Influence of finishing systems on hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) in beef

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ABSTRACT

The aim of this research was to: (1) develop a reliable extraction procedure and assay to determine antioxidant activity in meat products, and (2) assess the effect of beef finishing system (forage-finished: alfalfa, pearl millet or mixed pastures vs. concentrate-finished) on longissimus muscle antioxidant activity. The effect of extraction method (ethanol concentration and extraction time), protein removal, and sample preparation method (pulverization or freeze drying) were first evaluated to develop an antioxidant assay for meat products. Beef extracts prepared with low ethanol concentrations (20%) demonstrated higher hydrophilic ORAC. Protein removal prior to extraction reduced hydrophilic ORAC values. Sample preparation method influenced both hydrophilic and lipophilic ORAC, with pulverized samples containing higher hydrophilic and lipophilic ORAC values. Beef cattle finishing system (Forage: alfalfa, pearl millet, or natural pasture vs. concentrates) had little impact on muscle hydrophilic ORAC, but muscle from forage finished beef contained greater lipophilic ORAC. In addition, broiling of steaks reduced hydrophilic ORAC.

1. Introduction

Evidence suggests that reactive oxygen species (ROS) play an important role in the aging process and occurrence of many diseases (Cerutti, 1985; Packer, Rimbach, & Virgili, 1999). As a result, interest in the measurement of antioxidant capacities of foods has increased substantially in recent years; however, most studies have focused on vegetables, fruits and spices (Lee & Shibamoto, 2002). Limited research has been conducted to investigate the antioxidant activities in beef from forage-finished cattle in the US.

Previous studies showed that beef produced on pasture in Argentina had a higher level of total ferric reducing antioxidant power (FRAP) than meat from grain-fed animals. However, there was no difference in their ability to reduce ABTS+ (2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) when using an adapted Trolox equivalent antioxidant capacity (TEAC) assay. It was suggested that the total antioxidant activity was due more to the reduction potential than to the quenching capacity of the beef homogenates (Descalzo et al., 2007).

Several antioxidant assessment methods such as FRAP, TEAC, oxygen radical absorbance capacity (ORAC), and 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH), have been used to determine in vivo antioxidant activities. ORAC assay has been widely used in academics and the food and supplement industry as a method of choice to assess antioxidant capacity. ORAC assay uses a biological relevant radical source and is the only method that measures both inhibition time and degree of inhibition for an antioxidant (Huang, Ou, Hampsch-woodill, Flanagan, & Prior, 2002; Huang, Ou, & Prior, 2005). In addition, a small modification of this ORAC assay can be used to analyze the lipophilic antioxidants (Prior et al, 2003). DPPH assay is technically simple and applies to determine the free radical scavenging capacities in many plant extracts (Chung, Chang, Chao, Lin, & Chou, 2002; Wu et al, 2006).

Previous antioxidant status in beef from pasture-fed Argentina cattle were obtained using FRAP and TEAC, and the method of extraction for beef was not studied in the research (Descalzo et al., 2007). Little information is available on the antioxidant activity of grass-fed beef using ORAC and DPPH assay. In this paper, we studied the extraction procedure to prepare a beef extract with high antioxidant capacity when determined by ORAC and DPPH assay, and compared the hydrophilic and lipophilic antioxidant status of beef from different grass-finished cattle and high concentrate diet finished cattle using ORAC assay. The objectives of this research were to: (1) develop a reliable extraction procedure and assay to determine antioxidant activity in meat products, and (2) assess the effect of beef finishing system (forage-finished: alfalfa, pearl millet or mixed pastures vs. concentrate-finished) on longissimus muscle antioxidant activity.
2. Materials and methods

2.1. Reagents and chemicals

Ethanol in HPLC grade was obtained from Fisher Scientific (Fair-lawn, NJ). Chemical of 2,6-di-tert-butyl-4-methylphenol (BHT, 99%) was purchased from ACROS (New Jersey, USA). Randomly methylated β-cyclodextrin (RMCD) (Trapposol, pharmaceutical grade) was obtained from Cyclodextrin Technologies Development Inc (High Springs, FL). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from EMD bioscience (LosAlta, CA), and fluorescein was obtained from Riedel-deHäen. The 2,2'-Azobis-(2-amidinopropane) dihydrochloride (AAPH) was from Cayman Chemical Company (Ann Arbor, MI).

2.2. Sample shipment and rib composition

Forty-seven Angus-crossbred steers were used to evaluate the effects of forage species grazed in the last 41 d of the finishing period on antioxidant status in forage-finished beef and compared to traditional high concentrate finished beef. Steers grazed naturalized pasture (bluegrass/white clover/orchardgrass/tall fescue) for 93 d and then grazed alfalfa (A; n = 12), pearl millet (PM; n = 12), or naturalized pasture (N; n = 12) pastures for the final 41 d of finishing. Steers (n = 11) were also finished on a high concentrate, feedlot diet (F) for 134 d. At 24 h postmortem, the whole beef rib (NAMP 107; 10.16 cm tail; untrimmed) was removed from each carcass. The ribs were shipped via refrigerated semi-truck to Clemson University Meat Laboratory. Upon arrival, ribs were maintained at 4 °C until 14 d of postmortem aging was complete. Two steaks (2.54 cm thick) were removed from the posterior end of the beef rib for antioxidant activity. Steaks were trimmed of all external fat and connective tissue, kept at −70 °C prior to freeze drying or pulverization in liquid nitrogen, and then kept at −20 °C.

2.3. Method development

2.3.1. Ethanol concentration and extraction time

Muscle samples from pearl millet finished beef had higher α-tocopherol content than concentrate finished (Wu and Duckett, unpublished data). Therefore, two randomly selected longissimus muscle samples from PM were freeze dried and used to study the effect of extraction time and ethanol concentration on antioxidant activities using both DPPH and hydrophilic ORAC assay. Muscle samples (0.25 g) were extracted in a 50 mL centrifuge tube with 10 mL of hexanes, vortexed for 10 min., and hexane layer removed and evaporated under nitrogen. The residue was extracted with 10 mL 20% ethanol (v/v) or 80% ethanol (v/v) for 1 or 24 h at room temperature on orbital shaker at 160 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min, and the supernatant removed. One milliliter of supernatant was again centrifuged at 12,000 rpm for 10 min. and the supernatant removed. One milliliter of supernatant was centrifuged at 12,000 rpm for 5 min. The supernatant, with or without protein removal, was used for subsequent hydrophilic ORAC assays. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with temperature and finishing system in the model. Least squares means were generated and separated using the PDIFF option of SAS.

2.3.2. Protein removal and ethanol concentration

Two randomly selected samples from each of two finishing systems (pearl millet and high concentrate, feedlot diet) were used to study the effect of protein removal and ethanol concentration on hydrophilic ORAC. Muscle samples (freeze dried, 0.25 g) were extracted with hexane as above. The residue was extracted with 10 mL of 20% or 80% ethanol (v/v) for 1 h at room temperature on orbital shaker at 160 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min, and the supernatant removed. One milliliter of supernatant was again centrifuged at 12,000 rpm for 10 min. For protein removal, 400 μL of supernatant was added with 400 μL trichloroacetic acid (50%, v/v), and the sample was centrifuged at 12,000 rpm for 10 min. The supernatant, with or without protein removal, was used for subsequent hydrophilic ORAC assays. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with protein removal, ethanol concentration, finishing system and all interactions in the model. Least squares means were generated and separated using the PDIFF option of SAS.

2.3.3. Extraction temperature

The same muscle samples were used to study the effect of extraction temperature on hydrophilic ORAC. Muscle samples (0.25 g) were extracted with hexane as above. The residue was extracted with 10 mL of 20% ethanol (v/v) for 1 h at 22 °C or 60 °C on orbital shaker at 160 rpm. The extracted samples were then centrifuged at 3000 rpm for 5 min, and the supernatant removed. One milliliter of supernatant was again centrifuged at 12,000 rpm for 10 min. The supernatant was used for subsequent hydrophilic ORAC assays. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with temperature and finishing system in the model. Least squares means were generated and separated using the PDIFF option of SAS.

2.3.4. Sample preparation

Six samples, selected at random, from each of four finishing systems (alfalfa; high concentrate, feedlot diet; native pastures; pearl millet) were used to test the effect of sample preparation (freeze drying or pulverization) on hydrophilic and lipophilic ORAC. Adjacent steaks were either pulverized in liquid nitrogen or freeze dried and stored at −20 °C. Freeze dried samples (0.25 g) or pulverized muscle samples (0.5 g) were extracted using 20% ethanol for 1 h at room temperature without protein removal as determined above as optimum extraction methods for ORAC. For lipophilic ORAC, the hexane fraction was dried under nitrogen. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with protein removal, ethanol concentration, finishing system, and two-way interaction in the model. Least squares means were generated and separated using the PDIFF option of SAS.

2.4. Effect of finishing systems and cooking on ORAC

The influence of beef finishing system (alfalfa; high concentrate, feedlot diet; native pastures; pearl millet) on hydrophilic and lipophilic ORAC was determined. Freeze dried samples were prepared as described above with the final extraction method using 20% ethanol for 1 h at room temperature without protein removal. For lipophilic ORAC, the hexane fraction was dried under nitrogen. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) with finishing system in the model. Least squares means were generated and separated using the PDIFF option of SAS.

To test the cooking effect on antioxidant status of beef steaks, four randomly selected steaks from each of four finishing system were broiled on Sanyo indoor barbecue grills (Model HPS-SG3; China) to an internal temperature of 71 °C as monitored using copper constantan thermocouples and a Digi-Sense temperature logger (Cole Parmer, Niles, IL). After cooking, samples were removed for ORAC determination using the final extraction method.
2.5. Hydrophilic ORAC assay

The hydrophilic ORAC assay was modified based on application note (Held, 2005) from BioTek (Winooski, Vermont, USA). The supernatant was diluted 10 times with phosphate buffer (pH 7.4) for hydrophilic ORAC analysis. An aliquot (25 μL) of the diluted sample or blank (phosphate buffer) or Trolox calibration solutions (6.25, 12.5, 25, 50, 100 μM) was added to a well in 96-well bottom reading microplate. The 150 μL of fluorescein solution at 0.004 μM was added to each well of plate, and then the microplate was incubated at 37 °C for 30 min before an aliquot of 25 μL AAPH solutions (153 mM) was added to each well as peroxyl generator to start reaction. The microplate reader from BioTek Instruments, Inc (Winooski, Vermont, USA) was programmed to record the fluorescence reading with an excitation wavelength of 485 nm and an emission wavelength of 520 nm at 1 min interval for 1 h using software Gen 5™.

2.6. Lipophilic ORAC assay

The lipophilic ORAC assay was modified based on Prior et al. (2003). The dried hexane extract was dissolved in 250 μL of acetone and then diluted with 750 μL of a 7% RMCD solution (50% acetone/50 water, v/v). Any further dilution was made with the 7% RMCD solution to dissolve the Trolox standards (6.25, 12.5, 25, 50, 100 μM) for the lipophilic assay. The fluorescein solution and AAPH solutions were added in the same manner as that for hydrophilic ORAC assay, and followed the same procedure used for hydrophilic ORAC assay.

The final hydrophilic and lipophilic ORAC values were calculated by using a linear regression model (Y = aX + b) between Trolox concentration (μM) and the net area under the fluorescence decay curve. Data are expressed as micromoles of Trolox equivalents (TE) per gram of dry weight of beef sample (μmol of TE/g DM). The area under curve (AUC) was calculated using the following equation:

\[
\text{AUC} = 1 + \left( \frac{R2}{R1} + \frac{R3}{R1} + \ldots + \frac{R61}{R1} \right)
\]

where \( R1 \) = the initial fluorescence reading, \( R61 \) = the last fluorescence reading at the 61th minute. The net AUC was obtained by subtracting the AUC of the blank from that of a sample.

2.7. DPPH Assay

The DPPH assay was modified based on Hinneburg and Neubert (2005). An aliquot of 200 μL of BHT (100 μg/mL), or centrifuged meat extract was mixed with 50 μL DPPH (100 μg/mL) in each well of a 96-well microplate. To avoid the color (red) interference from the meat extract, a sample blank was performed without adding DPPH solution for each extract. The mixture was shaken vigorously and left in the dark at room temperature for 20 min. Absorbance of the resulting solutions was measured at 517 nm by spectrophotometer from BioTek (Winooski, Vermont, USA). The antioxidant capacity to scavenge the DPPH radical was calculated by the following equation:

\[
\text{Scavenging effect (SE) (%) } = \left(1 - \frac{A_{517 \text{ sample}} - A_{517 \text{ blank}}}{\text{abs. of control at 517 nm}}\right) \times 100
\]

2.8. Statistical analysis

Each extraction was repeated twice, and ORAC analysis was conducted in duplicate. Data were given as means ± standard error. Data were analyzed in a completely randomized design using GLM procedures of SAS (SAS Inst. Inc., Cary, NC) as described in method development. Least squares means were generated and separated using the PDIF option of SAS. Differences among all sample means were determined at \( p < 0.05 \). When no interaction was observed between finishing systems and method development parameter, data were given as least squares means of pooled data ± standard error of at least 4 independent samples.

3. Results and discussion

3.1. Effect of ethanol concentration and extraction time on antioxidant activities

Mixtures of ethanol and water were chosen because of their nontoxic and environmentally friendly properties, which were effective to prepare extract from several buckwheat herbs and other medicinal plant for antioxidant activity tests (Hinneburg & Neubert, 2005; Wu et al., 2006).

Based on statistical analysis of DPPH assay data, no interaction was found between time and ethanol concentration (Table 1). Ethanol concentration had significant effect on DPPH capacity of beef sample (\( p < 0.01 \)), and 20% ethanol extract demonstrated higher capacities than 80% ethanol extract (\( p < 0.01 \)). No significant effect was determined between 24 h and 1 h extraction (\( p = 0.2275 \)).

Based on ORAC assay, no interaction was found between time and ethanol concentration. Ethanol concentration significantly affected ORAC capacity of beef sample (\( p < 0.01 \)), and 20% ethanol extract demonstrated higher capacities than 80% ethanol extract (\( p < 0.01 \)). No significant effect was determined between 24 h and 1 h extraction (\( p = 0.0868 \)). All these results were in agreement with those obtained from DPPH assay data. Based on these results, 20% ethanol was chosen for the extraction solvent and 1 h for extraction time.

In addition, ORAC was a more sensitive method to determine the antioxidant activities for beef samples compared to DPPH assay. Although DPPH assay is technically simple, the free radical DPPH is long-lived nitrogen radical and bears no similarity to highly reactive and transient peroxyl radical involved in lipid peroxidation (Huang et al., 2005). Compared to DPPH, AAPH used in ORAC assay is an azo radical initiator and can generate peroxyl radicals by thermal decomposition. Based on above reasons, ORAC was used for all the subsequent analysis.

3.2. Effect of protein removal and ethanol concentration on hydrophilic ORAC

Beef muscle contains about 20% crude protein. Prior et al. (2003) has shown that some proteins demonstrate certain antioxidant activities. Therefore, the effect of protein removal and ethanol concentration was investigated (Fig. 1). The interaction between protein removal and ethanol concentration was significant (\( p < 0.05 \)). Adding trichloroacetic acid to remove protein significantly reduced the ORAC activities (\( p < 0.01 \)), which indicated some proteins in beef muscle had ORAC activity. Without protein

<table>
<thead>
<tr>
<th>Ethanol Conc., %</th>
<th>Extraction Time, h</th>
<th>SE (%)</th>
<th>HydroORAC (μmol of TE/g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1</td>
<td>59.72 ± 5.32a</td>
<td>29.98 ± 0.36a</td>
</tr>
<tr>
<td>80</td>
<td>1</td>
<td>23.95 ± 2.52b</td>
<td>21.75 ± 1.02b</td>
</tr>
<tr>
<td>20</td>
<td>24</td>
<td>60.33 ± 0.27a</td>
<td>35.57 ± 0.82a</td>
</tr>
<tr>
<td>80</td>
<td>24</td>
<td>33.32 ± 3.86b</td>
<td>22.83 ± 0.07b</td>
</tr>
</tbody>
</table>

Table 1  
Influence of ethanol concentration and extraction time on scavenging effects (SE) and hydrophilic oxygen radical absorbance capacity (HydroORAC (μmol of trolox equivalent [TE]/g DM)) of beef muscle samples (means with different superscripts (a,b) in the same column differ significantly at \( p < 0.05 \))
removal, the 20% ethanol extract demonstrated higher capacities than 80% ethanol extract (p < 0.01), which was in good agreement with previous study. As a result, 20% ethanol beef extract without removal of protein possessed highest ORAC. Beef finishing system did not alter hydrophilic ORAC level.

3.3. Effect of extraction temperature on hydrophilic ORAC

Extraction temperature affected hydrophilic ORAC of beef samples (p = 0.0031), with lower ORAC determined in extract obtained at 60°C (24.58 ± 0.95 µmol TE/g DM, n = 4) than at 20°C (28.96 ± 0.95 µmol TE/g DM, n = 4). It was suggested that some antioxidant compounds could be oxidized at higher temperatures, and more research should be conducted to verify the hypothesis. Based on these results, room temperature was chosen for extraction of beef samples.

3.4. Effect of sample preparation on ORAC

There was no significant interaction among sample preparation method and finishing systems (p > 0.05). Sample preparation, freeze drying and pulverization, had significant impact on hydrophilic and lipophilic ORAC (p < 0.01). Pulverized samples demonstrated higher ORAC activities, with hydrophilic ORAC at 31.88 ± 0.82 and lipophilic ORAC at 1.29 ± 0.03 µmol TE/g DM (n = 24), compared to hydrophilic ORAC at 27.66 ± 0.82 and lipophilic ORAC at 0.98 ± 0.03 µmol TE/g DM (n = 24) for freeze dried samples. Similarly, sample preparation influenced fat-soluble vitamin concentrations, with higher α-tocopherol content in pulverized samples (Wu and Duckett, unpublished data). Lower amount of antioxidants in freeze dried sample such as α-tocopherol might contribute to lower lipophilic ORAC in freeze dried sample. Furthermore, freezing damages cell structure by expansion of the intracellular fluid. Gradual oxidation of some antioxidant compounds can occur when the beef samples are exposed to air through ineffective packaging when freeze drying (Ball, 2006), although this should undergo further study.

3.5. Effect of finishing treatment on hydrophilic and lipophilic ORAC

Hydrophilic ORAC did not differ among forage-finished (alfalfa, pearl millet and naturalized pasture) and high concentrate finished animals (p > 0.05; Fig. 2). These results agree with other research on the reduction of ABTS+ cation in beef produced on pasture in Argentina (Descalzo et al., 2007) and Charolais bovine meat fed with pasture in France (Gatellier, Mercier, & Renerre, 2004). Both studies suggested no difference in radical scavenging capacity against ABTS+ cation between grain and pasture fed cattle. Both previous beef extracts were prepared with neutral phosphate buffer solution.

Beef extract from alfalfa finished steers showed higher lipophilic ORAC than those finished on naturalized pasture and high concentrate diets (p < 0.01; Fig. 2). The lipophilic ORAC of beef from alfalfa finished steers (1.10 ± 0.05 µmol TE/g DM) was comparable to that from pearl millet finished (1.01 µmol ± 0.05 TE/g DM) (Fig. 2).

Our ORAC method only determined the peroxy radical scavenging abilities. Finishing system of beef did not alter hydrophilic compounds that could scavenge the peroxyl radical, but altered lipophilic ORAC. Some forage crops (pearl millet and alfalfa) could deposit more lipophilic antioxidant compounds into animal muscle than grain could, which was observed in previous study on α-tocopherol concentration between grass-fed animal and grain-fed animals (Descalzo et al., 2005; Zhou, Dickinson, Yang, & Decker, 1998). Our extraction procedure was comparable to reported procedure to extract carnosine in beef (Park et al., 2005), and thus very likely to obtain carnosine in final extract. Shown in
Fig. 3. Hydrophilic ORAC (HydrORAC) Standard Curves of Carnosine and Ferulic acid.

Fig. 3. Carnosine demonstrated hydrophilic ORAC, and the capacity was linear between 0 and 500 μM. These results suggest that carnosine could contribute to our hydrophilic ORAC of beef samples. All beef samples, regardless of finishing system, would contain carnosine and this is likely contributing to the antioxidant status of beef.

Compared with another antioxidant compound ferulic acid, carnosine showed approximately 50 times lower ORAC at the same concentration of ferulic acid (Fig. 3). It is interesting to note that relatively lower concentrations of phenolic compounds could make more impact on ORAC in beef than carnosine levels.

3.6. Effect of cooking on hydrophilic and lipophilic ORAC

No significant difference was detected between beef sample from cattle finished on different systems (p > 0.05), and no significant interaction existed between cooking and finishing systems (p > 0.05). The hydrophilic ORAC of broiled beef steaks was decreased in all samples of beef, regardless of finishing system (p < 0.01), with 28.11 ± 0.74 μmol TE/g DM (n = 16), compared to 33.04 ± 0.74 μmol TE/g DM (n = 16) for raw samples. Cooking can denature some proteins, and degrade or oxidize some antioxidant compounds. All these may count for the loss of the hydrophilic antioxidant activity of beef samples after cooking. On the other hand, the lipophilic ORAC of cooked beef was increased in all samples of beef, regardless of finishing system (p < 0.01), with 1.58 ± 0.03 μmol TE/g DM (n = 16), compared to 1.30 ± 0.03 μmol TE/g DM (n = 16) for raw samples. Significantly increased lipophilic ORAC and hydrophilic ORAC were observed in cooked tomatoes, which were believed to relate with increased bioavailability of carotenoids in processed tomatoes (Wu et al., 2004). Our increased lipophilic ORAC of cooked could also result from increased bioavailability of lipophilic antioxidants such as β-carotene.

4. Conclusion

Using our developed extraction procedure and modified ORAC methods, the peroxyl radical scavenging abilities of hydrophilic and lipophilic compounds in beef sample can be determined. Finishing system (pearl millet, alfalfa, nature pasture and feedlot) had little impact on hydrophilic ORAC, but significantly affected lipophilic ORAC. Sample preparation, freeze drying or pulverization, alters both hydrophilic and lipophilic ORAC levels. In addition, cooking had significant negative impact on beef hydrophilic ORAC.

Beef is a complex matrix. Its antioxidant status consists of non-enzymatic hydro and lipid-soluble compounds such as α-tocopherol, β-carotene, polyphenols, carnosine and enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPX). Descalzo et al. (2007) found that beef produced on pasture in Argentina had higher level of FRAP than meat from grain-fed animals. However, in the same study, meat from pasture-fed cattle presented a similar level of glutathione peroxidase and catalase when compared with meat from grain-fed animal. These results suggest that not a single method can fully detect the antioxidant status of beef, as also proposed by other researchers for biological systems (Prior & Cao, 1999). As a result, it becomes difficult to assess the influence of finishing system on antioxidant status in beef. In the future, additional antioxidant assays such as FRAP and identification of antioxidant compounds like carnosine in beef will be studied.

References


antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. *Journal of Agricultural and Food Chemistry*, 51, 3273–3279.

