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The effect of carbon monoxide pretreatment exposure time on the colour stability and quality attributes of vacuum packaged beef steaks

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ABSTRACT

The effect of 5% CO pretreatments prior to vacuum packaging of beef striploin steaks (*Longissimus thoracis et lumborum*, LTL) on quality attributes, primarily colour stability was investigated. The aim was to determine the optimum pretreatment that would induce the desirable red colour, while allowing discoloration to occur by the end of a 28-day display period (2 °C), so as to not mask spoilage. A range of pretreatment exposure times (1, 3, 5, 7, 9, 15 and 24 h) were applied to steaks using a gas mixture of 5% CO, 60% CO_2 and 35% N_2. The 5 h CO pretreatment exposure time achieved the desirable colour and discoloration reached unacceptable levels ($a^* = 12, C^* = 16$) by the use-by date (28 days), thus ensuring consumers' of a reliable visual indication of freshness and addressing concerns about safety. The 5% CO pretreatment had no negative effect on microbiological safety, lipid oxidation, cooking loss and WBSF measurements at the end of storage ($P > 0.05$).

1. Introduction

Meat packaging innovations are driven by an effort to meet increased consumer demand and expectations of high quality. Consumers initially evaluate meat quality at point of purchase based on meat colour, as other quality attributes cannot be assessed prior to meat consumption. Meat colour is perceived by consumers as a strong indication of freshness or wholesomeness (Kropf, 1980). However, for eating experience tenderness is considered the most important palatability attribute (Grobbel, Dikeman, Hunt & Milliken, 2008a; Miller, Huffman, Gilbert, Hamman & Ramsey, 1995). This has highlighted the need for value-added meat packaging technologies which improve colour and tenderness.

Currently the meat industry employs a two-stage packaging system where primals are aged in vacuum packs (VP) ("wet aged") and then transferred to vacuum packaging (VP), vacuum skin packaging (VSP) or modified atmosphere packaging (MAP). Since meat colour is the primary quality trait desirable to consumers, high-oxygen MAP is the most commonly applied second-stage fresh packaging technology used to promote the desirable bright red colour (oxymyoglobin) desirable to consumers. Unfortunately, the disadvantages of this packaging technology include limited shelf life, reduced juiciness and increased oxidation leading to reduced tenderness and promotion of off-flavours. MAP packs are also a more bulky than VSP packs. With increasing demand for more tender aged meat, VP and VSP could be an alternative solution to MAP. VP is an anoxic technology that prevents lipid oxidation, prolongs shelf-life, reduces microbial spoilage and is the most commonly applied ageing method (wet ageing) for the tenderisation of primals. Wet ageing is also more cost effective than dry ageing and produces much higher yields (Obuz, Akkaya, Gök & Dikeman, 2014). More recently Eastwood, Arnold, Miller, Gehring and Savell (2016) showed the potential benefit of cutting steaks and individually ageing steaks in the pack instead of subprimal ageing as consumer panellists preferred steaks aged as individual steaks as opposed to subprimal ageing. However, VP and VSP are still largely limited due to the dark purple appearance (deoxymyoglobin) of the meat. Consumers perceive the purple colour of meat as unattractive and are less likely to purchase meat presented in this form (Carpenter, Cornforth & Whittier, 2001).

Carbon monoxide (CO) induces a bright red colour (carboxymyoglobin) similar to oxymyoglobin but more stable. CO is also naturally synthesised within the human body due to the breakdown of haemoproteins and an average concentration of 1.2–1.5% HbCO is endogenous in non-smokers (European Commission, 2001). CO has a long history of application within the meat industry for its colour stabilizing effect coupled with its antioxidant abilities. In the USA, low concentrations of CO (0.4%) have been GRAS (Generally Recognised As Safe) approved by the FDA and CO is permitted as a primary packaging gas in case-ready packaging systems (FDA, 2004). New Zealand and Australia also regulate low concentrations of CO in centralised packaging systems and it is considered a processing

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aid (Federal Register of Legislative Instruments, 2014). Similarly, Canada also allows the application of 0.4% CO as a secondary packaging gas (USDA-FSIS, 2016). However, in the EU, CO has not yet been approved as a packaging gas even though the application of low concentrations of CO to meat packaging systems have been reported to be consumer friendly and have no toxic effect (Sørheim, Aune & Nesbakken, 1997). An important concern which has been raised by regulatory authorities is that CO might be used to mask meat spoilage so that meat might be sold beyond its sell-by date due to the bright red colour being retained (Cornforth & Hunt, 2008). If spoilage is masked, consumers are led to falsely perceive the meat as fresh and wholesome (Hunt et al., 2004) and this is unacceptable for food safety.

Previous researchers have investigated applying 5% CO pretreatments prior to vacuum packaging (Aspé, Roeckel, Martí & Jiménez, 2008; Jayasingh, Cornforth, Carpenter & Whittier, 2001; O'Connor & Allen, 2011). These researchers applied a 5% CO pretreatment for 24 h to beef steaks prior to vacuum packaging. Spoilage was masked as colour was retained beyond microbiological spoilage. The optimum pretreatment exposure time which allows discoloration to occur by a use-by date of 28 days (2 °C so as to not mask meat spoilage, has not yet been determined. Lentz (1979) reported further research is required to establish the length of exposure of meat to CO. Furthermore, reducing exposure time to CO pretreatment may reduce process time thus increasing profitability, productivity and efficiency if applied in meat packaging plants. The addition of CO pretreatments prior to vacuum packaging may be beneficial to allow a desirable colour to be induced while allowing ageing to occur within the package and increase meat tenderness.

Therefore the objective of this study was to determine the pretreatment exposure time for 5% CO prior to vacuum packaging striploin steaks that would give an attractive red colour that would become unacceptable after 28 days display (2 °C) so as to not mask spoilage. Microbiological analysis, lipid oxidation, tenderness and cooking loss were also examined at 28 days storage to determine if the pretreatment had any effect on meat quality.

2. Materials and methods

2.1. Sample preparation and pretreatment procedure

Two *Longissimus thoracis et lumborum* (LTL) muscles (normal pH 5.41–5.57) were excised from the 10th rib to last lumbar vertebrae from one Charolais-cross (CHX) heifer (21–29 months of age) and obtained from a commercial meat producer. At 6–8 days post-mortem a total of 24 striploin steaks (2.5 cm in thickness) were cut from the two muscles and pooled. To account for any possible systematic differences between the left and right muscles and between steaks due to their position within the muscle, three steaks (one for colour and microbiological analysis, one for cooking loss and WBSF, and one for TBARs) were randomly assigned to each of eight CO exposure treatments; CO1 (1 h), CO3 (3 h), CO5 (5 h), CO7 (7 h), CO9 (9 h), CO15 (15 h) CO24 (24 h), and a control (untreated vacuum packaged steak). Three steaks assigned to the same treatment were immediately vacuum packaged together (New Diamond Vac J-V006W, Heavy Duty Automatic Vacuum Machine, Jaw Feng Machinery Co., LTD, Taiwan; vacuum pressure < 0.01 Torr held for 32 s) in a pouch (5-layer coextruded film with PA/Tie/PE/Tie/PE (OTR: <− 70 cm^3 O_2/m^2/24 h at 23 °C and 50% RH, Versatile Packaging, Ltd., Castleblayney, Co. Monaghan, Ireland) for 30 min as a reducing step to minimise the amount of oxymyoglobin prior to CO pretreatment. The pouch was then filled with the calibration-grade gas mixture of 5% CO, 60% CO_2 and 35% N_2 (Air Products and Chemicals, Inc.,), to give a large (at least 20:1) headspace to meat volume ratio. The pouches were then stored in chill rooms at 2 °C for the allocated CO exposure times. Steaks were then rapidly removed from the pouch to minimise potential O_2 exposure and rapidly individually vacuum packaged using 5-layer coextruded film with PA/Tie/PE/Tie/PE (OTR: <− 70 cm^3 O_2/m^2/24 h at 23 °C and 50% RH, Versatile Packaging, Ltd., Castleblayney, Co. Monaghan, Ireland). This whole experiment was repeated on three separate occasions using a different heifer for each replicate.

2.2. Display and storage conditions

Steaks were randomised and placed in an upright open front-display cabinet (Cronos fan-assisted cabinet, Criosbanc, Padova, Italy) at 2–2.5 °C with permanent fluorescent lighting (600 lx, 58 W deluxe cool white bulbs, temperature of 420 K, Philips, Eastern Electric, Dublin, Ireland) to simulate retail conditions. The display cabinet temperature was monitored at the meat surface on each of three shelves every 5 min using dataloggers (EasyLog-USB, Lascar Electronics Ltd., Salisbury, UK). The display cabinet had four 35 min defrost cycles each day reaching a maximum temperature of (8 °C) for 1 min. The simulated lighting was continuous throughout the display period of 28 days (2–2.5 °C) with an insulated blind which was pulled down throughout storage.

2.3. Instrumental colour analysis

Instrumental colour analysis was carried out using a HunterLab UltraScan Pro (Hunter Associates Laboratory, Inc., Reston, VA) with a viewing port of 25.54 mm and illuminant D_{65}, 10°. The specular component was excluded. Calibration was carried out using a white standard tile (L = 100) and a light trap (L = 0). The white tile was covered with the vacuum packaging film to eliminate any effect on the colour readings (AMSA, 2012). Steaks were measured within the vacuum packages and three independent measurements were taken in separate locations avoiding intramuscular fat, an average was then calculated to obtain CIELab L* (lightness), a* (redness) and b* (yellowness) values. CIELab a* and b* values were used to calculate Hue ($\tan^{-1}(b^*/a^*)$) and Chroma ($C^* = (a^{*2} + b^{*2})^{1/2}$) values (Hunter & Harold, 1987). Surface colour analysis was measured over 28 days of storage (2 °C) at 0, 7, 14, 21 and 28 days.

2.4. Determination of cooking loss

Cooking loss was determined according to the method of Shackelford et al. (1991) LTL steaks which had been displayed in the retail display cabinet for 28 days storage (2 °C) were then removed and frozen (−20 °C) until the day of analysis. Frozen samples were thawed in a circulating water bath (Model No Y-38, Grant Instruments Ltd., Cambridge, UK) set at 20 °C. Once the steaks were thawed, they were trimmed of any fat and the raw weight of each pretreated steak was recorded. Following this, steaks were placed in vacuum bags and cooked in a water bath (Model No Y-38, Grant Instruments Ltd., Cambridge, UK) set at 72 °C, until an internal temperature of 70 °C was reached for each steak. The internal temperature of the steaks were monitored using a temperature probe (Hanna Foodcare Digital thermometer, Hanna Instruments, Eden Way, Pages Industrial Park, Leighton Buzzard, Bedfordshire, LU7 8TZ, UK) which was placed in the geometric centre of each steak. Following cooking, any excess juices and moisture were removed from the steaks and the cooked weight of the steaks was recorded. The percentage cooking loss was determined according to the following equation:

$$\%\text{cook loss} = \left(\frac{X-Y}{X}\right) * 100$$

where X = raw weight of steak and Y = cooked weight of steak.

2.5. Warner Bratzler shear force (WBSF)

Warner Bratzler shear force analysis was carried out on cooked day 28 samples used for the determination of cooking loss which were cooled for 24 h at 4 °C, following the procedure by Shackelford et al.

(1991). Measurements were performed using an Instron Universal Testing Machine (Instron Model 5543 (UK) Ltd., High Wycombe, UK). Eight representative cores (1.25 cm in diameter) were obtained from each sample, parallel to the muscle fibre direction and sheared using a 500 N load cell at a crosshead speed of 5 cm/min^{-1} according to AMSA (1995) and Wheeler, Shackelford, and Koohmaraie (1996). Bluehill software was used and results were expressed in Newtons (N) using the mean of 6 cores of each sample (excluding the minimum and maximum shear force values).

2.6. Lipid oxidation (TBARS)

To confirm that the vacuum packaging was effective in maintaining a virtually anaerobic atmosphere during the entire display period Thiobarbituric acid reactive substances (TBARs) values were determined, as an index of lipid oxidation, according to the filtration method of Siu and Draper (1978) with minor modifications. Two representative (i.e. including the outer surface) samples of 2.5 g were taken from each pretreated steak after displaying for 28 days at 2 °C. Briefly, each sample was added to 10 ml of 5% (w/v) trichloroacetic acid (TCA) and 0.01 g of butylhydroxytoluene (BHT) and homogenised for 2 min using an Ultra-Turrax T25 homogeniser (Janke & Kunkel GmbH, IKA Labortechnik, Staufen, Germany) while kept on ice. The mixture was centrifuged (MSE Mistral 3000i, Sanyo, Leicestershire, UK) for 40 min at 3000 rpm at 4 °C and then filtered through Whatman No.4 filter paper. Filtrates (3 ml) and 3 ml of 0.03 M thiobarbituric acid (TBA) were added to glass screw cap tubes and heated for 1 h at 100 °C using a hot plate (Techne Dri-block DB-3D, Cambridge, UK). Following cooling, duplicate absorbance measurements (from each of the two representative samples per steak) were recorded and averaged using a spectrophotometer (Shimadzu UV-1700 Pharma Spec UV–visible spectrophotometer, Shimadzu Scientific Instruments, MD, USA) at 532 nm against a blank containing all reagents except samples. TBARs values were calculated using a standard curve (0.39–12.5 μm) of 1,1,3,3-tetramethoxypropane (TMP) and expressed as mg malonaldehyde (MDA)/kg sample and this was repeated for each of the three replicates.

2.7. Microbiological analysis

Sterile carcass swabs (Technical Service Consultants Ltd., Lancashire, UK) were used to swab the surface area of the LTL steaks at 28 days storage (2 °C). The surface area swabbed was the entire cut surface area of each pretreated steak, measured using graph paper prior to surface swabbing. Carcass swabs were then placed in a stomacher bag containing 40 ml Maximum Recovery Diluent (Oxoid LTD, England, CM0733) (MRD) and homogenised using a Colworth Stomacher (Model BA6024, Seward, London, UK) at a speed of 250 cycles/min for 2 min to obtain the initial suspension. Following this, serial dilutions were prepared by aspirating 1 ml aliquot of the initial suspension and dispensing into 9 ml MRD to obtain 1:10 (10^{-1}) dilution, vortexed and serial diluted to achieve appropriate dilutions. Aliquots of 100 μl of appropriate sample dilutions were spread plated in duplicate on the following agar to enumerate total viable counts (TVC) for aerobic mesophiles, anaerobic mesophiles, aerobic psychrotrophiles, anaerobic psychrotrophiles and *Pseudomonas spp*. For Lactic acid bacteria (LAB) and total *Enterobacteriaceae* counts (TEC), aliquots of 1 ml of appropriate sample dilutions were pour plated in duplicate on the following agar plates:

The TVC for aerobic mesophiles and anaerobic mesophiles were enumerated following ISO 4833 (2003) using Standard Plate Count Agar (SPCA, Oxoid Ltd., England, CM0463). Anaerobic mesophiles were placed into anaerobic jars to create a microaerobic atmosphere and both aerobic mesophiles and anaerobic mesophiles were incubated (30 °C, 72 h). For aerobic psychrotrophiles and anaerobic psychrotrophiles, TVC were enumerated following (ISO 4833, 2003; ISO 17401, 2001) using Standard Plate Count Agar (SPCA, Oxoid Ltd., England, CM0463). Anaerobic psychrophiles were placed into anaerobic jars to create a microaerobic environment and both aerobic psychrophiles and anaerobic psychrophiles were incubated (6.5 °C, 10 d). Lactic acid bacteria (LAB) were obtained following ISO 15214 (1998) on de Man Rogosa Sharpe agar (MRS, Oxoid, Ltd., England, CM0361) and incubated at (30 °C, 72 h). Total *Enterobacteriaceae* counts (TEC) were determined following ISO 21528-2 (2004) on Violet Red Bile Glucose Agar (VRBGA, Oxoid Ltd., England, CM0485) and incubated at (37 °C, 24 h). *Pseudomonads* were enumerated following ISO 13720 (2010) on *Pseudomonas* Agar Base (CFC, Oxoid Ltd., England, CM0559) and incubated (30 °C, 72 h). Results were expressed as the log of colony forming units (CFU) per cm^2 of the surface area of the steak ($\log_{10}$ cfu/cm^2).

2.8. Statistical analysis

The data were analysed using two separate forms of analyses using GenStat (Release 14.1 Copyright 2011, Lawes Agricultural Trust, Rothamsted Experimental Station, Hertfordshire, UK). Repeated measures ANOVA (rANOVA) was used to determine if the CO exposure pretreatment (treat) and storage duration (day) had any effect on colour variables (L*, a*, b*, Hue, C*) measured on one steak per CO exposure treatment at each of the display times (repeated measure) for each of three replicates. Where a significant difference was detected, post hoc tests for means comparisons using F-protected LSD was used and significance was defined as ($P < 0.05$). One-way ANOVA with Tukey's multiple comparisons test was used to determine if the pretreatment had any effect on WBSF measurements, cooking-loss, lipid oxidation and microbiological analysis. Significance was defined at ($P < 0.05$).

3. Results and discussion

3.1. Instrumental surface colour analysis

All CO exposure times increased the surface redness of LTL steaks at day 0 compared to the untreated control ($P < 0.001$) with the effect increasing with exposure time (Table 1) as expected from preliminary work (O'Connor & Allen, 2011) and previous studies investigating 5% CO-pretreatments (Aspé et al., 2008; Jayasingh et al., 2001). The effect of varying exposure to CO pretreatment compared to the control (untreated vacuum packaged) on lightness (L*), redness (a*), yellowness (b*), Hue and Chroma (C*) mean values over 28 days storage are presented in Table 1. There were CO pretreatment exposure time (treat) × display day (day) interactions for a*, b*, Hue and Chroma values ($P < 0.001$) but not for L* values ($P > 0.05$). A display day effect occurred with L* values ($P < 0.05$) increasing with display time, which is consistent with the myoglobin concentration decreasing with time (MacDougall, 1982). Hue values indicate meat browning and discoloration. Hue values significantly increased over storage ($P < 0.05$) and higher hue values were associated with reduced exposure times to CO pretreatment. Yellowness (b*), redness (a*) and chroma values decreased over storage due to discoloration ($P < 0.05$) and higher values were related to increased exposure time to CO pretreatment.

Colour stability and deterioration was measured primarily through a* and chroma values and as an index of colour discoloration. Redness (a*) on the meat surface is taken by consumers as an indicator of freshness. An exposure time × display day interaction was observed for a* values ($P < 0.05$) (Table 1). Jayasingh et al. (2001) observed colour stability for 5 weeks storage (2 °C) in beef steaks using the same CO pretreatment (5% CO, 60% CO_2 & 35% N_2) for 24 h prior to vacuum packaging. Similarly, Aspé et al. (2008) applied CO pretreatment (5% CO & 95 N_2) for 24 h followed by vacuum packaging and obtained a colour stability of 11 weeks and a microbial shelf-life of 7 weeks before exceeding microbial spoilage.

Surface redness (a*) values decreased over storage ($P < 0.05$) for all CO exposure times but not for the control. The threshold which was used to detect discoloration from the instrumental colour analysis was

Table 1
Summary of colour analysis (L*, a*, b*, Hue & C*) of CO pretreated steaks and effect of pretreatment exposure time (Treat), storage (Day) and their interaction (Day × Treat).

Dependent variable	Display (days)	Independent variable (CO pretreatment)								Pooled S.E.M.	*P* value		
		Control	CO1	CO3	CO5	CO7	CO9	CO15	CO24		Treat	Day	Day × Treat
L*	0	39.2^{A}	41.0^{A}	41.9^{A}	40.9^{A}	41.3^{A}	41.0^{A}	41.2^{A}	43.5^{B}	1.7	0.996	0.017	0.752
	7	41.3^{B}	41.5^{AB}	41.6^{A}	41.9^{B}	42.3^{B}	41.9^{B}	41.7^{AB}	42.7^{A}				
	14	41.3^{AB}	41.4^{A}	40.7^{A}	42.2^{AB}	41.8^{AB}	41.8^{AB}	42.1^{B}	43.3^{AB}				
	21	41.6^{B}	41.8^{B}	41.0^{A}	42.0^{B}	42.1^{B}	41.8^{B}	42.0^{B}	42.2^{A}				
	28	42.0^{B}	42.0^{B}	42.0^{A}	43.0^{B}	42.4^{B}	41.6^{B}	42.4^{B}	43.3^{A}				
a*	0	9.4^{Aa}	15.4^{Cb}	17.4^{Cc}	19.7^{Cd}	21.7^{Def}	20.9^{De}	22.7^{Df}	23.9^{Dg}	0.4	<0.001	<0.001	<0.001
	7	8.7^{Aa}	10.8^{Bb}	12.3^{Bc}	13.7^{Bd}	15.5^{Ce}	16.1^{Cf}	18.1^{Cg}	19.6^{Ch}				
	14	8.7^{Aa}	9.9^{Aab}	10.9^{Ab}	12.5^{Ac}	13.9^{Bd}	14.2^{Bd}	16.6^{Be}	17.6^{Be}				
	21	8.8^{Aa}	9.7^{Aa}	11.5^{Ab}	11.7^{Abc}	12.8^{Ac}	12.8^{Ac}	14.8^{Ad}	15.7^{Ad}				
	28	9.4^{Aa}	9.6^{Aa}	11.0^{Ab}	12.2^{Ac}	12.9^{Ac}	12.9^{Ac}	14.3^{Ad}	15.6^{Ae}				
b*	0	8.5^{Aa}	10.2^{Bab}	10.7^{Bb}	11.9^{Ab}	12.2^{Bb}	12.0^{Bb}	12.4^{Bb}	13.4^{Bb}	0.7	0.167	<0.001	<0.001
	7	8.6^{Aa}	9.4^{Aab}	9.7^{Aab}	10.5^{Bb}	10.7^{Ab}	10.9^{Ab}	10.0^{Ab}	11.9^{Ab}				
	14	9.0^{ABa}	9.5^{Aab}	9.1^{Aa}	10.1^{Bab}	10.7^{Aab}	10.2^{Aab}	11.1^{Aab}	11.3^{Ab}				
	21	9.4^{BCa}	9.7^{Ba}	10.4^{Aa}	10.5^{Ba}	10.4^{Aa}	10.3^{Aa}	10.9^{Aa}	11.2^{Aa}				
	28	9.9^{Ca}	10.2^{Ba}	10.8^{Aa}	10.8^{Ba}	10.9^{Aa}	10.5^{Aa}	10.9^{Aa}	11.3^{Aa}				
Hue	0	42.0^{Ab}	33.2^{Aa}	31.6^{Aa}	31.1^{Aa}	29.4^{Aa}	29.9^{Aa}	28.7^{Aa}	29.3^{Aa}	1.8	<0.001	<0.001	<0.001
	7	44.3^{Bc}	40.9^{Bbc}	38.0^{Bb}	37.4^{Bb}	34.6^{Bab}	34.1^{Bab}	31.3^{Ba}	31.1^{ABa}				
	14	46.0^{Bc}	43.7^{Cbc}	39.8^{Bb}	38.9^{Bb}	37.4^{Cab}	35.7^{Bab}	33.7^{Cab}	32.7^{Ba}				
	21	46.8^{Bc}	44.6^{CDc}	42.1^{Cbc}	41.7^{Cbc}	39.1^{Cab}	38.6^{Cab}	36.3^{Da}	35.4^{Ca}				
	28	46.0^{Bc}	46.2^{Dc}	44.3^{Dbc}	41.6^{Cbc}	40.1^{Dab}	39.1^{Cab}	37.2^{Dab}	35.8^{Ca}				
C*	0	12.7^{Aa}	18.5^{Bb}	20.4^{Cc}	23.0^{Cd}	24.9^{Dd}	24.2^{Dd}	25.9^{Dde}	27.4^{De}	0.6	<0.001	<0.001	<0.001
	7	12.3^{Aa}	14.3^{Ab}	15.7^{Bbc}	17.3^{Bcd}	18.8^{Cde}	19.5^{Cef}	21.2^{Cfg}	22.9^{Cg}				
	14	12.5^{Aa}	13.8^{Aab}	14.3^{Aab}	16.0^{Ab}	17.6^{Bb}	17.5^{Bb}	19.9^{Bc}	20.9^{Bc}				
	21	13.0^{ABa}	13.8^{Aab}	15.6^{Bbc}	15.7^{Ac}	16.5^{Ac}	16.5^{Ac}	18.4^{Ad}	19.2^{Ad}				
	28	13.7^{Ba}	14.1^{Aa}	15.4^{Bab}	16.3^{Abc}	16.9^{ABbc}	16.6^{ABbc}	17.9^{Acd}	19.3^{Ad}				

Different uppercase letters (A–D) in each column (different display days) indicate significant differences ($P < 0.05$).
Different lowercase letters (a–h) in each row (different pretreatments) indicate significant differences ($P < 0.05$).
Pooled S.E.M.: pooled standard error of means.
Treat: effect of carbon monoxide pretreatment.
Day: effect of display day.
Day × treat: interaction between CO pretreatment and display day.

a* > 12. An a* value of >12 was selected as a threshold for the limit of acceptability as an a* value of 12 is comparable to a C* value of 16 which was the limit of acceptability reported by MacDougall et al. (1986) as these authors also used a HunterLab and an illuminant D. A pretreatment exposure time of 5 h (CO5) is of particular interest in this study as a* values decreased over storage and had a mean value of 12.2 by day 28. This result indicates a CO pretreatment exposure time of 5 h is sufficient to enhance meat colour while allowing discoloration to occur by use-by date. In addition CO may not mask spoilage and continue to be used as a reliable indication of freshness. While previous researchers have investigated 5% CO pretreatment (Aspé et al., 2008; Jayasingh et al., 2001), these authors reported the colour was retained beyond the use-by date. CO pretreatment may have masked microbial spoilage as the colour stability exceeded the use-by date. Therefore reducing exposure time to CO pretreatment to 5 h may enhance colour stability while discoloring by use-by date as to not mask spoilage and address consumer concerns. In addition, an advantage of shorter CO pretreatment exposure times such as 5 h may be less time consuming and more efficient during meat pretreatment. However visual sensory evaluation would be necessary to confirm CO5 as the most appropriate treatment.

Chroma values followed a similar trend to a* values. Chroma determines the colour intensity and higher values represent a more vivid colour. Chroma values >16 are considered the limit of acceptability as these authors also used a HunterLab and an illuminant D (MacDougall, Down & Taylor, 1986). A meat surface that has been affected by 20% of metmyoglobin can affect the purchase decisions of consumers and discrimination may occur (MacDougall, 1982). Meat with metmyoglobin levels above 40% can lead to purchase rejection at point of sale (Greene, Hsin & Zipser, 1971). Mean chroma values on day 0 ranged from 18.5 to 27.4 for CO1–CO24, respectively while the control had a chroma value of 12.7. Chroma values decreased over storage as expected corresponding to a* values (28 days). The mean chroma values for CO pretreatments on day 28 ranged from 14.1 to 19.3 for CO1–CO24, respectively. The mean chroma value for CO5 was 16.3 on day 28 which was just above the limit of acceptability according to MacDougall et al. (1986) in agreement with the a* value being just above the acceptable limit of 12 at this time. There was a slight increase in a* and chroma values evident on day 28 compared to day 21 for all treatments including the control. Even though this was not statistically significant ($P > 0.05$) it merits consideration due to the consistency over all treatments. Although packaging conditions were anaerobic and packaging material had low permeability to oxygen, a probable cause for this slight increase could be attributed to oxidation or reoxygenation. This may have occurred due to the absence of a CO ligand over storage with respect to the decrease in a* and chroma values. Thus oxygen may have occupied the 6th ligand of myoglobin leading to reoxygenation and possibly oxidation (Hunt et al., 2004). Further research is necessary to gain a clearer understanding of the myoglobin redox state and the mechanism of action.

3.2. Warner Bratzler shear force (WBSF)

Tenderness is the most important palatability quality trait affecting the overall eating experience (Grobbel, Dikeman, Hunt & Milliken, 2008b). Tenderness can also affect consumer perception of meat quality and value (Holman, Fowler & Hopkins, 2016) as well as the likelihood to re-purchase (Hur, Jin, Park, Jung & Lyu, 2013). Therefore, WBSF is widely used as an instrumental proxy for sensory measurements to determine meat tenderness (Holman et al., 2016). There was no significant difference ($P > 0.05$) in WBSF values from varying the CO pretreatment exposure times compared to the untreated vacuum packaged control (Table 2.). WBSF values ranged from 26.6 N to 35.4 N for CO1–CO24 in comparison to the control which had a WBSF value of 35.5 N. Three treatments (CO1, CO3, CO15) had means which are considered 'very tender' (WBSF < 31.4 N) while all other treatments including the control had 'tender' (31.4 N < WBSF < 38.2 N) values (Belew, Brooks,

Table 2
Effect of 5% CO pretreatment exposure time on the WBSF, cooking loss and lipid oxidation of beef steaks after 28 days storage (2 °C).

Dependent variable	Independent variable (CO pretreatment)								S.E.M.	*P* value
	Control	CO1	CO3	CO5	CO7	CO9	CO15	CO24		
Warner Bratzler (N)	35.5	26.6	27.3	33.5	34.0	35.4	27.0	31.8	5.4	0.821
Cooking loss (%)	28.4	29.0	32.4	30.4	29.7	31.2	30.4	31.6	1.4	0.537
TBARs mg MDA/kg	0.14	0.08	0.10	0.11	0.08	0.11	0.12	0.12	0.04	0.916

NS: Not significant ($P < 0.05$).
Pooled S.E.M.: Pooled standard error of means.

McKenna & Savell, 2003). The low WBSF values are attributed to the 34–36 days vacuum 'wet' ageing period the samples experienced (6–8 days primal vacuum ageing postmortem before CO pretreatment, followed by 28 vacuum packed display period), as vacuum packaging is widely reported to increase tenderness. Anaerobic environments decrease protein oxidation and promote proteolysis (Clausen, Jakobsen, Ertbjerg & Madsen, 2009; Lund, Lametsch, Hviid, Jensen & Skibsted, 2007). The results also demonstrate that while CO pretreatments did not increase meat tenderness when compared to the control ($P > 0.05$), there was no negative effect on meat tenderness due to CO pretreatment. Previous studies have reported the benefits of CO-MAP increasing meat tenderness when compared to high oxygen MAP due to the reduction of protein oxidation preventing the formation of intermolecular cross linkages in myosin and the promotion of proteolytic enzymes (Cornforth & Hunt, 2008; Grobbel et al., 2008b). In contrast, this was not evident in this present study and the researchers speculate that all treatments including the control had low WBSF values because the relatively long ageing time was in an oxygen deficit environment in all cases. In addition, it should be noted that the standard method used to measure tenderness was limited as tenderness was only measured in the centre of the steak and did not measure any possible surface effect of CO pretreatment on tenderness. Nevertheless, since CO pretreated samples did not have significantly lower WBSF values than the control we conclude that CO treatment per se did not have any effect on tenderness.

3.3. Percentage cooking loss

The results of percentage cooking loss as an index of moisture retention are presented in Table 2. When heat is applied during the cooking processes, myofibrillar proteins and collagen denature forming pores and gaps within the meat matrix and as a result increased moisture loss occurs (Sørheim, Ofstad & Lea, 2004). Mean cooking loss values ranged from 29.0 to 32.4% for CO pretreatments and 28.4% for the control (Table 2.). No significant differences were evident from varying the exposure time to CO pretreatment or compared to the untreated control for cooking loss values ($P > 0.05$) (Table 2). Similarly, Wicklund et al. (2006) reported CO-MAP had no significant effect on cook loss values of pork chops enhanced with phosphates, when compared to high oxygen MAP. Likewise, Stetzer et al. (2007) found no differences in cooking loss between CO-MAP and high-oxygen MAP after 14 days storage for beef steaks enhanced with phosphates, salt and natural flavourings. In contrast, Grobbel et al. (2008a) reported beef steaks packaged in high oxygen MAP had lower cooking loss values than CO-MAP, while vacuum packaged steaks were intermediate and cooking loss values did not differ compared to CO-MAP and high oxygen MAP. However, it should be noted that the differences reported by these authors may also have been influenced by the inclusion of enhancement solutions within their studies. Any effect observed may not be an effect of CO but may be an interaction with CO and enhancement solutions. Nevertheless, the evidence from this present study indicates CO had no negative effect on meat quality as an increased cooking loss was not observed.

3.4. Lipid oxidation (TBARS)

Meat is susceptible to lipid oxidation due to the reaction of oxygen with unsaturated fats to form lipid peroxides and as a result off-flavours, rancidity and surface discoloration occur. TBARs is used as an index of lipid oxidation. CO pretreatment for different exposure times had no effect ($P > 0.05$) on lipid oxidation when compared to the untreated vacuum packaged control (Table 2.). This result is in agreement with Aspé et al. (2008) who also reported no significant difference in 5% CO pretreated vacuum packaged beef steaks compared to the control. The mean TBARs values ranged from (0.08–0.12 MDA/kg) for CO1–CO24 and 0.14 MDA/kg for the control (Table 2.). TBARs values for all treatments were below 1 mg/kg which is considered the threshold for detection of rancidity by a sensory panel (Jayasingh, Cornforth, Brennand, Carpenter & Whittier, 2002; Tarladgis, Watts, Younathan & Dugan, 1960). Low TBARs values were expected due to the anaerobic packaging storage conditions (vacuum packaging) which can retard lipid oxidation as oxygen is widely reported to promote lipid oxidation.

CO has been reported to have antioxidant properties and to be an enzyme inactivactor, thus delaying lipid oxidation (Besser & Kramer, 1972; Silliker & Wolfe, 1980). It has been reported that CO-MAP can reduce lipid oxidation when compared to high oxygen MAP (John et al., 2004, 2005; Luno, Beltran & Roncales, 1998; Luno, Roncales, Djenane & Beltran, 2000; Martinez, Djenane, Cilla, Beltran & Roncales, 2005; Sørheim, Nissen & Nesbakken, 1999; Wadhwani, Cornforth, Murdia & Whittier, 2011). While there was no effect of a 5% CO pretreatment on lipid oxidation inhibition observed in this study compared to a VP control, Lyu, Shen, Ding, and Ma (2016) reported that a 100% CO pretreatment for 1.5 h prior to vacuum packaging significantly reduced TBARs values compared to the VP control. Based on previous research and the results of this study it may be that relatively high CO pretreatment gas concentrations are necessary to inhibit lipid oxidation. Nevertheless, 5% CO pretreatments had no negative influence on increasing lipid oxidation or meat quality.

3.5. Microbiological analysis

Mean TVC for anaerobic and aerobic mesophilic and psychrotrophic bacteria, Lactic acid bacteria (LAB) counts, *Pseudomonas* and total *Enterobacteriaceae* counts (TEC) are shown Table 3. The addition of varying exposure time to CO pretreatment time had no effect on microbial shelf-life compared to the untreated VP control ($P > 0.05$). All microbial counts enumerated did not exceed the upper microbiological limit to be considered "spoiled". Previous researchers have reported that the limit of acceptability at which meat spoilage can occur is 7–8 log CFU/-cm^2 (Ayres, 1960; James & James, 2000; Lavieri & Williams, 2014). Conversely, for *Enterobacteriaceae* counts (TEC) the upper microbiological limit which is considered unsatisfactory is 4.0 log CFU/cm^2 (FSAI, 2014) which all CO pretreated steaks were below (<3.5 log CFU/cm^2) in comparison to the untreated control which was 4.5 log cfu/cm^2.

Previously, CO pretreatments have been reported to inhibit microbial growth at gas concentration above 5% (>5% CO) (Concollato et al. 2015; Gee & Duane Brown, 1981). However, microbial inhibition is proportional to the concentration of CO used. Clark, Lentz, and Roth (1976)

Table 3
Effect of 5% CO pretreatment exposure time on the microbiological safety of beef steaks at 28 days storage (2 °C).

Microorganism (log cfu/cm^2)	CO pretreatment								S.E.M.	*P* value
	Control	CO1	CO3	CO5	CO7	CO9	CO15	CO24		
Aerobic mesophiles	6.4	6.1	6.2	5.9	5.9	6.1	6.2	6.3	0.64	1.000
Anaerobic mesophiles	6.0	6.3	6.0	6.0	5.9	6.0	6.4	6.2	0.62	0.999
Aerobic psychrophiles	7.7	7.9	7.8	7.9	7.4	7.6	7.8	7.8	0.28	0.827
Anaerobic psychrophiles	7.6	7.9	7.9	7.8	7.3	7.6	7.8	7.8	0.27	0.739
Lactic acid bacteria	6.0	6.0	5.8	5.6	5.7	6.0	6.1	5.9	0.50	0.998
Enterobacteriaceae	4.5	3.5	3.2	3.3	3.1	3.1	3.1	2.9	0.74	0.832
Pseudomonas spp.	5.2	4.2	4.3	3.1	3.5	3.8	3.7	3.2	0.60	0.280

NS: not significant ($P < 0.05$).
Pooled S.E.M.: pooled standard error of means.

showed increased CO-pretreatment concentrations (balance gas being N_2) inhibited the growth of psychotropic bacteria on beef rump steaks due to CO having the ability to increase the lag phase and reduce the log phase. Additionally, Brewer, Wu, Field & Ray (1994) showed CO had a bacteriostatic effect in beef steaks pretreated with 100% CO for 30 min prior to vacuum packaging in comparison to untreated vacuum packaged steaks. However, the results in this present study agree with previous studies which have shown that low concentrations of CO have no effect on the meat micro flora (Clark et al. 1976; Gee & Brown, 1978; Sørheim et al., 1999). Aspé et al. (2008) showed that 5% CO pretreatment for 24 h prior to vacuum packaging had no microbiological effect when compared to an untreated VP control ($P > 0.05$), in agreement with the results of this study. Since a detailed microbiological analysis was not performed throughout storage and only analysed at the end of storage (28 days display) it is difficult to determine the exact micro flora growth rates or phases which occurred. However, the initial bacterial counts of an untreated steak were estimated on day 0 to confirm that the microflora and were of an acceptable level and were below (2.0 log CFU/cm^2) (data not shown). In summary, while there was no treatment effect ($P > 0.05$), the results show that the optimum exposure time of 5 h (CO5) would not mask meat spoilage at the end of shelf-life (28 days) as the colour reached an unacceptable level. Therefore colour could be used as a reliable indication of freshness. In addition, 5% CO pretreatment had no negative effect on the microbiological safety of LTL steaks ($P > 0.05$) at the end of shelf-life (28 days).

4. Conclusion

In summary, all the CO pretreatment exposure times improved the colour stability of beef steaks without any adverse effects on the microbiological status after 28 days storage. The CO5 pretreatment appears to be the most appropriate as the discoloration reached an unacceptable level by the use-by date of 28 days. Thus ensuring the consumer of a reliable visual indication of freshness and addressing previous concerns about consumer safety as CO did not mask spoilage. Exposure time to CO pretreatment was greatly reduced compared to previous studies, which applied a 5% CO pretreatment for 24 h, thus reducing process time (Jayasingh et al., 2001, Aspé et al., 2008). In addition, this anoxic packaging technology should prevent any negative quality issues related to high oxygen MAP packaging coupled with allowing ageing to occur within the package. There was no negative effect on quality attributes including lipid oxidation, cooking loss, tenderness and microbiological safety. Therefore applying 5% CO pretreatments may be a potential innovative solution to current packaging issues within the meat sector adding value, safety and enhancing meat quality while facilitating exports to distant markets.

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