

EFFECTS OF NATURAL ANTIOXIDANTS ON COLOUR STABILITY, LIPID OXIDATION AND METMYOGLOBIN REDUCING ACTIVITY IN RAW BEEF PATTIES*

Fang Liu, Qian Xu, Ruitong Dai, Yuanying Ni✉

College of Food Science and Nutritional Engineering, China Agricultural University
17 Qinghua East Road, Beijing, **China**

ABSTRACT

Background. Minced meats undergo oxidative changes and develop rancidity more quickly than intact muscle since grinding exposes more of the muscle surface to air and microbial contamination. Due to concerns about toxicological safety of synthetic antioxidants, recent studies have put more focus on natural antioxidant compounds derived from food components.

Material and methods. The effects of four natural antioxidants (vitamin E, carnosine, grape seed extract and tea catechins) on oxidative processes and metmyoglobin reducing activity in raw beef patties during refrigerated (4°C) storage were investigated and the results were compared with butylated hydroxyanisole treatment patties. The correlation of lipid oxidation, colour and metmyoglobin reducing activity of beef patties were also studied.

Results. Samples treated with carnosine had the highest redness values on the eighth day. Tea catechins, vitamin E and grape seed extract showed higher protective effect against lipid oxidation than carnosine. Metmyoglobin reducing activity increased greatly in all samples during the storage. Significant correlation between redness value and lipid oxidation was demonstrated, while a weak correlation between metmyoglobin reducing activity and any other parameters was shown.

Key words: beef patties, natural antioxidation, colour, lipid oxidation, metmyoglobin reducing activity

INTRODUCTION

Obtaining a stable red or purple colour of ground beef is a challenge, since the color is influenced by a number of factors. Lipid oxidation and discoloration are major problems for meat products during storage and distribution. Oxidative deterioration in any type of meat manifests in form of discoloration, development of off flavour, formation of toxic compounds,

poor shelf life, nutrient and drip losses, respectively (Falowo et al., 2014; Shah et al., 2014). Minced meats undergo oxidative changes and develop rancidity more quickly than intact muscle since grinding exposes more of the muscle surface to air and microbial contamination (Alp and Aksu, 2010). Antioxidants are regarded as compounds that are able to delay, retard

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✉niyycau@hotmail.com (Y.Y.Ni), phone +86 10 6273 7514, fax +86 10 6273 7514

or prevent oxidation processes (Lorenzo et al., 2014). Adding of antioxidants is one of the typical methods to inhibit oxidation reactions in meat products (Kim et al., 2013).

The potential of synthetic antioxidants causing toxicological effects has created demand for natural antioxidants by consumers and the meat industry (Falowo et al., 2014). Recent studies have put more focus on natural antioxidant compounds derived from food components, such as vitamin E, carnosine, grape seed extracts, and tea catechins.

Vitamin E, especially as α -tocopherol, is widely used as an antioxidant, reducing lipid oxidation, drip losses and providing colour stability as consequence of the close relation between oxidation of lipids and oxymyoglobin in muscle tissues (Ripoll et al., 2011). Carnosine is a compound of naturally-occurring dipeptide that synthesized by the carnosine synthetase from β -alanine and L-histidine. Carnosine inhibits the formation of thiobarbituric acid reactive substances in various oxidative model systems. Because of its antioxidant properties, carnosine has been suggested as a useful natural food antioxidant. Grape seed extracts contain a number of polyphenols including procyanidins and proanthocyanidins and are powerful free radical scavengers. It has been investigated *in vitro*, such as mutton slices (Falowo et al., 2014), cooked beef (Ahna et al., 2007) and pork patties (Lorenzo et al., 2014) and burgers (Garrido et al., 2011). Tea catechins are polyphenolic antioxidants which possess a range of health promoting properties. Tea catechins have demonstrated significant antioxidant, anti-carcinogenic, anti-mutagenic properties in numerous human, animal and *in vitro* studies. Tea polyphenols are metal chelating agents and also act on free radicals, since their benzene rings, inhibit chain reactions during lipid oxidation (Tang et al., 2002).

The objectives of this study were to investigate (I) the effect of vitamin E, carnosine, grape seed extracts and tea catechins in preventing lipid oxidation, myoglobin oxidation, and the loss of colour, in raw beef patties during 8 days of storage under 4°C. (II) The correlation of metmyoglobin reducing activity, meat colour stability and lipid oxidation.

MATERIAL AND METHODS

Reagents

L-carnosine ($C_9H_{14}N_4O_3$), tea catechins (polyphenols P 98%, catechins P 90%), grape seed extracts, vitamin E and BHA were all purchased from Qinghua Kechuang Food Additives Co. Ltd. (Beijing, China). NADH and equine Myoglobin were obtained from Sigma (St. Louis, USA). All other chemicals and solvents used were of analytical grade and purchased from Lanyi Chemical Articles Co. Ltd. (Beijing, China).

Sample preparation

Beef strip loins (*Longissimus dorsi* muscle, LD) were obtained at 48 h post mortem. The animals were stunned and slaughtered in a commercial plant following the industry practices. After dressing, the carcasses were held at $4 \pm 1^\circ\text{C}$ for 48 h. The average ultimate pH was 5.70 ± 0.05 .

Beef LD muscles were chopped into small pieces after removal of visible fat and connective tissues. Chopped meat samples (180 g) were ground using a food processor (CombinMax600, China). Meat samples were sprinkled with the appropriate antioxidants during grinding (tea catechins, vitamin E, grape seed extract and carnosine at the concentration of 0.3 g/kg meat, while BHA of 0.03 g/kg meat and control samples with no added antioxidant ingredients. Meat patties were formed using a meat former and stored at 4°C. Two samples were taken from each group at 0, 2, 4, 6 and 8 days of storage. All analyses were performed in triplicate.

TBARS values

Lipid oxidation was assessed in beef patties during refrigerated ($4 \pm 1^\circ\text{C}$) storage. Thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Liu et al. (2010) with little modification. Pattie samples (10 g) were mixed with 45 ml of stock solution (25 ml 20% TCA – 20 ml water) and homogenized for 30 s with a superfine homogenizer (F6–10, Fluko, Shanghai, China) and centrifuged at 1000 g for 20 min at 4°C. The supernatant was filtered through Whatman No. 1 filter paper. The solution (3 ml supernatant – 3 ml 0.02 M TBA) heated in a boiling water bath for 30 min to develop the pink colour. Samples were cooled under running tap water, centrifuged at 4500 g

for 25 min and the absorbance of the supernatant was measured at 532 nm using a Unicam UV4 spectrometer (Unicam Ltd., China). TBA number was calculated as mg malondialdehyde/kg sample following the formula:

$$\text{TBA value, mg/100 g} = (A_{532} - A_{600}) / 155 \cdot (1/10) \cdot 72.6 \cdot 100$$

Percentage of metmyoglobin

Pattie samples (5 g) were homogenized in 25 ml ice-cold 40 mM phosphate buffer (pH 6.8) for 10 s using a superfine homogenizer (F6–10, Fluko, Shanghai, China; 13,500 rpm). The homogenate was allowed to stand for 1 h at 4°C and centrifuged at 4500 g for 30 min at 4°C. The supernatant was filtered through Whatman No. 1 filter paper and the absorbance was measured at 572, 565, 545 and 525 nm using a Unicam UV4 spectrometer (Unicam Ltd., China). The percentage of Metmyoglobin (MetMb%), was determined as described by Krzywicki (1982) using the formula:

$$\text{MetMb\%} = \{-2.514(A_{572}/A_{525}) + 0.777(A_{565}/A_{525}) + 0.8(A_{545}/A_{525}) + 1.098\} \cdot 100$$

Metmyoglobin-reducing activity

Metmyoglobin reductase was extracted from freeze-stored samples (–80°C) as described by Mikkelsen et al. (1999). A 12 g sample and 20 ml phosphate buffer, pH 7.0, was homogenized for 30 s with a Polytron PT 3100 (Polytron, Littau, China) at 13,500 rpm. The homogenate was centrifuged at 35,000 g for 30 min at 4°C and the supernatant filtered through Whatman No. 1 filter paper to remove fat. Oxy-hemoproteins were oxidized with excess $\text{K}_3\text{Fe}(\text{CN})_6$ and the solution was dialyzed (14,000 MW cut-off) against 2.0 mM phosphate buffer pH 7.0 at 4°C. The solution was centrifuged at 15,000 g for 20 min at 4°C and the volume of the supernatant adjusted to 20 ml with 2.0 mM phosphate buffer pH 7.0.

Metmyoglobin-reducing activity was determined as described by Mikkelsen et al. (1999) using a Unicam UV4 spectrometer (Unicam Ltd., China). The standard assay mixture contained 0.1 ml 5 mM EDTA, 0.1 ml 50 mM phosphate buffer (pH 7.0), 0.1 ml 3.0 mM $\text{K}_4\text{Fe}(\text{CN})_6$, 0.1 ml water, 0.2 ml 0.75 mM Mb Fe(III) in 2.0 mM phosphate buffer (pH 7.0), 0.3 ml muscle extract, and 0.1 ml 2.0 mM NADH. The final

pH of the assay mixture was 6.4 and the assay was carried out at 25°C. The reaction was initiated by adding NADH and was determined by following the change in absorbance at 580 nm. Blanks contained all the additions except NADH which was replaced by water. Metmyoglobin-reducing activity was measured in units of min^{-1} and is calculated as MetMb-reducing activity = $\Delta A_{580} / \Delta t$. ΔA_{580} is the change in absorbance at 580 nm per min during the period where the rate of change is linear with time; Δt is the time (min) between two measurements of the absorbance.

Colour

Colour of ground beef patties was determined using a reflectance colorimeter (WSC-S, China) and expressed as CIE Lab a^* (redness). Three replicate measurements were taken for each sample directly after blending and, subsequently, every 24 h until the meat was visually unacceptable.

STATISTICAL AND DATA ANALYSIS

Data analysis was done with the ANOVA function in the Statistical Program for Social Sciences (SPSS 12.0, Chicago, IL, USA) software, and significant differences were determined with a Duncan multiple range test. Relationships among measurement variables were studied using standard correlation, r values being the correlation factor.

RESULTS AND DISCUSSION

Colour

Redness (a^*) values decreased ($p < 0.05$) progressively during 8 days of storage (Table 1). Antioxidant treatments significantly ($p < 0.05$) slowed the patties discoloration. Treatment with grape seed extract and carnosine significantly retarded discoloration ($p < 0.05$) compared with other treatments. The effectiveness of the antioxidants in preserving the redness value was in the following order: carnosine > grape seed extract > vitamin E > tea catechins > BHA.

Although many factors can influence the stability of meat colour, the oxidation of Myoglobin by free radical lipid peroxidation products is predominant. The biochemistry of myoglobin and its interactions with small biomolecules in a complex muscle food

Table 1. Effect of antioxidants on redness a^* value of raw beef patties during refrigerated storage ($4 \pm 1^\circ\text{C}$)

Time day	Control	BHA	TC	VE	GSE	CR
0	27.14 \pm 0.24xa	26.66 \pm 0.666xa	26.99 \pm 0.65xa	27.3 \pm 0.53xa	27.21 \pm 0.31xa	26.667 \pm 0.568xa
2	24.28 \pm 0.21ybe	25.03 \pm 0.58ye	21.56 \pm 1.22yc	21.49 \pm 0.44yc	20.9 \pm 0.14yc	22.43 \pm 0.41ydc
4	17.45 \pm 0.36zc	20.05 \pm 0.94za	17.01 \pm 0.35zc	19.71 \pm 0.46za	18.58 \pm 0.49zb	19.74 \pm 0.11za
6	15.87 \pm 0.31hb	16.21 \pm 0.22hb	17.18 \pm 0.11zb	18.57 \pm 0.33hb	26.002 \pm 0.744ha	26.928 \pm 0.411xa
8	14.986 \pm 0.277we	15.99 \pm 0.65hd	16.27 \pm 0.37zd	20.173 \pm 0.466zc	27.293 \pm 0.0929xb	28.893 \pm 0.178ha

x-w – means in same row with the same letter are not significantly different at $p < 0.05$.

a-e – means in same column with the same letter are not significantly different at $p < 0.05$.

TC – tea catechins, VE – vitamin E, GSE – grape seed extract, CR – carnosine.

matrix govern the colour of meat, and numerous other factors affect the color stability of meat from livestock and poultry (Suman et al., 2014). The low temperature ($4 \pm 1^\circ\text{C}$) and effect of antioxidation during storage most likely slowed the deterioration of the colour observed in these patties.

Mielnik et al. (2006) demonstrated that grape seed extract could marginally increase redness values of raw pork patties with increasing concentration which is similar with our research. This colour stabilizing effect is due indirectly to delayed oxidation of MbO_2 via direct inhibition of lipid oxidation. Carnosine exerted significant efficacy in maintaining an acceptable visual red colour during storage of ground beef and raw patties. Badr et al. (2007) reported that 0.5% or 1% carnosine exerted significant efficacy in maintaining an acceptable visual red colour post-irradiation

and during storage of ground beef and raw patties. In the present study, carnosine exhibited a significantly higher ($p < 0.05$) redness value than the control. Carnosine may be involved in the regeneration of NADH.

Percentage of metmyoglobin

The storage time and antioxidant treatment both affected the MetMb%. MetMb% increased ($p < 0.05$) progressively during 6 days of storage (Table 2). There were significant ($p < 0.05$) differences in the content of MetMb of all samples, and beef patties treated with antioxidants had lower MetMb% than control groups after 8 days of storage. The data suggest that the efficiency of the examined antioxidations in inhibiting myoglobin and/or oxymyoglobin oxidation is in the following order: carnosin > grape seed extract > vitamin E > tea catechins > BHA.

Table 2. Effect of antioxidants on MetMb% in raw beef patties during refrigerated storage ($4 \pm 1^\circ\text{C}$)

Time day	Control	BHA	TC	VE	GSE	CR
0	3.915 \pm 0.0495xa	5.910 \pm 0.184xb	7.845 \pm 1.492xc	8.005 \pm 0.785xc	8.580 \pm 0.311xc	6.095 \pm 0.163xb
2	20.050 \pm 0.269ya	17.445 \pm 0.148yb	20.365 \pm 0.233ya	20.685 \pm 0.0212yac	21.635 \pm 0.530yc	17.700 \pm 0.990yb
4	27.000 \pm 0.0283za	26.765 \pm 0.0636za	28.565 \pm 0.629zb	31.935 \pm 0.0919zb	24.495 \pm 0.0212zc	24.695 \pm 0.106zc
6	38.065 \pm 1.704ha	32.355 \pm 0.0778hb	36.205 \pm 0.219hc	32.745 \pm 0.361zhb	34.540 \pm 0.0141hc	35.605 \pm 0.00707hc
8	38.905 \pm 0.0354ha	35.830 \pm 0.255wb	36.455 \pm 0.459hc	33.260 \pm 0.141hd	27.345 \pm 0.0353we	24.695 \pm 0.0495zf

Means in same row with the same letter (x-w) are not significantly different at $p < 0.05$.

Means in same column with the same letter (a-e) are not significantly different at $p < 0.05$.

In agreement with the present results, retarded the formation of the MetMb in beef due to the effect of carnosine, was also reported by other researchers. Das et al. (2006) observed that meat samples containing 1.0% and 1.5% carnosine significantly inhibited MetMb formation. However, Bekhit et al. (2003) found that carnosine accelerated the accumulation of MetMb and after 6 days of storage, MetMb% was 50% higher than that of the control group. This might be due to the different concentration of carnosine used in the experiment.

Lipid oxidation

Lipid oxidation levels increased in all beef patties as storage time increased (Table 3). Antioxidants treated patties showed more lipid stability than control groups, but there were no significant ($p < 0.05$) differences among those antioxidants-treated patties. On the 8th day, the inhibition rate (Δ inhibition (%)) of lipid oxidation of beef patties treated with various natural antioxidants was 34.03%, 20.0%, 14.30%, 11.60% and 13.40% for BHA, tea catechins, vitamin E, grape seed extract and carnosine, respectively (Table 3). It is also interesting to point out that tea catechins had a similar antioxidation effect with BHA on the 8th day.

Bekhit et al. (2003) found carnosine-treated meat exhibited higher TBARS values compared to that of control group. But Djenane et al. (2004) found that

carnosine exerted a significant ($p < 0.05$) inhibition effect on TBARS formation. And Badr et al. (2007) found that addition of carnosine significantly ($P < 0.05$) reduced the oxidative processes and MetMb formation (proportionally to the used concentration) in samples post-irradiation and during storage of ground beef and raw patties. The different results on the effect of carnosine in lipid oxidation might be explained by the different species of materials used in the research.

Brannan and Mah (2007) have reported that grape seed extract was very effective inhibitor of primary and secondary oxidation products in various muscle systems even at concentrations as low as 0.1%. As discussed earlier, tea catechins (300 mg/kg meat) were also reported to be more effective compared to vitamin E (300 mg α -tocopherol/kg meat) in retardation of lipid oxidation in cooked beef and chicken meat and in raw red meat, poultry and fish muscle (Tang et al., 2001a). Phenolic -compounds interrupt the propagation of the free radical auto oxidation chain by contributing a hydrogen atom from a phenolic hydroxyl group ($-OH$), with the formation of a relatively stable free radical that does not initiate or propagate further oxidation processes (Falowo et al., 2014).

Metmyoglobin reducing activity (MRA)

The results of MRA are shown in Table 4. Results clearly demonstrated that after 7 days of storage, MRA

Table 3. Effect of antioxidants on TBARS value (mg MDA/kg meat) in raw beef patties during refrigerated storage ($4 \pm 1^\circ\text{C}$)

Time day	Control	BHA	TC	VE	GSE	CR
0	0.162 \pm 0.00778xa	0.165 \pm 0.00707xa	0.149 \pm 0.0283xa	0.161 \pm 0.0113xa	0.156 \pm 0.00424xa	0.158 \pm 0.00636xa
2	0.202 \pm 0.0184ya	0.159 \pm 0.00141xc	0.155 \pm 0.00141xc	0.175 \pm 0.00354xbc	0.168 \pm 0.0283ybc	0.188 \pm 0.00212yab
4	0.232 \pm 0.00212ya	0.177 \pm 0.00283xc	0.213 \pm 0.0109yb	0.22 \pm 0.0141yab	0.224 \pm 0.00354zab	0.216 \pm 0.0307zb
6	0.318 \pm 0.0197za	0.212 \pm 0.0134yd	0.249 \pm 0.0990zc	0.268 \pm 0.0148zbc	0.288 \pm 0.0144hab	0.265 \pm 0.00354hbc
8	0.335 \pm 0.00141za	0.221 \pm 0.00636yd	0.268 \pm 0.0134zc	0.287 \pm 0.00495zbc	0.296 \pm 0.00212hab	0.290 \pm 0.0156wbc
Δ Inhibition %	–	34.03	20.00	14.30	11.60	13.40

x-w – means in same row with the same letter are not significantly different at $p < 0.05$.

a-e – means in same column with the same letter are not significantly different at $p < 0.05$.

Δ Inhibition, % = $\text{TBARS}(\text{control})_8 - \text{TBARS}(\text{antioxidant})_8 / \text{TBARS}_8(\text{control}) \cdot 100$.

Table 4. Effect of antioxidants on MRA (nmol·min⁻¹·g⁻¹) in raw beef patties during refrigerated storage (4 ±1°C)

Time day	Control	BHA	TC	VE	GSE	CR
0	0.198 ±0.00849xa	0.195 ±0.0127xa	0.184 ±0.00849xa	0.177 ±0.00424xa	0.186 ±0.00424xa	0.180 ±0.00846xa
2	0.207 ±0.0212yc	0.225 ±0.0212xd	0.282 ±0.0594ya	0.309 ±0.00415ybc	0.282 ±0.00848yc	0.291 ±0.0297ybc
4	0.306 ±0.00101zb	0.324 ±0.00849yab	0.354 ±0.0170yza	0.345 ±0.0212yza	0.321 ±0.00424zab	0.303 ±0.0205yb
6	0.333 ±0.00424zhb	0.351 ±0.0127yzb	0.372 ±0.00849za	0.357 ±0.0211yzb	0.339 ±0.00425zb	0.348 ±0.0424yb
8	0.354 ±0.00848hb	0.360 ±0.0170zb	0.375 ±0.0127zb	0.381 ±0.0297zab	0.405 ±0.0212ha	0.381 ±0.0127zab

x-w – means in same row with the same letter are not significantly different at $p < 0.05$.

a-e – means in same column with the same letter are not significantly different at $p < 0.05$.

were significantly increased ($p < 0.05$) with all the patties than the first day. A greater increase was observed with carnosine, tea catechins, vitamin E and grape seed extract treatments compared to BHA.

Bekhit et al. (2001) also observed a 20% increase in MetMb reducing activity in ovine muscle after vacuum storage for 6 weeks, supporting the results in this research. On the other hand, Pong et al. (2000a) also found Tuna muscle MetMb reducing activity decreased to less than 20% of its original activity after 3 days of cold storage.

While the techniques and assay conditions used in determining MetMb reducing activity might explain some of the different results from different researchers, the controversy regarding the effect of storage time on MetMb reducing activity cannot be attributed solely to the methodology employed (Bekhit and Faustman, 2005). There is still a need to understand the factors which influence the activities of MetMb reductase.

The correlation between TBARS values, a^* and MetMb%

There was a significant ($p < 0.01$) positive correlation ($r = 0.880$) between lipid oxidation and MetMb%, while a significant ($p < 0.01$) negative correlation ($r = -0.886$) between MetMb% and a^* (Table 5). To predict the TBARS value, a regression equation ($Y = -273.65X - 30.288$) was established, where $Y = \text{MetMb\%}$ and $X = \text{TBARS value}$ or ($Y = -98.533X + 40.988$), where $Y = a^*$ and $X = \text{TBARS value}$ (Fig. 1).

Das et al. (2006) also found that MetMb% showed significant ($p < 0.01$) positive correlation ($r = 0.66$) with TBARS values. The reason behind such positive

correlation may be that lipid oxidation produces peroxide material and free radicals which promote the pigment oxidation, resulting in meat discoloration (Cheng et al., 2007). On the other hand, pigment oxidation produces iron ion and promotes lipid oxidation (Grunwald and Richards, 2006). But not all studies that have measured lipid oxidation and myoglobin oxidation in meat have produced results demonstrating that the two processes are linked (Faustman et al., 2010). Hayes et al. (2009) found that sesamol addition to porcine and bovine meat systems led to decreased lipid oxidation but enhanced OxyMb oxidation; addition of ellagic acid and olive leaf extract led to decreases in both oxidative processes. Faustman et al. (2010) found that atmospheres containing very high or very low concentrations of oxygen provide conditions in which the oxidative interaction between lipid and myoglobin is not tightly linked.

Table 5. Correlation coefficients (r -value) of MRA, MetMb(%), TBARS value and a^* in raw beef patties as affected by application of different antioxidants during refrigerated storage (4 ±1°C)

	MetMb%	a^*	TBARS	MRA
MetMb%	1	-.886**	.880**	-0.27
a^*	-.886**	1	-.685**	0.17
TBARS	.880**	-.685**	1	-0.03
MRA	-0.27	0.17	-0.03	1

* $p < 0.05$, ** $p < 0.01$.

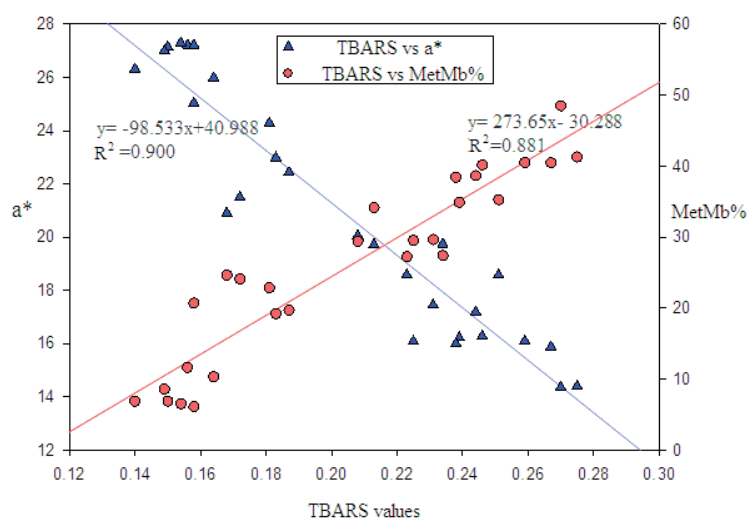


Fig. 1. Relationship of TBARS values, MetMb% and a^* in beef meat patties pre-blended with different antioxidants during refrigerated storage ($4 \pm 1^\circ\text{C}$)

The correlation between TBARS values, MRA and a^*

A weaker ($p > 0.05$) correlation was found between MRA and TBARS values ($r = -0.03$), and there was also a weak correlation between MRA and a^* ($r = 0.17$).

Few studies have demonstrated an association between MRA in meat and its colour stability. Bekhit and Faustman (2005) believed that the biochemical MetMb reducing system in post-mortem muscle was the most important factor regulating colour but found no significant correlation between MRA and meat discoloration. Bekhit et al. (2001) also found no correlation between metmyoglobin reducing activity and colour stability parameters in lamb loin meat, whereas factors influencing oxygen diffusion and consumption and the auto-oxidation of myoglobin have been shown to impact lamb meat colour during display (Jose, 2011).

The oxidation of Mb could generate free radicals that promote lipid oxidation and lead to meat discoloration, since total MRA (sarcolemmal and myofibrillar) was highly correlated with lipid oxidation (Bekhit and Faustman, 2005). So a hypothesis could be made from the present studies that antioxidants protected the red colour of fresh meat by protecting enzyme-based MetMb reducing systems from damage by lipid oxidation products.

CONCLUSION

It was concluded that addition of antioxidants to beef meat patties inhibited lipid oxidation and retarded the formation of metmyoglobin. Lipid oxidation was positively and strongly correlated ($r = 0.880$) with MetMb% and negatively ($r = 0.886$) with redness values. Tea catechins and grape seed extract effectively retard the oxidative processes in raw beef patties and their antioxidant ability is superior to carnosine. Tea catechins, grape seed extract and carnosine had a better protection effect on colour stability than vitamin E. The present approach is expected to provide a crucial guideline to use various antioxidants for retardation of oxidation of other meat products.

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