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The effect of vitamin D_3 supplementation on texture and oxidative stability of beef loins from steers treated with zilpaterol hydrochloride

Stine Hansen [a], Lorinda Frylinck [b], Phillip E. Strydom [b,*]

[a] Food Chemistry, Department of Food Science, Faculty of Life Sciences, University of Copenhagen, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark
[b] Animal Production Institute, Agricultural Research Council of South Africa, Private Bag X2, Irene, 0062, South Africa

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ABSTRACT

In this study, 10 young steers received no beta agonist (C), 50 animals all received zilpaterol hydrochloride (Z), with 1 group receiving Z while the other 4 groups received Z and vitamin D_3 at the following levels (IU/animal /day) and durations before slaughter: 7 million for 3 days (3D7M) or 6 days (6D7M), 7 million for 6 days with 7 days no supplementation (6D7M7N) and 1 million for 9 days (9D1M). LD samples were vacuum-aged 14 days *post mortem*, and repacked in high-oxygen modified atmosphere (70% O_2/30% CO_2) for a further 7 days. Parameters included Warner Bratzler shear force (WBSF), myofibril fragment length, thiobarbituric acid reactive substances, free thiol levels, muscle fat, and instrumental colour parameters. When allowing for a conversion period of vitamin D_3 to its active metabolites, supplementing the zilpaterol treated steers with a high dosage of vitamin D_3 (6D7M7N) resulted in improved colour stability, higher stability towards protein oxidation and lower stability towards lipid oxidation.

1. Introduction

It is widely known, that β-agonists supplemented animals produce tougher meat and that this increase in toughness, in part, is caused by a lower ageing potential due to a reduction in calpain activity and an increase in calpastatin activity (Koohmaraie, Shackelford, Mugglicockett, & Stone, 1991; Strydom, Frylinck, Montgomery, & Smith, 2009).

Supplementation with ultra high levels of vitamin D_3 over the final days before slaughter has been used to improve meat tenderness (Montgomery et al., 2002). This method is motivated by the suggestion that an increased calcium ion level, stimulated by high vitamin D_3 levels (Swanek et al., 1999), contributes to meat tenderization directly by weakening of myofibrillar structures (Takahashi, 1992) as well as indirectly through activation of μ-calpain (Koohmaraie, 1992).

High-oxygen modified atmosphere packaging (MAP) of fresh red meats has become widely used in many developed countries. MAP is usually comprised of 70–80% O_2 and 20–30% CO_2, which prolong the shelf life and provide a stable bloomed meat colour, both of which are attractive to the consumer (McMillin, 2008). However, storing meat in a high-oxygen atmosphere, promotes oxidative changes, and thus, negatively affect the meat quality, including both colour stability and eating quality (Kim, Huff-Lonergan, Sebranek, & Lonergan, 2010; Tørngren, 2003). Oxygen-dependant deterioration has been expressed as reduced juiciness, tenderness, flavour and odour as well as increased warmed-over flavour and rancidity in beef loin, which again have been associated with both protein and lipid oxidation (Clausen, Jakobsen, Ertbjerg, & Madsen, 2009; Sørheim, Wahlgren, Nilsen, & Lea, 2004; Tørngren, 2003).

Supplementation with the beta-agonist zilpaterol has previously been reported to increase the ratio of fast glycolytic (white) fibres (Strydom et al., 2009). Hence, meat from zilpaterol supplemented animals should be less prone to oxidation. Supplementation of vitamin D_3 could also improve antioxidative capacity of loin muscle (Lahucky et al., 2007), thereby extending colour shelf life and support meat and fat quality (Wiegand, Parrish, Morrical, & Huff-Lonergan, 2001).

The present work was a subpart of a study set up to establish which vitamin D_3 supplementation strategy most effectively improves beef tenderness of zilpaterol treated steers without negatively affecting tissue residues. Strydom, Hope-Jones, Frylinck, and Webb (2011) compared the effect of supplementation on the hardness (WBSF) of unaged (3 days) and vacuum aged (14 days) meat from carcasses that were either non-stimulated or subjected to electrical stimulation within 30 min of killing. In this part of the study, the effect of supplementation in relation to oxidative stability of beef loins from carcasses subjected to electrical stimulation has been investigated.

The objective was to determine how different doses and administration times of vitamin D_3 and subsequent chill storage in oxygen-rich atmosphere affected myofibrillar fragmentation, hardness as determined by WBSF, colour properties, oxidative stability as determined by

* Corresponding author.
E-mail address: pstrydom@arc.agric.za (P.E. Strydom).

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a decrease in free thiol groups (protein oxidation) and an increase in thiobarbituric acid reactive substances (TBARS; lipid oxidation) of zilpaterol supplemented beef loins.

It is hypothesized that meat from zilpaterol supplemented steers is less prone to oxidation and that supplementation with very high levels of vitamin D_3 over the final days before slaughter will increase the oxidative stability and improve colour stability and meat tenderness.

2. Materials and methods

2.1. Animals and feeding strategies

Sixty Bonsmara steers approximately 9 months of age were raised on a commercial feedlot diet for 120 days. The animals were divided into six groups ($n = 10$) so that the average weight and variation was the same for all groups. The groups represented six treatments, namely a control (C), which received the feedlot diet only, while the five remaining groups were supplemented with a beta agonist, zilpaterol hydrochloride, (Intervet/Schering-Plough Animal Health, South Africa) at 0.15 mg/kg live weight for 30 days during the final weeks of finishing. One of the 5 groups only received zilpaterol (Z), while the other 4 groups received zilpaterol and vitamin D_3 (He-Ro Chemicals Ltd, Hong Kong) at the following levels and durations before slaugher: 7×10^6 IU/animal/day for 3 days prior to slaughter (3D7M); 7×10^6 IU/animal/day for 6 days prior to slaughter (6D7M); 7×10^6 IU/animal/day 6 days followed by seven days of no supplementation prior to slaughter (6D7M7N) and 1×10^6 IU/animal/day for 9 days prior to slaughter (9D1M). The weight of vitamin D_3 supplement (500 IU/g) was calculated per 10 animals (one pen) needed for each day according to the treatment group, then mixed with coarsely grounded maize to increase the volume and applied as top dressing with the daily supply of the feedlot diet. A clean bunk approach was followed in the feedlot to facilitate complete and equal consumption of the supplement for all 10 animals per pen. To this end, the daily amount of feed was limited so that the feed troughs were empty in the morning before feeding. Sufficient bunk space was available so that all ten animals could feed at the same time. The zilpaterol was withdrawn 4 days prior to slaughter.

2.2. Slaughter and sampling procedures

The animals were slaughtered at the abattoir of the Animal Production Institute (Agricultural Research Council, Irene, Gauteng Province). Carcasses were split and the left sides were electrically stimulated for 30 seconds (400 V peak, 5 ms pulses at 15 pulses per second) within 30 min of exsanguination. Carcass sides were then chilled at 0–5 °C (chiller temperature at loading). All samples were collected from the LL of the stimulated side (left) after *rigor mortis* (1 day *post mortem*), depending on the purpose of the sample. The following tests were conducted: a) Warner Bratzler shear force resistance, b) myofibril fragment length (MFL), c) free thiol content, d) muscle fat content and TBARS and e) instrumental colour measurement.

2.3. Packaging and aging

Steaks were cut perpendicular to the long axis of each LL muscle. Two steaks 30 mm thick were aged in vacuum bags for 14 days at 2 °C and frozen at −20 °C for WBSF and MFL measurements. Two steaks of 25 mm and 50 mm, respectively, were aged in vacuum bags for 14 days. The 25 mm steak was used for instrumental colour measurement and then repacked under vacuum and frozen at −20 °C for measurement of free thiol content and TBARS. The 50 mm steak was divided in 2 × 25 mm steaks and packed in modified atmosphere containing 70% oxygen and 30% carbon dioxide using an ULMA Arctic Flow Wrapper (ULMA Packaging Systems (SA) (Pty) LTD, Sandton, South Africa). The steaks were placed in plastic trays (Cryovac UBRT 1621; 205 × 160 × 48 mm in dimension; Cryovac, subsidiary of Sealed Air Africa Pty Ltd, Kempton Park, South Africa) and heat sealed (Cryovac CJ62) with 25 μm polyolefin extruded film (Cryovac BDF 8050; O_2 transmission rate = 25 cm^3/m^2/24 h/bar, CO_2 transmission rate = 50 cm^3/m^2/24 h/bar, water-vapour transmission rate = 12 g/m^2/24 h; Sealed Air Africa Pty Ltd, Kempton Park, South Africa) giving a headspace-to-meat ratio of more than 4:1. The steaks were stored in the dark at 2–4 °C for 7 days (until 21 days *post mortem*). One steak was then vacuum packed and frozen at −20 °C for WBSF measurement and the other was used for instrumental colour measurement and then frozen for MFL, TBARS and free thiol measurement.

2.4. Warner Bratzler shear force (WBSF)

Frozen steaks were thawed at 4 °C for 24 h and cooked using an oven-broiling (Mielé, model H217, Mielé & Cie, Gütersloh, Germany) method with direct radiant heat (American Meat Science Association (AMSA), 1995). The steaks were broiled at 260 °C (pre-set) to 70 °C internal temperature and cooled down to 18 °C. Six round cores (12.7 mm diameter) were removed from the steaks parallel to the muscle fibres (American Meat Science Association (AMSA), 1995). Each core was sheared once through the centre, perpendicular to the fibre direction, by a Warner Bratzler shear device mounted on an Universal Instron apparatus (Model 4301, Instron Ltd, Buckinghamshire, England; cross head speed = 200 mm/min) and the mean value of the six recordings used as a shear value.

2.5. Myofibrillar fragmentation

Myofibril fragment lengths (MFL) of LL were measured by means of video image analysis and expressed in μm. Myofibrils were extracted according to Culler, Parrish, Smith, and Cross (1978) as modified by Heinze and Bruggemann (1994). One hundred myofibrillar fragments per sample were examined and measured with an Olympus BX40 system microscope (Olympus, Tokyo, Japan) at a 400× magnification. The mean values were used for statistical analysis.

2.6. Free thiol group determination

Protein oxidation was measured as the amount of free thiol groups in proteins determined as μM thiol per mg protein (high values indicate low oxidation). Homogenates were prepared by homogenizing 1.0 g of minced sample in 25 mL 5.0% sodium dodecylsulfate (SDS) in 0.10 M tris buffer, pH 8.0 (tris(hydroxymethyl) aminomethane) using an Ultra Turrax (T25, IKA-Labortechnik, Janke & Kunkel, Staufen, Germany). The homogenates were then heated in an 80 °C water bath for 30 min and the supernatant was filtered through a paper filter (3HW; Munktell & Filtrak GmbH, Bärenstein, Germany). The protein concentration of the filtrate was determined by measuring absorbance at 280 nm using a standard curve prepared from 0 to 3 mg/mL bovine serum albumin. No absorbance was detected at wavelengths above 300 nm, hence, myoglobin did not interfere with the assay. Free thiol determination was carried out by diluting the filtrates to a concentration of 1.5 mg/mL with 5% SDS in 0.10 M tris buffer. Then the assay was prepared by mixing 0.50 mL diluted filtrate, 2.0 mL tris buffer (0.10 M, pH 8.0) and 0.5 mL of 10 mM DTNB (5,5′-Dithiobis (2-nitrobenzoic acid)) in 0.10 M tris buffer. After 30 min, the absorbance of the samples was measured at 412 nm against an aqueous reference solution of 0.50 mL 5% SDS in 0.10 M tris buffer, 2.0 mL tris buffer. Duplicate homogenates and triplicate measurements on each homogenate were made for each meat sample and the mean values used for statistical analysis.

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2.7. Thiobarbituric acid reactive substances (TBARS)

Lipid oxidation was evaluated using 2-Thiobarbituric acid (TBA; 4,6-dihydroxy-2-mercapto-pyrimidin) as described by Raharjo, Sofos, and Schmidt (1992). Briefly, 4.0 g of meat was homogenized in 15 mL 5.0% (w/v) aqueous solution of trichloroacetic acid for 1 min using an Ultra Turrax homogenizer (T25, IKA-Labortechnik, Janke & Kunkel, Staufen, Germany). The meat slurry was centrifuged at 10,000 *g* for 10 min and the supernatant was filtered through a paper filter (MN615; Macherey-Nagel, Düren, Germany). 2.0 mL of filtrate was mixed with 2.0 mL 40 mM TBA and incubated at 94 ± 1 °C in a waterbath for 10 min. The absorbance of the red pigment formed was scanned from 400 to 600 nm (DU 7500 Beckman, Beckman Instruments Incorporated, Fullerton, California, USA). Results are expressed as 2-thiobarbituric acid reactive substances (TBARS) in mg malondialdehyde per kg meat using a standard curve prepared from 1,1,3,3-tetraethoxypropane (malonaldehyde). A 78% recovery value of malondialdehyde-TBA complex was used in the final calculations. Mean values of two independent determinations were used for statistical analysis.

2.8. Meat colour (CIE L*a*b*)

Instrumental meat colour was measured with a Minolta meter (Model CR200, Osaka, Japan; 8 mm diameter measuring area, diffuse illumination and 0° viewing angle). The 25 mm vacuum packed steaks (Section 2.1) were allowed to bloom for at least 30 min at 4 ± 2 °C after removal from the vacuum bags. One of the 25 mm steaks stored under modified atmosphere for 7 days was removed from the packaging and colour recordings were done immediately. Colour measurements followed the CIE colour convention, where the three fundamental outputs are L*, a* and b*. L* is lightness on a scale of 0 (all light absorbed) to 100 (all light reflected); a* spans from +60 (red) to −60 (green) and b* spans from +60 (yellow) to −60 (blue). Saturation index is calculated as the square root of $a^{*2}+b^{*2}$, also known as chroma (MacDougall, 1977). Mean values were used for statistical analysis.

2.9. Muscle fat

A sample of the LL (without external fat) was ground and frozen (−20 °C) for proximate analyses of fat (Association of Official Analytical Chemists (AOAC), 1995).

2.10. Statistical analysis

Data of WBSF, MFL, instrumental colour attributes, TBARS and free thiol were subjected to analysis of variance for a split-plot design (Payne, Murray, Harding, Baird, & Soutar, 2007) with the six treatment groups (C, Z, 3D1M, 9D1M, 6D7M, 6D7M7N) as whole plots and the two *post mortem* storage periods (14 days and 21 days) as sub-plots. For colour and thiol data only 14 and 21 days were used. Means for the interactions between the whole plot and sub-plot were separated using Fisher's protected *t*-test least significant difference (LSD) at the 5% level (Snedecor & Cochran, 1980).

3. Results

3.1. Gas composition

For most of the MA packages, the oxygen-enriched environment was well maintained throughout the display period. The average gas composition of each modified atmosphere after seven days of display was 65.8 ± 1.4% O_2.

3.2. Warner Bratzler shear force (WBSF)

The effects of supplementation strategy and storage time and condition on WBSF are shown in Table 1. Storage time and conditions *post mortem* had significant effects on WBSF ($P<0.001$) in that WBSF decreased significantly from day 14 (vacuum ageing) to day 21 (14 days vacuum + 7 days MAP, except for 3D7M). Zilpaterol, with and without vitamin D_3 supplementation, resulted in harder meat ($P<0.001$) than meat from steers fed the control diet. No difference in WBSF was observed between the different vitamin D_3 strategies. Thus, oral administration of vitamin D_3 in combination with zilpaterol treatment did not seem to decrease instrumental hardness of the meat.

3.3. Myofibrillar fragment length (MFL)

At 14 days *post mortem*, the zilpaterol treatment without vitamin D_3 supplementation exhibited the longest myofibril fragments (Table 1). The control and 6D7M7N treatments showed similar MFL values, which differed ($P<0.05$) from the zilpaterol, 9D1M and 3D7M treatments. The latter 3 treatments resulted in a decrease ($P<0.001$) in MFL values after 7 days in MAP conditions. At 21 days *post mortem*, meat from steers receiving the zilpaterol and 3D7M treatments still exhibited longer myofibrillar fragments ($P<0.05$) than meat from control steers. The MFL values from the remaining vitamin D_3 supplementation strategies did not differ from the control group.

3.4. Free thiol group determination

The content of free thiol groups was significantly affected by the feeding strategy ($P<0.001$) both after storage in vacuum (non-oxidative environment) for 14 days and in MAP for an additional 7 days (Table 2). Control samples exhibited the lowest level of free thiol groups compared to supplemented samples ($P<0.001$) for both storage conditions. After vacuum storage, samples treated with zilpaterol without

Table 1
Warner Bratzler shear force (WBSF) and myofibril fragment lengths (MFL) of *M. longissimus lumborum* from steers treated with zilpaterol and differing dosages of vitamin D_3.

			Vitamin D_3: duration and dosage (IU/day)				SEM[1]
	Control	Zilpaterol	9 days, 1 million	3 days, 7 million	6 days, 7 million	6 days, 7 million + 7 days withdrawal	
WBSF (kg)							
14 d (vac[2])	3.5^{aB}	4.3^{bB}	4.6^{bB}	4.3^{bA}	4.7^{bB}	4.6^{bB}	0.2419
21 d (14 d vac + 7 d MAP[2])	2.4^{aA}	3.7^{bA}	4.0^{bA}	4.1^{bA}	4.0^{bA}	4.0^{bA}	
MFL (μm)							
14 d (vac)	26.1^{aA}	36.7^{dB}	34.1^{cdB}	33.0^{bcB}	30.7^{abA}	28.9^{aA}	0.990
21 d (14 d vac + 7 d MAP)	24.8^{aA}	28.8^{bA}	27.3^{abA}	28.6^{bA}	28.3^{abA}	26.6^{abA}	

[1] SEM = standard error of mean.
[2] vac = vacuum packed storage; MAP = modified atmosphere packed, 70% O_2/30% CO_2.
[a,b,c,d] Means within the same row with different superscripts, differ significantly ($P<0.05$).
[A,B,C] Means of WBSF and MFL, respectively, within the same treatment group with different superscripts, differ significantly ($P<0.05$).

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Table 2
Oxidative stability of *M. longissimus lumborum* from steers treated with zilpaterol and differing dosages of vitamin D_3. Data are expressed by the level of free thiol (protein oxidation) and TBARS (lipid oxidation).

			Vitamin D_3: duration and dosage (IU/day)				SEM[3]
	Control	Zilpaterol	9 days, 1 million	3 days, 7 million	6 days, 7 million	6 days, 7 million + 7 days withdrawal	
Free thiol (μM/mg protein)							
14 d (vac[2])	67.8^{aB}	73.4^{cB}	70.5^{abcB}	72.7^{bcB}	68.7^{abB}	71.0^{abcB}	1.609
21 d (14 d vac + 7 d MAP[2])	54.6^{aA}	62.8^{bA}	61.3^{bA}	63.3^{bcA}	62.4^{bA}	67.5^{cA}	
Δ Thiol	-13.2^{c}	-10.6^{bc}	-9.2^{abc}	-9.4^{bc}	-6.3^{ab}	-3.5^{a}	1.987
TBARS (mg MDA[1]/kg meat)							
14 d (vac)	0.25^{A}	0.36^{A}	0.30^{A}	0.30^{A}	0.30^{A}	0.27^{A}	0.0738
21 d (14 d vac + 7 d MAP)	0.58^{abB}	0.51^{aA}	0.58^{abB}	0.74^{bcB}	0.52^{aB}	0.82^{cB}	
Δ TBARS	$+0.33^{ab}$	$+0.15^{a}$	$+0.27^{a}$	$+0.45^{ab}$	$+0.22^{ab}$	$+0.55^{b}$	0.1072
Muscle fat (%)	1.50	1.26	1.30	1.29	1.25	1.35	0.1370

[1] MDA = malondialdehyde.
[2] vac = vacuum packed storage; MAP = modified atmosphere packed, 70% O_2/30% CO_2.
[3] SEM = standard error of mean.
[a,b,c] Means within the same row with different superscripts, differ significantly ($P<0.05$).
[A,B] Means of free thiol and TBARS, respectively, within the same treatment group with different superscripts, differ significantly ($P<0.05$).

vitamin D_3 had the highest content of free thiol groups followed by samples treated with both zilpaterol and vitamin D_3. The free thiol content of the control samples decreased the most (13.2 units), giving the poorest stability towards protein oxidation after MAP. Supplementation with a high vitamin dosage for a short time (3D7M) and low dosage for a long time (9D1M) resulted in similar declines in the level of free thiol groups (9.5 and 9.2 units, respectively) which was only marginally lower than the decline of zilpaterol treated samples (10.6 units). However, supplementation with a high dosage of vitamin D_3 for six days (6D7M) showed lower decline in the free thiol content (6.3 units) and even more so if the supplementation was withdrawn seven days prior to slaughter (6D7M7N; 3.5 units). After 7 days in an oxidative environment, 6D7M7N was least oxidized (lowest decrease in free thiol) and the remaining supplementation groups showed similar levels of free thiol groups (larger decrease in free thiol).

3.5. Thiobarbituric reactive substances (TBARS)

Storage time and condition *post mortem* had a significant effect ($P<0.001$) on lipid oxidation (Table 2). The TBARS values after storage in vacuum (non-oxidative environment) for 14 days were significantly lower for all treatments (except zilpaterol) than the values from meat stored in high-oxygen MAP for an additional seven days (21 days *post mortem*; 14 days vac + 7 days MAP). The control samples showed a 2.3 times increase in TBARS during MAP storage. After storage in high-oxygen modified atmosphere for seven days, the zilpaterol treated samples exhibited the lowest increase in TBARS (1.4×) followed by 6D7M (1.7×), 9D7M (1.9×), 3D7M (2.5×) and 6D7M7N (3.0×). This showed a clear tendency towards increased lipid oxidation in meat from steers supplemented with vitamin D_3.

3.6. Muscle fat content

Muscle fat content was generally very low (<2.0%) and control samples had higher fat levels than treated samples (Table 2).

3.7. Instrumental colour characteristics

Storage time and condition affected all colour measurements significantly (Table 3). The values for redness (CIE a*) and saturation index were highest for the control samples and lowest for the zilpaterol samples ($P<0.05$) at both 14 and 21 days *post mortem*. All vitamin D_3 supplementation strategies gave higher scores for redness after 14 days of vacuum ageing compared with the zilpaterol group. After seven days of storage in a high-oxygen atmosphere, giving 21 days of storage in total, there was a significant improvement in redness ($P<0.05$) by supplementing vitamin D_3, regardless of dosage and duration of the supplementation. Although redness values and saturation indexes decreased from 14 days (vacuum) to 21 days (14 days vacuum + 7 days MAP) for all the groups, the decrease was markedly smaller for the 6D7M7N group, where the high dosage of vitamin D_3 was withdrawn seven days prior to slaughter. Yellowness (CIE b*) values were clearly separated into two groups: a) zilpaterol + high vitamin D_3 dosage for 3 and 6 days (Z, 3D7M, 6D7M; low end)

Table 3
Colour properties of *M. longissimus lumborum* from steers treated with zilpaterol and differing dosages of vitamin D_3.

			Vitamin D_3: duration and dosage (IU/day)				SEM[1]
	Control	Zilpaterol	9 days, 1 million	3 days, 7 million	6 days, 7 million	6 days, 7 million + 7 days withdrawal	
Light reflection (L*)							
14 d (vac[2])	41.1^{abA}	42.4^{bcdA}	42.8^{cdA}	40.4^{aA}	43.2^{dA}	41.5^{abcA}	0.5302
21 d (14 d vac + 7 d MAP[2])	44.0^{aB}	44.2^{abB}	45.5^{bB}	44.4^{abB}	44.2^{abB}	45.0^{abB}	
Redness (a*)							
14 d (vac)	18.3^{bB}	15.8^{aB}	17.2^{abB}	16.5^{aB}	16.9^{abB}	17.0^{abA}	0.498
21 d (14 d vac + 7 d MAP)	16.5^{bA}	14.7^{aA}	15.9^{abA}	15.2^{abA}	15.5^{abA}	16.4^{bA}	
Yellowness (b*)							
14 d (vac)	10.5^{bA}	9.7^{aA}	10.5^{bA}	9.8^{aA}	9.8^{aA}	10.5^{bA}	0.2298
21 d (14 d vac + 7 d MAP)	11.7^{cB}	10.7^{aB}	11.5^{bcB}	10.9^{abB}	10.7^{aB}	11.5^{bcB}	
Saturation index							
14 d (vac)	21.1^{cA}	18.6^{aA}	20.1^{bcA}	19.2^{abA}	19.5^{abA}	20.0^{bcA}	0.503
21 d (14 d vac + 7 d MAP)	20.2^{cA}	18.2^{aA}	19.7^{bcA}	18.7^{abA}	18.8^{abcA}	20.1^{bcA}	

[1] SEM = standard error of mean.
[2] vac = vacuum packed storage; MAP = modified atmosphere packed, 70% O_2/30% CO_2.
[a,b,c,d] Means within the same row with different superscripts, differ significantly ($P<0.05$).
[A,B] Different superscripts between means (based on each colour property separately) within the same treatment group indicate significant differences ($P<0.05$).

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and b) control + high vitamin D_3 dosage including withdrawal or low dosage for a longer time (C, 6D7M7N, 9D1M; high end) at both 14 and 21 days.

4. Discussion

Strydom et al. (in press) reported on the effects of zilpaterol and vitamin D_3 supplementation on WBSF and MFL of vacuum aged samples (14 days *post mortem*). It was concluded that beta agonist (zilpaterol) treated loins exhibited increased hardness (WBSF) compared to control samples and that various combinations of vitamin D_3 supplement failed to overcome this negative effect. Consistently higher (at 1 and 24 h *post mortem)* calpastatin activity in zilpaterol treated samples was proposed as the main reason for the retarded tenderisation and was supported by Geesink et al. (1993), Koohmaraie and Shackelford (1991), Simmons et al. (1997) and Wang and Beermann (1988). Despite some variation in both calpastatin and μ-calpain activities (at 1 and 24 h) among vitamin D_3 treatments, no positive effect on WBSF was recorded after vacuum storage (14 days). In particular, the 3D7M treatment exhibited an initially higher μ-calpain (1.36 units) activity followed by a larger reduction in activity (0.53 units, similar to that of the control; 0.46 units) and a significant decrease in calpastatin activity (Strydom et al., in press). In contrast, MFL after vacuum storage showed some response (Table 2), in particular to longer supplemented high dosage vitamin D_3 treatments (6D7M and 6D7M7N). These resulted in MFL values closer to that of the control, which may indicate increased proteolytic activity. It should be noted that these two treatments showed higher retention of μ-calpain activity (slower rate of decline in activity). Dransfield (1996) regarded the reduced recovered activity of calpains during *rigor mortis* and subsequent storage as unknown but important in relation to tenderization. He accounted the reduction to either autolysis of activated calpain, a low extractability or post-rigor degradation. It is therefore tempting to relate the higher retention of μ-calpain of 6D7M and 6D7M7N to the larger reduction in MFL at 14 days *post mortem*. On the other hand, zilpaterol alone and 9D1M showed similar patterns in calpain activity, yet their changes in myofibril length did not match that of 6D7M and 6D7M7N. Despite the variable effects of vitamin D_3 treatments on MFL and calcium dependent proteienases, the calpastatin activity at 24 h of all vitamin D_3 treatments remained high relative to the control which probably explains the lack of improvement WBSF of zilpaterol treated meat at 14 days *post mortem*.

All meat samples showed continuous improvement in WBSF values throughout the storage period of 21 days. However, the improvement in WBSF for all zilpaterol treated samples, with and without vitamin D_3, tended to slow down during storage under oxidative conditions when compared to the control. Hence, larger differences in hardness after 21 days resulted (Table 1). Changes in MFL of the control, 6D7M and 6D7M7N treatments also slowed down, thereby resulting in MFL values approaching the same level among all treatments. Thus, although *post mortem* proteolytic degradation is slowed down in the zilpaterol treatments it was not completely inhibited. Protein oxidation (expressed as loss of free thiol groups) was not correlated to the slower decrease in hardness observed, although increased protein oxidation has been associated with decreased tenderness (Clausen et al., 2009; Kim et al., 2010; Lund, Lametsch, Hviid, Jensen, & Skibsted, 2007;). According to Table 2, the control group showed the lowest initial level of free thiol groups after 14 days in vacuum and oxidized at a higher rate during storage in an oxygen-rich environment than the zilpaterol treated samples with and without vitamin D_3. Thus, the lower WBSF values of the control samples did not correlate with the increased protein oxidation taking place. The increase in protein oxidation was not reflected in the MFL or WBSF values.

In the studies where correlations between protein oxidation and reduced tenderness have been reported, beef steaks (Clausen et al., 2009; Kim et al., 2010) and pork chops (Lund et al., 2007) were packaged in high-oxygen MAP at 1 or 3 days *post mortem*. Hence, inactivation of μ-calpain may have contributed to the lower tenderness scores (Rowe, Maddock, Lonergan, & Huff-Lonergan, 2004). Zakrys, Hogan, O'Sullivan, Allen, and Kerry (2008) did not find significant changes in WBSF, tenderness scores, and carbonyl formation after chill storage in MA and, hence, were unable to establish any definitive relationship between carbonyl formation and tenderness development when beef steaks were packaged at two weeks *post mortem* in high-oxygen MAP. Although μ-calpain is sensitive to oxidation, it is known to autolyse within three days *post mortem*, which eventually leads to loss of activity (Boehm, Kendall, Thompson, & Goll, 1998; Grobbel, Dikeman, Hunt, & Milliken, 2008). Thus, in the present study, inhibition of the μ-calpain through oxidation did not seem to be a likely explanation of the slower tenderness development taking place during high-oxygen chill storage. In fact, most of the ageing potential of zilpaterol treated samples (with and without vitamin D_3) had most likely been reached by the time the meat was stored in high-oxygen MAP at 14 days *post mortem*. This may indicate that the *post mortem* time of packaging in high-oxygen MA is important in relation to the tenderness development is in the subsequent storage period.

The zilpaterol group had a higher initial level of free thiol and exhibited higher stability against oxidation under MAP conditions than the control group. This was expected, as previous studies (Grobbel et al., 2008; Strydom et al., 2009) have shown that zilpaterol treatments increase the size and percentage of glycolytic (white) fibres in the loin muscle compared to intermediate and oxidative (red) fibres. In addition, Daun, Johansson, Onning, and Akesson (2001) reported that more white fibred muscles (glycolytic) such as the *m. longissimus* exhibit a higher defence system against oxidation than red fibred muscles like the *m. psoas*. The same applied for specific muscles in pork (more glycolytic) compared to beef (more oxidative).

At 14 days *post mortem* there was no difference in the level of lipid oxidation between treatments. After seven days in a high-oxygen atmosphere, the zilpaterol group differed significantly from 3D7M and 6D7M7N. However, if lipid oxidation is expressed as the change occurring during high-oxygen storage (from 14 to 21 days *post mortem*) then the zilpaterol group showed markedly lower lipid oxidation than the control group. The lower level of lipid oxidation may be caused by the change in muscle fibre distribution. According to Pearson and Young (1989), red fibres contain a higher amount of lipid and myoglobin. Strydom et al. (2009) found that treatment with zilpaterol resulted in meat with a higher percentage of white fibres and lower fat content. In this study, muscle fat content was generally low and the control samples had higher fat levels than zilpaterol samples. It is reasonable to believe that the lower fat content would result in lower availability of polyunsaturated fatty acids, which are substrate for lipid oxidation (Faustman, Sun, Mancini, & Suman, 2010). In addition, formation of hypervalent myoglobin in meat has been linked to increased levels of lipid oxidation (Baron & Andersen, 2002; Carlsen, Møller, & Skibsted, 2005). Thus, if the content of myoglobin is lowered, a concurrent reduction in lipid oxidation may take place. In conclusion, it seemed likely that the change in the fibre type composition obtained by treatment with zilpaterol was responsible for the lower lipid oxidation of the zilpaterol samples compared to the control samples.

High O_2 concentrations promote the formation of oxymyoglobin (O'Grady, Monahan, Burke, & Allen, 2000) which generally results in better initial colour (redness) compared to conventional PVC overwrap (Jayasingh, Cornforth, Brennand, Carpenter, & Whittier, 2002) and vacuum packaging (John et al., 2005; Kim et al., 2010). However, due to oxygen-dependent reactions colour starts to deteriorate after 3 to 7 days and could result in similar or poorer colour quality

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than conventional packed samples (Jakobsen & Bertelsen, 2000; Kim et al., 2010) after 8 to 10 days. In high-oxygen MAP, colour deterioration was often associated with increased lipid oxidation (TBARS) (Jayasingh et al., 2002; Kim et al., 2010; Zakrys et al., 2008;) and protein oxidation (Lund et al., 2007; Zakrys et al., 2008) which caused increased levels of metmyoglobin (Lagerstedt, Lundström, & Lindahl, 2011). In the present study, the contrast in redness and saturation index between the control and zilpaterol treatments also reflects the differences in muscle fibre composition which was supported by Avendano-Reyes et al. (2006) and Vestergaard, Sejrsen, and Klastrup (1994). Vitamin D_3 tended to improve the instrumental redness and saturation index of zilpaterol treated samples measured after vacuum storage. Although all samples deteriorated in redness under oxygen-rich conditions as expected, the vitamin D_3 samples maintained their advantage over the zilpaterol samples. In particular when vitamin D_3 was supplemented for 6 days at high levels and then withdrawn for 7 days, colour deterioration (redness and saturation index) was significantly less compared to all other treatments. Lahucky et al. (2007) (5 days PVC overwrapped display) and Wiegand et al. (2002) (vacuum-packed for 14 days) reported improved redness (CIE a*) values for vitamin D_3 supplemented pork loins (500,000 IU/day). Lahucky et al. (2007) related the improved colour to an increased anti-oxidative capacity by recording lower TBARS values after incubation of muscle homogenates with Fe^{2+}/ascorbate. They suggested that a higher level of Ca^{2+} (bivalent ion) in muscles caused the positive influence on the lipid oxidation. This is supported by the findings of Babizhayev (1988), who found that high concentrations of Ca^{2+} may prevent lipid oxidation through interaction with superoxide radicals. On the other hand, Bors, Buettner, Michel, and Saran (1990) found that the concentration of calcium ions reported by Babizhayev to have an inhibitory effect far exceeded those relevant to animal systems. In the present study vitamin D_3 did not inhibit lipid oxidation through elevated calcium ion level as hypothesized probably because the rise in calcium ion level, as induced by the vitamin D_3 supplementation strategies applied, was not sufficient.

The lipid and protein oxidation pattern of 6D7M7N and its relation to colour stability is contradictive. This treatment showed the highest resistance against protein oxidation under oxygen-rich conditions (Δ Thiol; Table 2), also reflected in better colour stability. At the same time, a significantly higher rate of lipid oxidation compared to the control, zilpaterol and 9D1M supplement treatments was recorded. The reason for the protein oxidative stability of 6D7M7N compared to 6D7M and other vitamin D treatments may be found in the need for vitamin D_3 to be converted to its final and active metabolite, 1, 25-dihydroxy-vitamin D_3, which then leads to increased blood calcium. This process takes approximately 3 to 5 days from initial treatment (Montgomery et al., 2004). This seemed to favour the raised calcium theory of Lahucky et al. (2007). It is also possible that this effect may have led to the slightly positive effect that these two treatments (6D7M7N and 6D7M) had on MFL during vacuum storage (14 days), although this was not reflected in a positive WBSF result. On the other hand, lipid oxidation was promoted by the 6D7M7N treatment, probably due to high vitamin D_3 concentration in the fat tissue. This may indicate direct cellular redox reaction of vitamin D_3. Koren et al. (2001) found that vitamin D_3 acted as pro-oxidant in breast cancer due to an increase in the overall cellular redox potential. It is a well known phenomenon that vitamins may act as antioxidants at low concentrations and pro-oxidants at high concentrations. This has been demonstrated for ß-carotene (provitamin A) (Ruiz, Perez-Vendrell, & Esteve-Garcia, 1998), α-tocopherol (vitamin E) (Bowry & Stocker, 1993) and ascorbate (vitamin C) (Monahan, 2000). Rungby, Mortensen, Jakobsen, Brock, and Mosekilde (1993) reported that tissue concentrations of $1,25(OH)_2D_3$ in pigs were three to sevenfold higher in adipose tissues than in plasma and that muscle tissue contained less of the metabolite. Hence, the 6D7M7N treatment may have increased the concentrations of $1,25(OH)_2D_3$ in the adipose tissue of the steers to a level which exhibit pro-oxidant behaviour. In addition, the concentrations of $1,25(OH)_2D_3$ in the muscle tissue may have been sufficiently elevated by this treatment to exhibit antioxidative behaviour towards proteins and hence towards oxymyglobin stability. Jorgensen and Skibsted (1993) found that the antioxidative behaviour of carotenoids was most pronounced at low oxygen partial pressure. Information of this nature concerning vitamin D_3 is, to our knowledge, not available. However, if something similar could be expected, then the pro-oxidant behaviour of the 6D7M7N treatment may be explained. The lack of antioxidative behaviour may in part have been influenced by the high oxygen pressure of the modified atmosphere in which the meat samples were stored.

While Jayasingh et al. (2002), Kim et al. (2010), Lagerstedt et al. (2011), Lund et al. (2007) and Zakrys et al. (2008) relate protein and lipid oxidation to colour deterioration due to increased levels of metmyoglobin, various factors could affect the role of each mechanism. Jakobsen and Bertelsen (2000) mentioned fat level and fat composition (fatty acid saturation) as parameters affecting oxymyoglobin oxidation, although no specific level was reported. In further support, O'Grady et al. (2000) and O'Grady, Monahan, and Mooney (2001) suggested that significant discoloration will only occur in high oxidative environments when lipid oxidation levels exceeded a threshold level suggesting certain TBARS values and amount of oxidated lipid that could have been below that threshold level in the present study. In the study of Lahucky et al. (2007) where a lipid oxidation vs. colour stability relation was reported, fat levels were more than double the levels recorded in this work. The combined evidence may suggest that lower amount of lipid and hence lower levels of oxidated lipids negated the effect of high TBARS values of 6D7M7N. Finally, Faustman et al. (2010) gave evidence where lipid oxidation was minimized by anti-oxidants without any affect on colour preservation or where anti-oxidants decreased lipid oxidation but enhanced oxymyoglobin oxidation and various other combinations of effects.

5. Conclusions

It was hypothesized that meat from zilpaterol supplemented steers would be less prone to protein oxidation due to an increase in white fibres. This was supported by the findings. It was also hypothesized that very high levels of vitamin D_3 would exhibit antioxidative behaviour through increased calcium ion content in the meat tissue. This hypothesis was found to be true in relation the 6D7M7N treatment concerning protein oxidation. Both level and duration of oral supplementation of vitamin D_3 seemed to be of importance in relation to the effectiveness of the vitamin. It seemed that a withdrawal period following vitamin D_3 supplementation was beneficial, probably due to an increase in the conversion of the vitamin to its active metabolite, 1,25-dihydroxy-vitamin D_3. This treatment was also found to improve the colour, however, the mechanism is not clear.

The hypothesis that vitamin D_3 would exhibit antioxidative behaviour in relation to lipid oxidation could not be supported. In contrast, the 6D7M7N treatment, which showed antioxidative capacity towards protein oxidation, was found to be.

None of the vitamin D_3 supplementation strategies were found to improve beef tenderness of zilpaterol treated steers, even though the 6D7M7N treatment exhibited similar myofibril fragment lengths as the control samples at 14 days *post mortem*.

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