using combinations of NaCl (1% or 5%) and pH (4 or 8). Because ribs were different lengths, protein content was determined such that hemoglobin concentration could be expressed as a function of protein content (mg Hb/g protein). Absorbance was determined spectrophotometrically (HP spectrophotometer 8453 and 845x UV-Visible software, Agilent Technologies 95-00, San Fran- cisco, Calif., U.S.A.) at 406 nm (metHb), 414 nm (oxyHb), and 430 nm (deoxyHb) as described by Lemberg and Legde (1949). The extraction solution that contained the most hemoglobin was 1% NaCl, pH = 4 (Figure 1). For subsequent hemoglobin determinations (after visual and instrumental color evaluations), all samples were extracted for 20 h at 4 °C in a 1% NaCl solution, pH 4.0.

Determination of hemoglobin concentration

Hemoglobin standard curves were prepared based on protocols by Miller (1998). Purified, lyophilized porcine hemoglobin was ob- tained from Sigma Aldrich (St. Louis, Mo., U.S.A.). Increasing con- centrations (0.25, 0.50, 0.75, 1.00, 1.50, 2.00 mg/mL) of methemo- globin (methHb) were mixed into 10 mL of 1/15 phosphate buffer, pH 7. To reduce methHb to deoxyhemoglobin (deoxyHb), 1 mL of this solution was removed (from the 10 mL originally prepared) and replaced with 1 mL of sodium dithionite (Sigma-Aldrich), a reducing agent. To obtain oxyhemoglobin (oxyHb), air was bubbled direct- ly into the reduced deoxyHb at an average flux of 207 cc/min. Spe- cific time required to convert all hemoglobin into oxyhemoglobin at each dilution varied: 7, 11, 18, 20, 25, or 35 min for 0.25, 0.50, 0.75, 1.00, 1.50, or 2.00 mg/mL, respectively. Hemoglobin concentration (mg/mL) was calculated from absorbance using standard curves.

Bone model system

The experimental design for the evaluation of the beef bone model is shown in Figure 2. Beef rib bones were obtained 24 h post- mortem from the Univ. of Illinois Meat Science Laboratory (Urbana, Ill., U.S.A.) from 3 animals. Animals (n = 3) were considered as blocks. Rib bones (4 bones/side × 2 sides/animal × 3 animals = 24 whole ribs) were randomly removed from each carcass. Two ribs/side/carass were frozen (−20 °C; n = 12) for 24 h and 2 ribs/side/carass were held at 4 °C for 24 h. Ribs were split as previously described to expose the marrow portion (2 halves/rib × 24 ribs = 48 halves; 24/temperature pretreatment). Bone pH was determined using a flat pH meter (Accu- meter nr 13-620-2890, Fisher Scientific). Bone pH was 7.4. Half the rib sections (n = 12) from each temperature pretreatment group were submerged in a simulated enhancement solution of 1000 mL water, 4% alkaline phosphate (Rhodia Inc. Food Ingredients, Cranbury, N.J., U.S.A.), and 4% NaCl (US Salt, Watkins Glen, N.Y., U.S.A.) before packaging. Solution pH was determined using a pH meter fitted with a glass body electrode (Accumet Research AR15, Fisher Scientific). Solution pH was 8.5. Split bones were submerged in the enhance- ment solution for 10 min at 4 °C. The remaining half (n = 12) served as the controls. Half the split bones from each temperature pretreat- ment/enhancement combination (n = 6) were placed in multilayer polypropylene, case-ready plastic trays (Cryovac, Saddlebrook, Va., U.S.A.) using oxygen impermeable multilayer over wrap plastic film (1 mil; Cryovac). The other half were packaged under normal atmosphere (air) with an oxygen-permeable poly vinyl chloride (PVC) film. All samples were displayed in a coffin- style retail case (Hill, Atlas Refrigeration and Equipment Sales, Baton Rouge, La.).

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Cream cheese pH and serum phase.

Visual color evaluation

During display, color was visually evaluated through the packaging material at 0, 3, 6, 10, and 24 h by trained panelists (n = 8) provided with 3 color scales (red, brown, green/back), each with 3 specific points of reference. Color name, scale values, and Minolta color values for references are shown in Table 1. Using a 15-cm line scale, panelists evaluated the color, based on the reference points for each color characteristic, of the marrow portion of the bone only.

Instrumental color evaluation

Because instrumental evaluation required opening the packages in order for the instrument to make contact with the meat surface, these evaluations were conducted at the end of the 24-h display period on the marrow portion of the bone using a reflectance spectrophotometer (Minolta CR300, Minolta Camera, Co., Kogaku, Japan) standardized using the white standardization tile. The spectral curve was determined over the 400- to 700-nm range, CIE L* (lightness), a* (redness), and b* (yellowness) values were measured, and hue angle (departure from the true red axis of the CIE color space in degrees) and chroma (color saturation) were calculated. Hue angle and chroma were calculated as follows (Hunter and Harold 1987):

\[
\text{Hue angle} = 57.3^\circ \arctan \left( \frac{b}{a} \right)
\]

\[
\text{Chroma} = \sqrt{a^2 + b^2}
\]

Statistical analyses

Data for the extraction solutions portion of this study were evaluated and were subjected to 2-way analysis of variance using the (MIXED) models procedure of SAS (2002) to assess the effects of salt, pH, and their interactions. Differences were considered significant at P < 0.05. Means were separated using probability of difference.

Temperature pretreatment, enhancement, and commercial packaging were simulated in the laboratory using a randomized complete block design (RCBD) with 3 factors (Figure 2): temperature (previously frozen or refrigerated), enhancement solution treatment (control or enhanced [phosphate + NaCl + water]), and packaging atmosphere (air or MAP [80% O2/20% CO2]). Instrumental data collected after 24 h were subjected to 3-way analysis of variance to evaluate temperature pretreatment, enhancement solution, packaging, and their interactions. Differences were considered significant at P < 0.05. Means were separated using probability of difference.

Sensory data were subjected to 3-way analysis of variance using a repeated measures design with the MIXED models procedure of SAS (SAS Inst. 2002) to evaluate temperature pretreatment, enhancement solution, packaging, and their interactions over time. Tukey’s Studentized Range (Honestly Significantly Different) was used to separate significantly different (P < 0.05) means.

Results and Discussion

Visual color

Before packaging, exposure to the enhancement solution slightly, but significantly (P < 0.05), reduced visual green/black color (Table 2). It increased (P < 0.05) hue angle (less true red) and increased the methemoglobin content of bone. These data indicate that while the bone was discoloring to some degree, probably due to

Table 1—Characteristics and scale values of color standards

<table>
<thead>
<tr>
<th>Visual color</th>
<th>Name and code</th>
<th>Scale value</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>L*</td>
</tr>
<tr>
<td>Red</td>
<td>Apple red (94074)</td>
<td>1.00</td>
<td>41.51</td>
</tr>
<tr>
<td></td>
<td>Red hot (94084)</td>
<td>7.50</td>
<td>43.02</td>
</tr>
<tr>
<td></td>
<td>Late tomato (94094)</td>
<td>14.00</td>
<td>50.47</td>
</tr>
<tr>
<td>Brown</td>
<td>Red potato (94113)</td>
<td>1.00</td>
<td>56.23</td>
</tr>
<tr>
<td></td>
<td>Mountain trail (96203)</td>
<td>7.50</td>
<td>17.98</td>
</tr>
<tr>
<td></td>
<td>True walnut (96174)</td>
<td>14.00</td>
<td>53.11</td>
</tr>
<tr>
<td>Green/black</td>
<td>Oiled Teak (96173)</td>
<td>1.00</td>
<td>45.58</td>
</tr>
<tr>
<td></td>
<td>Nut brown (96284)</td>
<td>7.50</td>
<td>39.36</td>
</tr>
<tr>
<td></td>
<td>Nightfall (96104)</td>
<td>14.00</td>
<td>35.13</td>
</tr>
</tbody>
</table>

to hemoglobin oxidation, visual darkening was somewhat suppressed or delayed due to exposure to enhancement solution. These findings support those of Lamkey and others (1986) and Miller and others (1986) regarding the effects of phosphate-containing enhancement solutions on the color of beef during frozen storage. However, it should be noted that surface dehydration of samples could also impact the color bone tissue. Scores for visual green/black color were dramatically higher ($P < 0.05$) for previously frozen samples than for refrigerated samples (Figure 3). This difference was apparent after 3 h. Green/black color increased ($P < 0.05$) over display time regardless of packaging atmosphere. Previously frozen samples held in MAP had the highest ($P < 0.05$) green/black color scores (Table 3). However, samples held at refrigeration temperature before packaging and display were less green/black, regardless of packaging atmosphere (Figure 3; Tables 3 and 4). Development of green/black color appeared to peak between 8 and 10h into the display period, regardless of prior temperature treatment or packaging atmosphere (Figure 3).

Red color intensity increased ($P < 0.05$) in MAP samples and decreased in the air-packaged samples during the 1st 5h of display, after which they decreased similarly (Figure 3). The initial increase in red color in MAP samples is likely due to the high oxygen concentration in these packages, which results in rapid oxygenation of hemoglobin. Scores for discoloration, brown, green/black, and red colors after 24h were not significantly higher than they had been at 10h ($P < 0.05$), regardless of gas atmosphere in the package (MAP or air; Figure 4).

Discoloration and brown color formation occurred during the 1st 10h of display for both previously frozen and fresh samples (Figure 4). Previously frozen samples were about 90% discolored after 10h, whereas refrigerated samples were about 65% discolored. Discoloration in MAP and air packaged samples occurred during the 1st 10h of display (Figure 4). Previously frozen samples had higher ($P < 0.05$) scores for brown and green/black colors after 6h of display. Frozen, thawed hemoglobin has been shown to have an increased oxidation rate, making color less stable than when it is fresh (Kinsman and others 1994; James 2002).

### Table 2—Enhancement solution effects on bone marrow color

<table>
<thead>
<tr>
<th></th>
<th>Enhancement</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Visual color</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green/black c</td>
<td>4.00a (0.41)</td>
<td>4.51b (0.43)</td>
</tr>
<tr>
<td><strong>Instrumental Color</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hue angle</td>
<td>20.85a (4.09)</td>
<td>18.36b (5.70)</td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>3.01a (1.47)</td>
<td>2.54b (0.93)</td>
</tr>
</tbody>
</table>

*aMeans with the same letter are not significantly different ($P < 0.05$).*

*bAverage over time (no interaction).*

c*Scale: 0 = green, 15 = black.*

*dDetermined after 24h (no interaction).*

*Standard deviation.*
Instrumental color and hemoglobin oxidation state

Freezing and thawing of beef bones before display had significant effects on CIE $L^*$ and $b^*$ values, chroma, metHb, and oxyHb concentrations (Table 4). Previously frozen samples had higher ($P < 0.05$) concentrations (mg/g) of metHb in the bone marrow after display. These samples were darker (lower CIE $L^*$) and more brown (Figure 4), which is likely due to the higher concentration of metHb on the surface. Using optical absorbance (OD) at 406 nm and 414 nm to assess MetHb and oxyHb, respectively, it is difficult to discriminate 1 from the other. The peaks are very close together, and any increment in metHb OD is reflected in an increment in oxyHb OD. However, this increment in oxyHb did not seem to affect the overall color of the bone. Previously frozen bone samples had lower ($P < 0.05$) CIE $L^*$ values (lightness) indicating that they were darker than those held at refrigeration temperature ($4^\circ C$) before display. Freezing then thawing beef (lean) has been shown to result in lower $L^*$ values (Farouk and others 2004). The increased brownness of lean tissue reported in previous studies has been attributed to the reduced activity of metmyoglobin-reducing enzymes during frozen storage (Ledward 1985). A similar situation may exist with respect to hemoglobin in bone marrow. The CIE $b^*$ value (yellowness) and chroma (color intensity) were both lower ($P < 0.05$) in previously frozen samples, indicating they were less yellow and that color was less intense.

Packaging atmosphere (MAP or air) had a significant effect on CIE $b^*$ value and deoxyHb concentration (data not shown). Samples packaged under MAP conditions had higher ($P < 0.05$) CIE $b^*$ values (5.14; more yellow) than those packaged in air (4.40). MAP-packaged samples also had lower deoxyHb concentrations (1.00 mg/g protein) than air-packaged samples (1.30 mg/g protein), indicating that they were less red/purple in color. However, visually, no difference ($P > 0.05$) existed due to gas atmosphere in the package indicating that these objectively measured differences were below the detection level visually. The decrease in deoxyMb in MAP samples may have been overridden by the dramatic effect of temperature on oxyMb and metMb. Nevertheless, the change at this point, while detectable instrumentally, was not apparent visually.

Exposure to the enhancement solution had a significant effect on hue angle and metHb concentration (Table 2). Samples that were exposed to the enhancement solution had higher ($P < 0.05$) hue angles (less true red) and metHb (brown/black pigment) concentrations. In terms of overall color, samples exposed to the enhancement solution were darker ($P < 0.05$) and less red. Again, these differences were insufficient to be detected visually.

Overall, previously frozen samples had lower ($P < 0.05$) CIE $a^*$ values (less red) than refrigerated samples (data not shown). Samples held at refrigeration temperature before exposure to the enhancement solution had lower CIE $a^*$ values (15.9) than their refrigerated, non-enhanced counterparts (18.0), indicating that they were less red while enhancement had essentially no effect ($P < 0.05$) on CIE $a^*$ values of previously frozen samples (8.2). Previously frozen samples packaged under MAP conditions had higher ($P < 0.05$) hue angles (less true red) than those packaged under air (Table 4). However, after display, refrigerated samples also had higher hue angles regardless of packaging.

Conclusions

Based on visual and instrumental color measurements, the temperature to which the bone was exposed before display was the major factor affecting discoloration of bone marrow. Previously frozen samples were visually more discolored, brown and black. They had lower CIE $L^*$ values, indicating they were darker, and higher MetHb (brown pigment) concentrations. Relative percent discoloration, green/black and brown colors increased and red color decreased over display time for both previously frozen and for refrigerated samples; however, values for previously frozen samples were significantly higher after 6 h of display. These attributes increased with time for regardless of packaging gas. At the end of the display period, no significant visual difference was observed between MAP and air-packaged samples. Instrumental evaluation indicated that MAP-packaged samples were more discolored; however, this difference was not visually apparent. The use of bone that has been previously frozen as a model system for investigating intervention strategies appears to be possible. It would be worthwhile to evaluate various antioxidant treatments applied soon after freezing and before packaging to prevent this discoloration.

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References

**Cream cheese pH and serum phase . . .**


