THE EFFECT OF CARBON MONOXIDE ON THE COLOUR STABILITY AND QUALITY OF YELLOWFIN TUNA (*THUNNUS ALBACARES*) MUSCLE

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DECLARATION

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The mediocre mentor tells. The good mentor explains. The superior mentor demonstrates. The great mentor inspires, encourages and takes you into the trenches - Navtaj Chandhoke

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SUMMARY

Processors face the problem of extending the shelf-life of yellowfin tuna, while still maintaining the desirable bright red colour. Methods which have commonly been applied to meats and fish for shelf-life extension, such as ultra-low temperature freezing and vacuum packaging, have proved ineffective for tuna as these methods result in undesirable colour changes. Another method is the use of a carbon monoxide (CO) treatment, which results in tuna muscle with a desirable cherry-red colour that is stable during freezing and vacuum packaging. It is generally used in conjunction with freezing and vacuum packaging and can be used as a single gas (100% CO) or at varying concentrations in a mixture of gases. Other benefits of the use of CO include the potential inhibition of protein and lipid oxidation which would result in shelf-life extension. Its use with tuna has been criticised as it could mask spoilage indicators such as discolouration which could be misleading to consumers.

Two pilot studies established that the tuna would be treated (+CO) for 150 min at 3 bar pressure to attain the desired surface colour development and colour penetration. Untreated samples were used as a control (-CO). In accordance to industry practices, the tuna was also subjected to both aerobic (overwrap) (OP) and anaerobic (OI) conditions and either one (Fx1) or two (Fx2) freeze/thaw cycles.

It was found that the CO treatment did enhance, maintain and stabilise the surface colour of the tuna muscle during freezing and thawing. The carboxymyoglobin of the OP samples, however, rapidly oxidised to metmyoglobin, resulting in an undesirable brown discolouration. The OI samples maintained the colour throughout the shelf-life trial. The enhanced damage caused by the second freeze/thaw cycle was not apparent in the OP +CO treatments but the effect was seen in the OI +CO treatments.

The CO treatment had no effect on either the lipid or protein oxidation. The number of freeze/thaw cycles also had no effect on the lipid oxidation but accelerated the protein oxidation to such an extent that the carbonyls being measured had reacted with other biological constituents and could no longer be detected. The packaging had an effect on both the protein and lipid oxidation with less lipid oxidation and retarded protein oxidation being observed in the OI treatments.

A correlation was observed between myoglobin oxidation and protein oxidation in the tuna muscle with all the treatments. In the OI +CO samples, however, the a* values remained high even as the b* values and TBARS values increased. Thus the CO treatment of the tuna masked the visible indicator (browning) of lipid oxidation.

It was concluded that overall the OI +CO Fx1 treatment resulted in the best quality product with regards to colour stability, colour maintenance, and lipid and protein oxidation. The results from this study reiterated the concerns regarding the use of CO with tuna as it can mask visible spoilage indicators which raise food safety concerns.

OPSOMMING

Prosesseerders staar die probleem om geelvintuna se raklewe te verleng en terselfdertyd die helder rooi kleur van die vleis te behou, in die gesig. Verskeie aanvaarde metodes, bv. die vries en vakuumverpakking van vleis teen ultra-lae temperature, wat gedurende die behandeling van ander soorte vleis en vis die gewenste uitwerking het, het nie die gewenste uitwerking op tuna nie. Beide laasgenoemde metodes veroorsaak ongewenste kleurverandering van die vleis. ‘n Alternatiewe metode is die gebruik van koolstofmonoksied (CO) behandeling wat tunaspier met 'n wenslike kersie-rooi kleur wat stabiel tydens bevriesing en vakuumverpakking is tot gevolg het. Dit word tipies in samewerking met bevriesing en vakuumverpakking gebruik en kan as 'n enkele gas (100% CO) of as deel van 'n mengsel van gasse by wisselende konsentrasies toegedien word. Ander voordele met die gebruik van CO behandeling sluit die potensiële inhibering van proteïen en lipied oksidasie in wat kan lei tot die verlenging van rakleeftyd. Die gebruik van CO met tuna word egter gekritiseer aangesien dit bederf aanwysers, soos verkleuring, kan verbloem wat misleidend vir verbruikers kan wees.

Twee loodstudies het gewys dat tuna vir 150 min teen 3 bar druk behandel moet word (+CO) om die gewenste ontwikkeling van oppervlak kleur en kleur penetrasie te bekom. As kontrole medium was onbehandelde toetsmonsters gebruik (-CO). In ooreenstemming met industrie standaarde was die tuna aan aerobiese (toegedraai) (OP) sowel as anaerobiese (OI) toestande teen óf een (Fx1) óf twee (Fx2) vries/ontdooi siklusse blootgestel.

Daar was gevind dat CO behandeling die oppervlakkleur van die tuna spiere gedurende die vries sowel as ontdooi siklusse bevorder, gehandhaaf en gestabiliseer het. Die karboksimioglobien van die OP monsters het egter vinnig tot metmioglobien geoksideer en ‘n ongewenste bruin verkleuring tot gevolg gehad. Die OI monsters daarenteen het hul kleur gedurende die duur van die raklewe toets behou. Die verhoogde skade wat deur die tweede vries/ontdooi siklus teweeggebring was, was nie kennelik sigbaar in die OP +CO behandelings nie, maar die effek was tydens die OI +CO behandelings waargeneem.

Die CO behandelings het op nóg die lipied nóg die proteïen oksidasie ‘n uitwerking gehad. Die aantal vries/ontdooi siklusse het ook geen effek op die lipied oksidasie gehad nie, maar het die proteïen oksidasie tot so ‘n mate versnel dat die karboniele wat gemeet was gereageer het met ander biologiese komponente en nie verder waargeneem kon word nie. Die verpakking het op beide die proteïen sowel as lipied oksidasie ‘n effek gehad, maar ‘n verlaagde lipied oksidasie en gestremde proteïen oksidasie is waargeneem tydens OI behandelings.

‘n Korrelasie tussen mioglobien oksidasie en proteïen oksidasie was in die tuna spiere gedurende al die behandelings waargeneem. In die OI +CO monsters het die *a waardes egter hoog gebly selfs terwyl die b* sowel as TBARS waardes gestyg het. Die CO behandeling het dus die sigbare aanwyser (verbruining) van lipied oksidasie verskans.

Daar was tot die gevolgtrekking gekom dat die algehele OI +CO Fx1 behandelings tot die beste produk ten opsigte van kleurstabiliteit en -handhawing sowel as lipied en proteïen oksidasie gelei het. Daar was bevind dat die resultate van dié studie die besorgdheid met die gebruik van CO op tuna beaam het, deurdat dit die sigbare aanwysers van bederf en onderliggende veiligheidskwessies kan verdoesel.

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CHAPTER 1

INTRODUCTION

Yellowfin tuna (*Thannus albacares*) is often referred to or marketed as *ahi* tuna. This species is a member of the scombroid family and occurs in pelagic, warm temperate and tropical oceanic waters around the world (Filippone, 2007). They are seasonally migratory, schooling fish that can grow to 239 cm in length and weigh up to 200 kg (Luna & Kesner-Reyes, 2012). They are sold both fresh and canned and are popular for use in sushi due to their desirable bright red colour and flavour (Filippone, 2007). As a result of over-fishing, this species it is currently listed as "*Lower Risk/near threatened"* (LR/nt) on the International Union for Conservation of Nature (IUCN) red list of threatened species (IUCN, 2012), with 4 359 372 tons of tuna being harvested worldwide in 2008, of which 1 160 872 tons was yellowfin tuna (FAO, 2012).

It has been shown that consumer preference, with regard to tuna and most other meat products, is mainly determined by colour (Garner, 2004; Mancini & Hunt, 2005). Consumers prefer tuna muscle that is bright red in colour, rather than brown. Besides being more aesthetically pleasing, the former is associated with tuna which is fresh and the latter with older, poorer quality tuna (Kropf, 1980; Livingston & Brown, 1981). The market value of yellowfin tuna is thus based on its colour, with fresh, bright red tuna having the highest market value (Otwell, 2006). This association was confirmed by Carpenter *et al.* (2001) who showed that there was a strong correlation between colour and purchase intent of the consumer. The main problem faced by most tuna distributers is maintaining the bright red colour during processing, transportation, frozen storage and display (Kristinsson *et al.,* 2008). The reason for this is that tuna muscle readily discolours from bright red to brown, especially when stored under chilled or frozen conditions even for short time periods, resulting in a loss of market value (Kropf, 1980; Chow *et al*., 1988; Chow *et al.,* 1989). In an attempt to maintain the market value, tuna can be sold as "fresh" for up to 3 weeks after being harvested due to the vast distances between where the tuna is caught and its end destination (Kristinsson *et al.,* 2008). Thus some of the tuna that is frozen directly after being harvested, can be of better quality than some of the "fresh" tuna available (Olson, 2006).

One way of maintaining the colour of tuna is rapidly freezing it to very low temperatures (-56°C) and storing it at these temperatures. The problem with this is that not only does the tuna rapidly discolour when thawed (accelerated oxidation of myoglobin), but it is not an economically viable process. Cost effective alternatives to prevent tuna discolouration during processing, transportation, frozen storage and display should thus be investigated (Balaban *et al.,* 2005).

One such alternative is the use of carbon monoxide (CO), where the resulting colour pigment formed is stable during freezing and thawing (Balaban *et al.,* 2005). The exposure of the muscle to CO causes a similar reaction to that of oxygen when bound to myoglobin but with the formation of a 240 times more stable, bright cherry-red pigment known as carboxymyoglobin

(Sørheim *et al*., 1997; Mancini & Hunt, 2005). The intensity of the colour and its duration depend both on the amount of CO exposure and the distribution of the myoglobin within the muscle (Otwell, 2006). Currently, vacuum packaging followed by refrigerated storage is the most effective method used for shelf-life extension of uncooked meats. Consumer acceptance of fresh, vacuum packaged tuna has however been low due to the resulting dark reddish-purple colour, known as deoxymyoglobin (Kristinsson *et al*., 2008). The undesirable colour changes, the brown, metmyoglobin, and purple, deoxymyoglobin, can be prevented by treating the tuna muscle with CO. It has also been suggested that the CO treatment of tuna may have other benefits such as decreasing the rate or onset of lipid and protein oxidation (Kristinsson *et al.,* 2006), as well as preservation of taste, texture and aroma (Yamoaka *et al.,* 1996).

The high resistance of carboxymyoglobin to autoxidation and thus discolouration, even under abusive conditions, raises concerns as the bright, cherry-red colour remains well beyond the microbial shelf-life of the tuna. Since consumers base the freshness and wholesomeness of tuna on the bright red colour (Mancini & Hunt, 2005), the use of CO on tuna could mask visual spoilage indicators such as discolouration. It could also mask other underlying safety concerns such as elevated histamine levels and pathogens which occur in thermally abused tuna (Kropf, 1980; Balaban *et al.,* 2005). For this reason its use on meat and fish is currently not legal in many countries (European Parliament and Council Directive, 1995). The United States Food and Drug Administration (FDA) has reviewed the use of CO on seafood under its generally recognised as safe (GRAS) notification program and allows the use of CO as a preservative for seafood in the USA, as long as it is frozen and correctly labelled (Hahn, 2000; Rulis, 2002).

Despite these concerns there is still a growing market demand for CO treated tuna, which has caused producers to branch out into a variety of new products and different methods of application (Kristinsson *et al.,* 2003; Otwell, 2006). The demand is mainly driven by convenience, appeal, lower cost, increase in revenue and the availability of both frozen and thawed products (Kristinsson *et al*., 2003; Anderson & Wu, 2005; Otwell, 2006).

Due to the negative connotations associated with use of CO to treat yellowfin tuna, the current study focussed not only on investigating whether, and to what extent the specific method of application that is used has an effect on the colour of the tuna, but also whether it has other advantageous quality benefits, such as a decrease in lipid and protein oxidation. The main objective of this study was to ascertain whether a 100% CO treatment of previously frozen tuna would result in an increased surface a* value (redness), to what extent it would increase the surface a* value and how stable the colour will be over time when stored under both refrigerated (4°C) and frozen (-20°C) conditions. The effect of aerobic and anaerobic conditions, as well as the effect of the number of freeze/thaw cycles was also investigated. The secondary objective was to investigate whether the CO treatment had an effect on the rate of lipid and protein oxidation of the same samples. A possible correlation between lipid oxidation and myoglobin oxidation was also investigated. It is hoped that the results obtained will improve the utilisation and market value of

yellowfin tuna, potentially of reducing the post-harvest wastage by increasing the colour stability, shelf-life and quality of frozen yellowfin tuna.

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CHAPTER 2

LITERATURE REVIEW

INTRODUCTION

Seafood, including tuna, is of major economic importance to many countries (Garner, 2004), including South Africa. As with most fish, tuna perishes rapidly and thus proper processing and storage is crucial in ensuring maximum shelf-life (Garner, 2004). Tuna muscle quality will rapidly deteriorate after it is harvested and will continue to deteriorate while being processed, during transportation, storage and retail display. The main factors affecting the quality deterioration of the tuna muscle are microorganisms, oxygen, lipid and protein oxidation (particularly oxidation of the haem proteins) and enzymes (Garner, 2004).

Common methods used with other meats and meat products to extend the shelf-life, such as freezing and vacuum packaging, have proved effective with tuna but have resulted in undesirable consequences (Kjærsgård *et al.,* 2006; Kristinsson *et al.,* 2008). Tuna readily discolours when frozen, from bright red to brown (Chow *et al.,* 1988; Chow *et al.,* 1989), and appears purple when vacuum packed (Kristinsson *et al.,* 2008). Consumers prefer the bright red colour associated with fresh tuna (Garner, 2004; Pivarnik *et al.,* 2011) and find the brown or purple colours associated with poorer quality and vacuum packed tuna undesirable (Kropf, 1980; Livingston & Brown, 1981; Mancini & Hunt, 2005). Since a strong link has been found between colour and purchase intent of consumers (Carpenter *et al.,* 2001; Otwell, 2006), it is important to maintain the colour of the tuna during processing, transportation, storage and retail display (Kristinsson *et al.,* 2008). The onus of maintaining the colour falls to the processor, with great monetary losses being incurred due to discolouration.

One solution to maintaining the colour, even under vacuum packed conditions, is the use of carbon monoxide (CO) (Balaban *et al.,* 2005). The treatment of tuna with CO results in a stable, bright cherry-red myoglobin derivative, known as carboxymyoglobin (Livingston & Brown, 1981; Mancini & Hunt, 2005). Carboxymyoglobin is stable during freezing and thawing (Kristinsson *et al.,* 2006a) and does not discolour under anaerobic conditions (vacuum packaging) (Kristinsson *et al.,* 2008). Although the use of CO would seem to be the ideal solution, it has sparked much controversy as the resulting carboxymyoglobin is highly resistant to oxidation under anaerobic conditions, with the bright, cherry-red colour remaining well after the tuna is no longer safe to consume (Olson, 2006). Thus its use on seafood is illegal or highly regulated in many countries (Otwell, 2006). It has also been suggested that the CO treatment may have a positive effect on the quality of tuna muscle by inhibiting lipid and protein oxidation by stabilising myoglobin and subsequently inhibiting its pro-oxidant effect (Kristinsson *et al.,* 2005).

COLOUR OF FISH MEAT

The colour of fish muscle can be influenced by several factors including: species; harvesting season; chemical composition; time after harvest (freshness); type of muscle; and type and quantity of haem proteins (Kristinsson *et al.,* 2006a). The factors which play the most important role are the haem proteins, haemoglobin and myoglobin. This is particularly true for the dark muscle fish species such as tuna where the colour of the muscle results from the presence of these proteins.

Haemoglobin and myoglobin are responsible for transporting oxygen through the body and muscle of living fish (Livingston & Brown, 1981). Myoglobin is found in the muscle whereas haemoglobin is found in the blood. In tuna which has been correctly exsanguinated, myoglobin will mainly be accountable for the muscle colour, as most of the haemoglobin would have been lost. The myoglobin concentration within muscles varies according to species, fibre type, activity, oxygen availability, blood circulation and age (Kristinsson *et al.,* 2006a). In the case of yellowfin tuna the concentration of myoglobin in the muscle is related to the tuna's age, physical activity and the way the meat is treated during processing (Gidding, 1974; Livingston & Brown, 1981).

Myoglobin

Myoglobin (M_r ± 18 000 $g.mol^{-1}$) is a monomeric, water soluble, globular haem protein containing 8 α-helices (no β-pleated regions). These α-helices are linked by non-helical sections. Myoglobin has a central haem ring, consisting of a porphyrin ring with a central iron atom. The iron atom can form six bonds of which four are used to link to pyrrole nitrogens' with the 5^{th} binding to a proximal histadine-93. The 6^{th} binding site is vacant and can reversibly bind ligands such as oxygen and CO. The type of ligand bound to the 6^{th} binding site and the valence of the iron atom will influence the colour of the meat (Mancini & Hunt, 2005; Campbell & Farrell, 2008). There are four major myoglobin derivatives responsible for meat colour: deoxymyoglobin; oxymyoglobin; metmyoglobin; and carboxymyoglobin (Fig. 1) (Mancini & Hunt, 2005).

Deoxymyoglobin

Deoxymyoglobin is the derivative of myoglobin where no ligand is bound to the 6^{th} vacant position on the iron atom and is thus in its ferrous form (Fe^{2+}). Meat that contains high proportions of deoxymyoglobin has a purplish-red or purplish-pink appearance usually associated with the interior of freshly cut meat or vacuum packaged meat i.e. meat under low oxygen conditions (Mancini & Hunt, 2005).

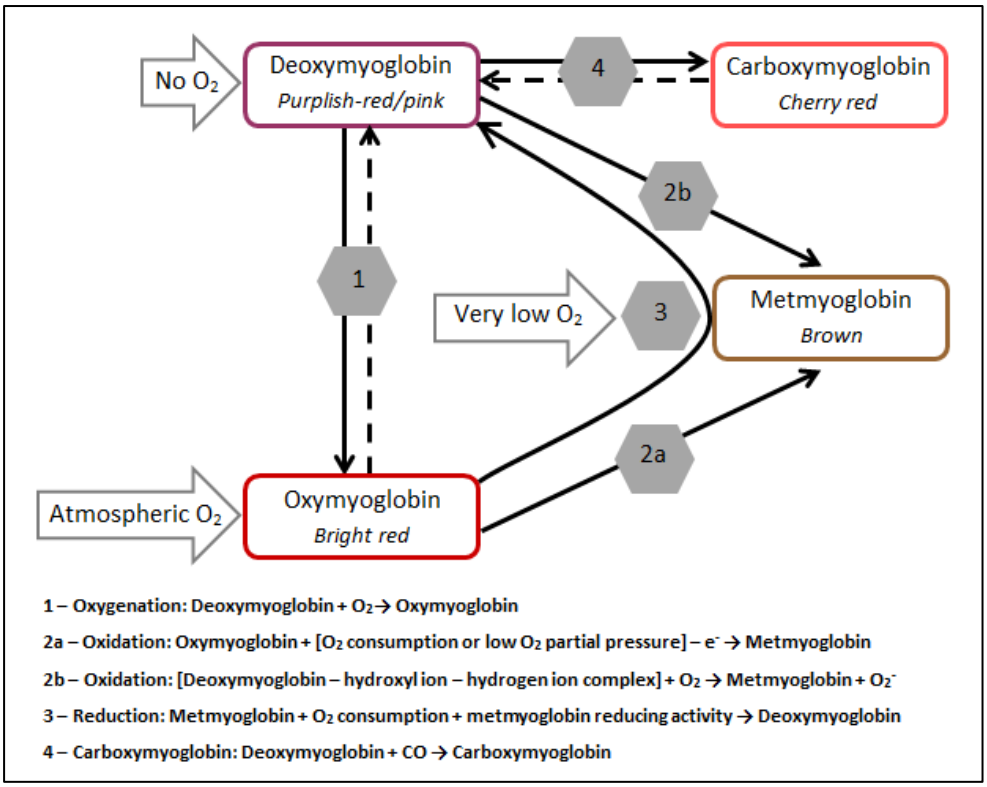

Figure 1 Myoglobin derivatives and their corresponding meat colour (adapted from Sørheim *et al.,* 1997; Mancini & Hunt, 2005).

Oxymyoglobin

Oxymyoglobin, is the diamagnetic ferrous form of myoglobin (Livingston & Brown, 1981). It is stable under high oxygen conditions characterised by the development of a bright red colour (Livingston & Brown, 1981; Mancini & Hunt, 2005). The oxygenation of myoglobin does not lead to a change in the valance of the iron atom (it remains as Fe^{2+}), there is only a diatomic oxygen bound to the 6th site of the iron atom. The distal histadine also interacts with the bound oxygen, altering the stability and structure of the myoglobin molecule (Mancini & Hunt, 2005).

As exposure to oxygen is increased, more oxymyoglobin will form deeper beneath the surface of the meat. Various factors such as temperature, oxygen partial pressure, pH and competition for oxygen by other processes play a role in the depth of oxymyoglobin penetration that will occur (Mancini & Hunt, 2005).

It is important to note that ferrous (Fe^{2+}) myoglobin is required to bind oxygen stably. Once oxidation has occurred the undesirable brown derivative of myoglobin, metmyoglobin, will irreversibly replace oxymyoglobin except under reducing conditions (Livingston & Brown, 1981).

Metmyoglobin

Metmyoglobin forms due to the oxidation of deoxymyoglobin or oxymyoglobin causing the formation of the undesirable brown colour of meat (Livingston & Brown, 1981; Mancini & Brown,

2005). Although exposure to oxygen initially results in the formation of oxymyoglobin, extended periods of exposure to oxygen eventually leads to oxidation of the ferrous iron (Fe^{2+}) to form ferric iron (Fe^{3+}) (Wallace *et al.,* 1982). The formation of metmyoglobin is influenced by numerous factors such as oxygen partial pressure, temperature, pH, reducing activity of meat and microbial growth (Mancini & Hunt, 2005).

The colour of red meat and red-fleshed fish species, such as tuna, plays an import role in the purchasing decisions of consumers as they use it as an indicator of freshness and wholesomeness (Garner, 2004; Mancini & Hunt, 2005). Consumers prefer the bright red colour of oxymyoglobin and dislike the brown colour of metmyoglobin (Mancini & Hunt, 2005). Studies have shown that approximately 60% conversion of myoglobin to metmyoglobin causes the meat product to become unacceptable to consumers (Lawrie, 2006). Since red-fleshed fish muscle reacts similarly to that of red-fleshed meat, it can be assumed that the same or a similar percentage of metmyoglobin would also cause the tuna flesh to become unacceptable to consumers. Thus the proportion of oxymyoglobin to metmyoglobin is of great importance to consumer acceptability of meat products (Lawrie, 2006).

Carboxymyoglobin

The exact mechanism which results in carboxymyoglobin is unclear. It is not known whether CO can displace oxygen from the 6^{th} binding site or whether it has a reducing ability on metmyoglobin to form a bright red colour. It has been noted that deoxymyoglobin more readily converts to carboxymyoglobin than oxy- and metmyoglobin (Mancini & Hunt, 2005). Research has shown that CO can readily bind to both oxy- and deoxymyoglobin (Lanier *et al.,* 1978). Carboxymyoglobin is more resistant to oxidation than oxymyoglobin due to the stronger binding of CO to the iron binding site (>240 times higher) (Sørheim *et al.,* 1997). Despite the stronger binding of CO to myoglobin, it is not stable and CO will dissociate from myoglobin in atmospheres free of CO (Mancini & Hunt, 2005). In the presence of oxygen the CO will slowly dissociate from the myoglobin and be converted to oxymyoglobin which in turn will be oxidised to metmyoglobin (Krause *et al.,* 2003; Anderson & Wu., 2005). This is contradictory to the fact that CO has a higher binding affinity for myoglobin compared to that of oxygen (Sørheim *et al.,* 1997). There are several possible explanations for this. The first is that myoglobin may have a predilection for oxygen rather than for CO, favouring oxygen rather than CO even though it has a higher binding affinity for the latter (Mancini & Hunt, 2005). It was further noted by Hunt *et al.* (2004) that discolouration of CO treated meat will occur under atmospheric conditions due to the loss of the CO ligand from the myoglobin. Once the CO ligand has been lost it will be followed by re-oxygenation and subsequent iron oxidation. Thus there will be a decrease in carboxymyoglobin with a concurrent increase in metmyoglobin. It was further surmised by Hunt *et al.* (2004) that carboxymyoglobin which was exposed to atmospheric oxygen, resulted in oxymyoglobin which was more liable to oxidation than

the oxymyoglobin of meat not previously exposed to CO. This accelerated oxidation could be due to longer storage times and the limited or absent reducing capacity remaining in the muscle.

Another mechanism was also proposed where metmyoglobin reduction leads to the formation of deoxymyoglobin that was less stable and more liable to autoxidation than native deoxymyoglobin that had not previously been in the ferric form (Lanier, 1978). Thus packaging CO treated meat and fish in oxygen permeable packaging (in atmospheres free of CO) will lead to a loss in colour (redness) and the formation of brown metmyoglobin over time.

Tuna myoglobin

Tuna myoglobin differs to mammalian myoglobin in that it has fewer amino acid residues and a lower molecular weight. It also contains cysteine which could influence the susceptibility of myoglobin to oxidation (Brown, 1961). The sulfhydryl group of cysteine is nucleophilic and is expected to be more reactive with lipid oxidation products than other less nucleophilic amino acids (Witting *et al.,* 2000). It is also possible that differences in the amino acid composition of the myoglobin between mammals and fish may influence the colour stability of the muscle post mortem. There has however, been very little work published regarding the oxidative stability of fish myoglobin and its relationship with lipid oxidation (Lee *et al.,* 2003).

Colour measurements

The colour of meat can be measured both subjectively and instrumentally (Honikel, 1998). In the current study only instrumental colour measurements (spectrophotometer) are of interest as colour intensity and stability are being investigated and not consumer acceptance or preference for the colour of the tuna muscle.

There are three main causes of colour variation (Honikel, 1998): concentration of myoglobin is specific to the muscle, which is dependent on primary production factors such as breed, age and nutritional status (low or high plane of nutrition); rate and extent of pH and temperature decline which is determined by the pre-slaughter period, slaughter process and subsequent processing; and the process of oxygenation and oxidation of myoglobin during storage, transportation and retail display.

The stipulations for correct colour measurement were set out by Honikel (1998). The measurement should only be taken after the final pH of the muscle has been reached post mortem. This is due to, as mentioned above, the colour being affected by the pH of the muscle. The muscle, from which the measurement is taken, should be clearly described and the location within the muscle noted. The sampling should be done in the cross-section, perpendicular to the long axis of the muscle with a minimum thickness of 1.5 cm but preferably 2 cm. At least triplicate measurements should be taken at three different points on the surface of the muscle. The

instrument used should be calibrated using a black standard with L*=0 and a white standard with L*=100 (Honikel 1998).

CARBON MONOXIDE AND MEAT COLOUR

Carbon monoxide is a colourless, odourless and tasteless gas which is slightly lighter than air (Sørheim *et al.,* 1997). It is formed by the incomplete combustion of organic materials. It is toxic, with exposure to ± 200 ppm resulting in a headache and overexposure resulting in death (Brown *et al.,* 2009).

Carbon monoxide treatment of meat and seafood

Over 100 years ago the first patent was granted for packaging meat in a carbon dioxide/carbon monoxide gas mixture for shelf-life extension (Church, 1994). Since then several patents have been granted for the use of CO on both meat and seafood (Woodruff & Silliker, 1985; Yomaoka *et al*., 1996; Kowalski, 1999). There are currently several different forms of CO treatment used including traditional wood smoking, pure CO (100% CO), CO as a mixture of gasses in modified atmospheric packaging (MAP) (usually about 4% CO), filtered wood smoke (usually about 18% CO) and tasteless smoke (7-30% CO) (Olson, 2006).

Several studies have shown that the use of CO significantly influences the red colour (a*) of muscle but does not have much of an effect on the lightness (L*) or yellowness (b*) values (Kristinsson *et al.,* 2003; Otwell *et al*., 2003; Garner, 2004; Balaban *et al.,* 2005; Mantilla *et al.,* 2008). There are several factors which impact the level of redness attained. One of these is the percentage CO used, as it influences the amount of CO available to be bound. The more CO bound, the higher the concentration of carboxymyoglobin and thus the higher the level of redness attained. Figure 2 shows the a* values obtained 48 h after yellowfin tuna was treated with varying CO concentrations. It is clear that the 100% CO treatment gave the highest a* values and the 4% CO treatment the lowest. Thus the increase in redness is directly proportional to the CO concentration in the muscle. It should be noted that there will be residual CO in the muscle, especially in the 100% CO treated muscle, which leads to extension of the colour during storage (Kristinsson *et al.,* 2006a).

Another important factor is application time. In Fig. 3 it can clearly be seen how varying application times for different CO concentrations influence the surface colour of the yellowfin tuna steaks. It has been found that lower concentrations of CO (4%) require longer exposure times than higher concentrations of CO (100%) (Ross, 2000).

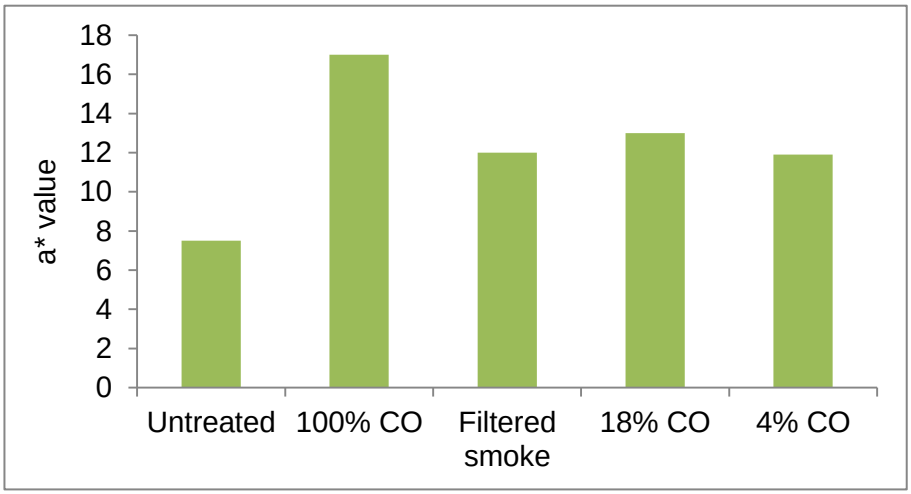

Figure 2 The increase in a* (redness) values of yellowfin tuna steaks after treatment for 48 h in different gas environments (filtered smoke treatment has an 18% CO concentration) (adapted from Kristinsson *et al*., 2006a).

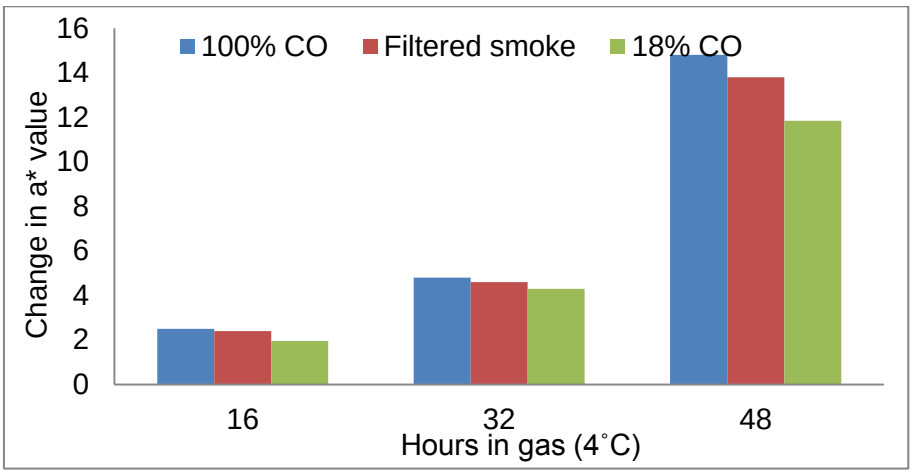

Figure 3 The influence of gas treatment time on increase in a* value of yellowfin tuna steak (filtered smoke has an 18% CO concentration) (adapted from Kristinsson *et al.,* 2006a).

Temperature also influences the redness of muscle with regard to CO treatment. Carbon monoxide has a very low solubility, which increases with a decrease in temperature. It would thus be expected that fish muscle, which consists of 60-80% water, at lower temperatures, would bind more CO, which would lead to an increase in the a* value. This, however, is not the case, and in fact the opposite has been shown to be true (Kristinsson *et al.,* 2005). Yellowfin tuna treated at varying temperatures and CO concentrations showed that the a* values are higher at 20˚C than at 4˚C (Fig. 4). This could possibly be due to the fact that, although the CO is more soluble at lower temperatures, tuna myoglobin is adapted to warmer water temperatures and will thus presumably have a higher binding affinity for CO at higher temperatures (Kristinsson *et al.,* 2006a). Higher temperatures, however, have the disadvantage of increasing the chance of protein oxidation (Kristinsson *et al.,* 2005), which would in turn retard CO binding.

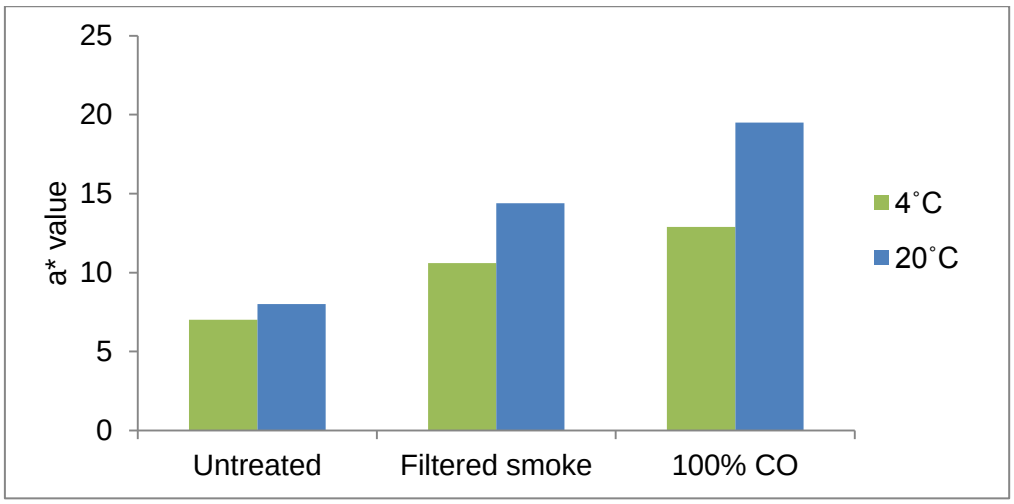

Figure 4 Influence of temperature during gas treatment with 100% and 18% (filtered smoke) CO on the a* values of yellowfin tuna steak (adapted from Kristinsson *et al.,* 2006a).

Colour stability during refrigeration, freezing and thawing of CO treated fish

Rapidly freezing tuna and then storing it at very low temperatures (-56°C) will stabilise its colour but upon thawing it will rapidly turn brown. This procedure is also costly and cost effective methods of maintaining tuna colour during freezing and thawing have thus been sought by industry. The use of CO was found to be effective in stabilising the tuna colour during freezing and thawing (Balaban *et al.,* 2005). Carbon monoxide treatment does not only increase the redness of the tuna muscle but also increases the stability of the colour during refrigerated and frozen storage, which is the main benefit of CO treatment of yellowfin tuna (Kristinsson *et al.,* 2006a). As mentioned, the main problem faced by distributers is maintaining the desirable bright red colour of tuna muscle during processing, transportation, frozen storage and display (Kristinsson *et al.,* 2008). Commercial freezing of tuna (-20°C) causes myoglobin to oxidise, resulting in a brown coloured muscle (Balaban *et al.,* 2005). The thawing process also causes accelerated browning by accelerating protein oxidation. It is also known that frozen and thawed muscles become brown quicker than unfrozen muscle during refrigeration (Chow *et al*., 1988; Chow *et al.,* 1989). Similar results have been found with various other meats and meat products (Leygonie *et al.,* 2012).

The data in Fig. 5 shows the results of yellowfin tuna steaks treated with varying concentrations of CO for 48 h and then subjected to 30 d of freezing at -30˚C, after which they were defrosted and kept at 4˚C. It is interesting to note that an initial increase in redness occurs in the treated tuna after thawing. This is due to the residual CO in the muscle binding to the remaining unbound myoglobin, resulting in increased redness. It can be seen that CO stabilises the colour of yellowfin tuna muscle during freezing and refrigerated storage (Kristinsson *et al.,* 2006a).

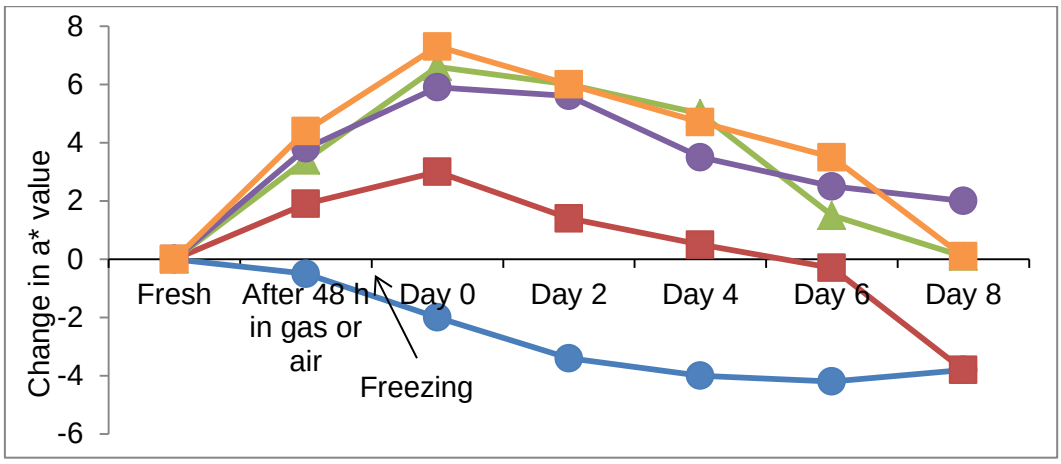

Figure 5 The effect of various CO treatments on the a* value of yellowfin tuna steaks after 48 h of exposure and 30 d of freezing – (●) untreated; (■) 4% CO; (▲) 18% CO; (●) filtered smoke (18% CO); and (■) 100% CO (adapted from Kristinsson *et al.,* 2006a).

REGULATIONS REGARDING THE USE OF CARBON MONOXIDE ON FISH

Due to the potential for CO treatment of fish to mask underlying safety problems, strict regulations are required regarding its use (Otwell, 2006). The intentional use of CO for colour retention in fish was employed prior to the existence of any regulations for this process (Otwell, 2006). The use of CO on fish is currently not legal in European Union (European Parliament and Council Directive, 1995). In South Africa the legality of the use of CO on fish is unclear as no regulations exits regarding its use on foodstuffs. It is however legal in the USA as long as it is correctly labelled (Rulis, 2002). Thus CO treated tuna can be sold in the USA but may not be legally sold within the European Union.

Since the regulatory status for the use of CO to retain the red colour of fish in commercial practices in the USA was initially unclear, clarification was sought. Initial discussions started in 1996 between the Food and Drug Administration (FDA), National Marine Fisheries Service and Hawaii's State Department of Health and others interested parties' regarding the clarification of the use of CO on tuna specifically. These discussions addressed issues such as the food additive status for CO; labelling requirements; and potential for use in adulterated of products. In 1999, the FDA issued an important bulletin (May 1999) regarding the use of tasteless smoke (TS) in the processing of tuna (FDA, 1999). The bulletin did not object to the use of CO or TS as long as the tuna is labelled as processed foods that had been treated with CO or TS; not misrepresented as fresh frozen seafood by their label; and near normal in fresh colour (FDA, 1999). It further stated that the minimum requirement for labelling, as part of the ingredients statement was "tasteless smoke (preservative to promote colour retention)", which is in compliance with the Code of Federal Regulations, Title 21 (Food and drugs), Part 101 (Food labelling), Section 22(j) (Anon., 2012).

The preliminary position of the FDA was then formally stated in the GRAS notification No. 0000015 (Oliver, 2000). This response was more specific and dealt with tuna treated with TS

which was frozen after treatment. It stated that TS was considered a preservative and as such, it required the declaration of both the common and unusual name in the ingredient list as well as a separate description of its function. A similar position was taken by the FDA regarding the use of CO on tuna with the recommended labelling as*: Tuna, Carbon Monoxide (as colour preservative)* (Olson, 2006). Recently the FDA decided that CO used as part of a mixture of gasses in MAP of meat is a "processing aid" and thus does not require product labelling declarations (Rulis, 2002; Tarantino, 2004).

The leading concern regarding CO treated fish is the masking of inferior quality products which may already have or may develop high levels of histamine. Tuna is one of the species prone to the development of high histamine levels associated with scombroid poisoning (FDA, 2001). Although it is important to address the concerns regarding the use of CO on fish products the potential safety benefits of such a process should also be evaluated. Tuna can be sold as "fresh" for up to three weeks after being harvested due to the vast distances between where the tuna is harvested and the end destination. Thus, in an attempt to market tuna as fresh, never frozen, to attain a high market value, the product quality and safety may be reduced (Olson, 2006). Since most of the tuna harvested is shipped to distant locations, freezing is the best method to use in preventing and controlling histamine levels in tuna and increasing its shelf-life. As mentioned, however, freezing and thawing of tuna results in colour loss (Chow *et al*., 1988; Chow *et al.,* 1989) and decreased market value (Kropf, 1980). The use of CO and similar processes allows for the freezing of tuna without reducing its market value as the colour is retained during freezing and thawing (Olson, 2006).

The fact remains that the use of CO can be used to mask poor quality and potentially unsafe tuna and is thus still not approved in most countries other than the USA. In Japan the use of CO on fish has been banned (Huang *et al*., 2006) and Canada does not allow the use of CO but the use of tasteless smoke is still under consideration (Prince, 1999; Andruckzk, 2000). The EU also does not allow CO as an additive in any food (European Parliament and Council Directive, 1995). As previously mentioned the legality of CO treatment of foodstuffs in South Africa is unclear as no laws exits regarding its use.

CARBON MONOXIDE TREATED MEAT CONSUMPTION AND HUMAN HEALTH

As previously discussed, CO has a more than 240 times stronger binding affinity for haem proteins (myoglobin and haemoglobin) than oxygen and can thus compete with oxygen for the haem binding site (Sørheim *et al.,* 1997). Thus the CO will competitively bind to the haem binding site by displacing oxygen. In the case where both oxygen and CO are present, the CO will displace the oxygen (Sørheim *et al.,* 1997). Although the binding of CO to haem proteins is reversible and concentration dependent, it results in a much slower dissociation from the haem proteins than oxygen. This means that CO will bind to more haem molecules and will saturate all available haem

binding sites by displacing oxygen at low concentrations and stay bound for a longer time (El-Badawi *et al.,* 1964).

Haemoglobin and myoglobin are the proteins responsible for transporting oxygen around the human body in blood and muscle, respectively (Kristinsson *et al.,* 2006a). There is thus some concern that during mastication and digestion of CO treated tuna that the released CO will be absorbed into the human blood. Davenport *et al.* (2006) showed that the consumption of CO treated tuna did result in a rapid but brief increase of exhaled CO, which is an indication of the amount of CO in human blood. The exhaled CO originated from blood absorption from the mucosal membranes of the mouth during mastication and the stomach during digestion. The amount of CO increase that is caused is still far below the blood CO safety limits and is rapidly removed from the blood by exhalation. It is thus not detrimental to human health to consume CO treated meat or tuna (Davenport *et al.*, 2006).

THE EFFECT OF CARBON MONOXIDE TREATMENT OF SEAFOOD ON MICROORGANISM GROWTH

Seafood has a very short shelf-life due to the impact of microbial and chemical processes (Kristinsson *et al.,* 2006b). There is very little known about the effect of CO on microbial growth. Studies which involved brief exposure of bacteria to 100% CO showed hardly any effect on the growth of *Staphylococcus aureus*, *Clostridium botulinum* or *Escherichia coli* (Kaffegakis *et al.,* 1969). It was however shown that CO could inhibit the growth of an aquatic *Streptomyces* (Fransisco & Silvery, 1971). Several studies have been conducted on red meats such as beef and goat using CO as a single gas or as part of a gas mixture. These studies have either shown that CO had an inhibitory effect on the microbial growth or that there was no inhibitory effect (Gee & Brown, 1978; Woodruff & Silliker, 1985; Hunt *et al*., 2004; Kristinsson *et al*., 2005). In some of the cases it was difficult to ascertain whether the CO was actually having an effect or if it was merely due to the exclusion of oxygen or the presence of CO as part of the gas mixture used (Kristinsson *et al.,* 2006b). Studies involving yellowfin tuna and other fish species showed that high CO concentrations did in fact lead to the reduction of microbial levels (Demir *et al.,* 2004; Balaban *et al.,* 2005). Again, it was not clear whether the CO had an effect on the microorganism or if the inhibition was due to the exclusion of oxygen.

In terms of histamine formation, CO treatment does not promote the formation of histamine provided the process is done correctly and followed by freezing directly after treatment (Kristinsson *et al.,* 2006a). In fact, Ross (2000) indicated that CO may retard the formation of histamine, with CO treated tuna showing lower histamine concentrations over time compared to untreated tuna.

LIPID OXIDATION

Lipid oxidation is one of the main causes of meat deterioration. It affects fatty acids, particularly polyunsaturated fatty acids (PUFAs) (Gray, 1978; Apgar & Hultin, 1982; Gordon, 2003; Munasinghe *et al.,* 2005; Kristinsson *et al*., 2006a). The products formed by lipid oxidation result in negative quality changes affecting colour, aroma, flavour, texture and nutritive value and possibly the development of toxic compounds (Eriksson, 1982; Love, 1983; Kanner, 1994). Lipid oxidation is the process by which oxygen reacts with unsaturated lipids forming lipid peroxides. It proceeds via an autocatalytic mechanism of 'free radicals' known as autoxidation involving three stages: initiation; propagation; and termination (Fig. 6) (Gray 1978; Raharjo & Sofos, 1993; Monahan, 2000).

Hydroperoxides have been identified as primary products of autoxidation. Decomposition of the hydroperoxides yield aldehydes, ketones, alcohols, hydrocarbons, volatile organic acids and epoxy compounds, known as secondary oxidation products. These compounds, together with free radicals, are used for measurement of lipid oxidation (Shahidi & Zhong, 2005). Hydroperoxides (LOOH) are considered to be the most important products produced during lipid oxidation. Hydroperoxides are highly reactive and transitory and undergo changes and deterioration with radicals causing secondary products such as malondialdehyde (MDA) (Raharjo & Sofos, 1993).

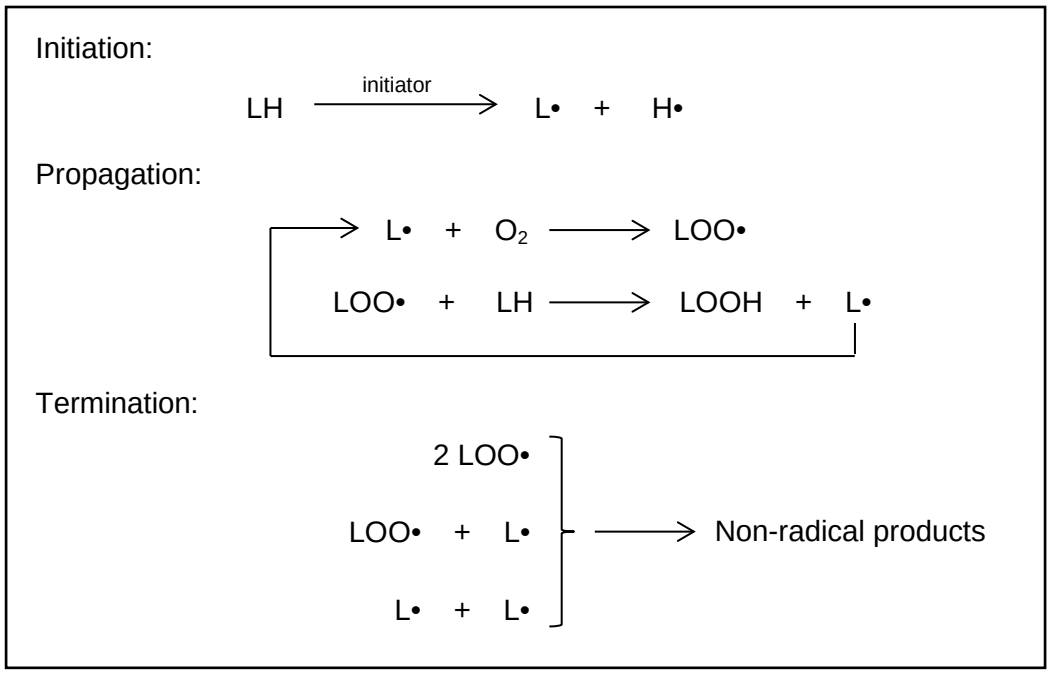

Figure 6 Mechanism of lipid oxidation (adapted from Shahidi & Zhong, 2005).

Lipid oxidation and carbon monoxide treatment

The quality deterioration of many fish species is directly related to lipid oxidation and reactions which occur from the by-products of lipid oxidation (Richards & Hultin, 2002). Lipid oxidation

results in various undesirable off-odours and flavours (Eriksson, 1982) and the by-products formed can react with proteins leading to deterioration in texture (Kristinsson *et al*., 2006b). Fish muscle is highly susceptible to lipid oxidation due to the high concentration of PUFAs. Fish muscle also contains various pro-oxidants that promote oxidation (Kristinsson *et al*., 2006a) including copper, iron and haem protein (haemoglobin and myoglobin) (Strasburg *et al.,* 2007). The haem proteins are believed to be the two main pro-oxidants in meat (Richards *et al.,* 1998; Undeland *et al.,* 2004). It can also be assumed that if exsanguination was performed correctly, only myoglobin will play a significant role in lipid oxidation (Kristinsson *et al.,* 2006a).

Myoglobin can cause lipid oxidation when oxygen is released from oxymyoglobin to form ferric metmyoglobin and super oxide anion radicals. Metmyoglobin can further oxidise to ferryl (Fe^{4+}) myoglobin which is very reactive. This oxidised form of myoglobin is thought to be the main catalyst of lipid oxidation (Richards & Hultin, 2002). Subsequently the by-products formed from lipid oxidation are implemented in haem protein oxidation, which further lead to muscle discolouration and deterioration (Faustman *et al.,* 1999). It has been found that antioxidants successfully retard lipid oxidation (Richards *et al.,* 1998) by retarding the activity of the pro-oxidants present such as the haem proteins (Richards *et al.,* 1998; Kristinsson 2002). In the case of myoglobin, when it is bound to CO to form carboxymyoglobin, it remains in the reduced state and does not readily oxidise (Kristinsson *et al.,* 2005). It is thus expected that the stabilisation of myoglobin with CO will reduce lipid oxidation. It can further be surmised that fish muscle treated with CO may be less prone to lipid oxidation and in fact several studies support this theory (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011).

Methods for determining lipid oxidation

Various analytical methods are used to determine lipid oxidation in foods. There is however no standard method for detecting all the oxidative changes in all types of food. It is thus important to select a suitable method for the specific application (Shahidi & Zhong, 2005). The current methods used to determine lipid oxidation in foods can be classified into four groups based on what is being measured: the absorption of oxygen; the loss of initial substrates; and the formation of primary (hydroperoxides) and secondary (decomposition of hydroperoxides) oxidation products (Dobarganes & Velasco, 2002; Shahidi & Zhong, 2005). Both physical and chemical tests have been employed for measurement of lipid oxidation (Dobarganes & Velasco, 2002).

One of the most commonly used methods to quantify lipid oxidation in meat products is the 2-thiobarbituric acid (TBA) test (Gray, 1978; Kishida *et al.,* 1993). It is based on the principle that the TBA reacts with the malondialdehyde (MDA) formed from lipid oxidation giving a colour reaction which can be quantified spectrophotometrically (Tarladgis *et al.,* 1960). According to Dobarganes and Velasco (2002), this method can be used for all samples but is specifically used for biological samples and fish oils. It is frequently employed to test the extent of lipid oxidation in

muscle foods even though it lacks specificity and sensitivity (Raharjo & Sofo, 1993; Shahidi & Zhong, 2005). It has also been noted that due to the interference, the TBARS method should only be used to assess the extent of lipid oxidation in general (Gray & Monahan, 1992). It should thus not be expected that the TBA test will give exact results regarding the amount of lipid oxidation which has occurred.

PROTEIN OXIDATION

Oxidation, in general, is one of the leading causes of quality deterioration in muscle foods (Xiong, 2000). The susceptibility of meat, poultry and seafood to oxidative processes is due to the relatively high concentrations of unsaturated fatty acids and oxidising agents in their muscles (Johns *et al.,* 1989). Although lipid oxidation has been extensively studied, protein oxidation has only been thoroughly investigated in recent years and as such the basic mechanisms involved are still being clarified (Lund *et al.,* 2011).

It is believed that protein oxidation proceeds via a free radical chain reaction, comparable to that of lipid oxidation although the higher complexity of the pathways leads to the production of more by-products (Lund *et al.,* 2011). Reactive oxygen species (ROS) have been found to play a role in the oxidation of proteins. In protein oxidation the reaction of radicals with proteins and peptides in the presence of oxygen causes alterations in both their backbone and their amino acid side chains (Dean *et al*., 1997; Lund *et al.,* 2011). These oxidative changes include cleavage of peptide bonds, modification of amino acid side chains and the formation of covalent intermolecular cross-linked protein derivatives. During the modification of amino acid side chains, carbonyl groups and protein hydroperoxides are formed (Lund *et al.,* 2008; Estévez *et al.,* 2009).

Although the implications of protein oxidation in quality deterioration has not yet fully been investigated, it has been found that the changes caused by ROS in muscle proteins could cause the loss of their functionality and thus, loss in quality of muscle foods (Xiong, 2000). Numerous mechanisms have been suggested for the impact that protein oxidation has on the texture of meat with regards to tenderness and juiciness (Rowe *et al.,* 2004; Huff-Lonergan & Lonergan, 2005; Lund *et al*., 2007; Kim *et al.,* 2010). Protein oxidation may also lead to changes in hydrophobicity, conformation and solubility of proteins. It may also lead to altered susceptibility of protein substrates to proteolytic enzymes (Wolff & Dean, 1986; Davies *et al.,* 1987). This altered susceptibility has been implemented as one of the major reasons for the low digestibility and consequently, lower nutritional value of oxidised proteins (Morzel *et al.,* 2006).

Protein oxidation and carbon monoxide treatment

In the same way that the binding of CO to myoglobin could retard lipid oxidation (mentioned above), it could possibly also retard protein oxidation (Kristinsson *et al.,* 2006b). However,

previous studies were inconclusive regarding whether protein oxidation of yellowfin tuna was in fact influenced by CO treatment and concluded that further research would have to be done (Demir & Kristinsson, 2005).

Methods for determining protein oxidation

There are various methods used to determine protein oxidation. Currently the most commonly measured products of protein oxidation are the carbonyls formed as by-products (Shacter, 2000). Many of the analyses involve reacting the carbonyl group with dinitrophenylhydrazine (DNPH), which leads to formation of a stable dinitrophenylhydrazone product (Levine *et al.,* 1990). Dinitrophenylhydrazone can then be quantified using various methods such as spectrophotometry, ELISA, HPLC and SDS electrophoresis (Shacter, 2000). Alternatively, specific carbonyls which have been found to be markers for protein oxidation (Daneshvar *et al*., 1997) have been used to quantify protein oxidation (Estévez, 2011). These specific carbonyls are α-aminoadipic and γ-glutamic semialdehydes, commonly referred to as AAS and GGS, respectively. After AAS and GGS are stabilised using various chemicals, they are quantified using various methods such as HPLC-MS, GC-MS and HPLC-ESI-MS (Shacter, 2000).

GENERAL CONCLUSIONS

Carbon monoxide has successfully been used to stabilise the colour of tuna by forming carboxymyoglobin (Kristinsson *et al.,* 2006a). The carboxymyoglobin is stable even under frozen storage conditions (Kristinsson *et al.,* 2006a) but the CO will dissociate from the myoglobin under atmospheric conditions to form brown metmyoglobin (Krause *et al.,* 2003; Anderson & Wu., 2005). There is also evidence to suggest that the treatment of tuna with CO has possible quality benefits by potentially inhibiting lipid and protein oxidation (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011).

There are two main areas of concern with tuna and the CO treatment of tuna. The first is that tuna discolours when frozen (Chow *et al.,* 1988; Chow *et al.,* 1989) which considerably reduces its market value (Kropf, 1980). Tuna often needs to be transported vast distances and freezing is the only effective method to prolong its shelf-life (Kristinsson *et al.,* 2008). Thus to maintain the quality, colour and market value of the yellowfin tuna CO treatment can be used as it is stable during frozen storage. Secondly, the potential for CO to mask underlying safety concerns, such as microbial spoilage, high histamine levels and thermal abuse raises concerns regarding its use with tuna (Balaban *et al.,* 2005). Thus other potential benefits, such as CO's potential to inhibit lipid and protein oxidation (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011) should also be investigated to improve the perception of its use with consumers, industry and regulatory bodies.

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CHAPTER 3

OPTIMISATION OF CARBON MONOXIDE PRESSURE AND EXPOSURE TIME DURING THE TREATMENT OF YELLOWFIN TUNA (*THUNNUS ALBACARES*) MUSCLE TO ENHANCE COLOUR STABILITY

ABSTRACT

The aim of these pilot studies was to determine the ideal carbon monoxide pressure and exposure time to use on yellowfin tuna after developing a lab-scale gas chamber. It was found that both pressure and exposure time played a significant role in the degree of colour development and penetration. A higher pressure and longer exposure time was found to produce more desirable results. It was established that 150 min exposure time at 3 bar pressure would be optimal for the treatment of yellowfin tuna in the main study as it resulted in the desired surface colour development and colour penetration.

KEYWORDS: Carbon monoxide; Yellowfin tuna; Carboxymyoglobin; Colour stability

BACKGROUND

Several studies have evaluated the use of carbon monoxide (CO) on yellowfin tuna (Balaban *et al.,* 2005; Huang *et al.,* 2006) as well as on other fish species (Kristinsson *et al.,* 2003; Garner, 2004; Anderson & Wu, 2005; Mantilla *et al.,* 2008) to enhance the colour stability. In these studies different application methods were used including flushing bags or chambers with varying concentrations of CO for up to 24 h (Kristinsson *et al.,* 2003; Garner, 2004; Anderson & Wu, 2005; Balaban *et al.,* 2005; Huang *et al.,* 2006) or euthanasia of live fish using CO (Mantilla *et al.,* 2008).

Processors usually receive the whole tuna frozen which is then subsequently thawed, processed and either sold as “fresh” or re-frozen for further transportation. Frozen storage is widely accepted as a good method to lengthen the shelf-life of fish and other meat products (Kjærsgård *et al.,* 2006). Unfortunately frozen tuna readily discolours when thawed (Chow *et al*., 1988; Chow *et al.,* 1989; Anderson & Wu, 2005) and the main challenge producers face is maintaining the colour of tuna during processing, transport, storage and retail display while maintaining the quality of the product (Kristinsson *et al.,* 2008).

Various concentrations and sources (filtered smoke, tasteless smoke and pure CO) of CO have been used to treat different fish species using several application methods (Kristinsson *et al.,* 2003; Garner, 2004; Anderson & Wu, 2005; Balaban *et al.,* 2005; Huang *et al.,* 2006; Mantilla *et al.,* 2008). In all these cases, an increase in the redness (a* value) of the muscle was noted. There are, however, various factors which influence the increase in redness, among these: the

concentration and source of the CO; the method of application; treatment/exposure time; and the fish species (as different fish species contain varying myoglobin content) (Kristinsson *et al.,* 2006). The aim of this study was to determine the ideal pressure and exposure time to use for preserving the colour of yellowfin tuna muscle.

EXPERIMENTAL PROCEDURE

Gas chamber

The yellowfin tuna was treated using a purpose built chamber (Fig. 1) which allowed for accurate and consistent treatment of the tuna in respect of pressure and exposure time. The samples to be treated were placed on the tray (Fig. 2) inside the chamber, which was then tightly sealed to prevent CO leakage. The chamber was vacuated to -1 bar and then flushed with CO (99.97% min, AFROX, Cape Town, South Africa) until the desired pressure (Fig. 6) was reached. The tuna was treated for the desired exposure time (Fig. 7). The CO was then released via a tube into a well-ventilated outdoor area. Once the pressure had normalised, the chamber was unsealed and the tray removed.

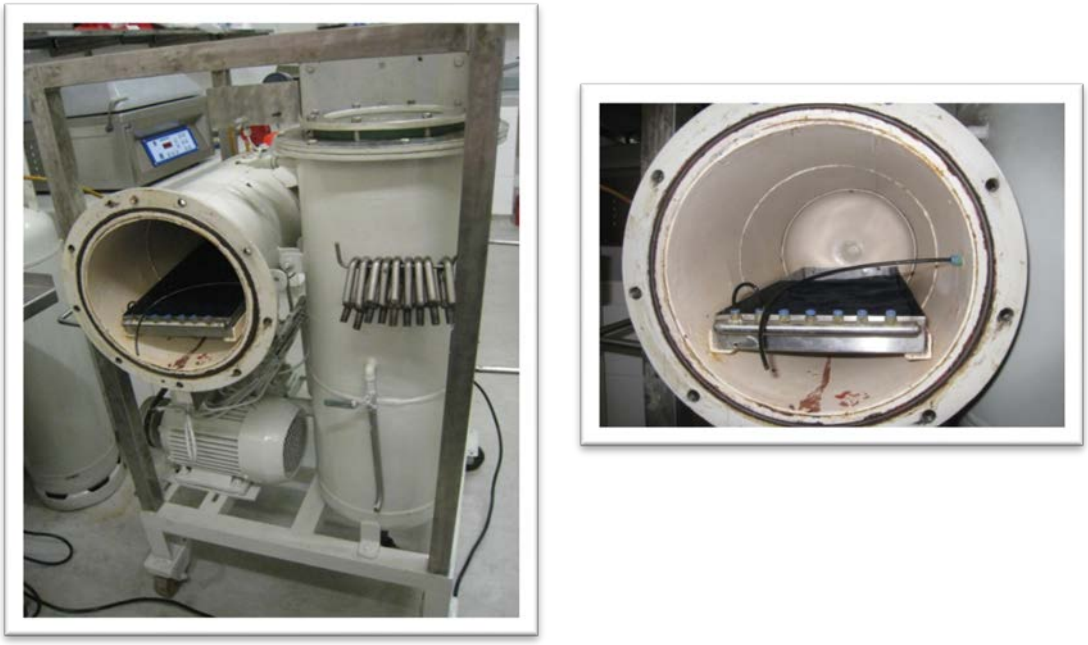

Figure 1 Purpose built gas chamber for the treatment of tuna samples with CO under pressure.

Figure 2 Tuna rounds prior to CO treatment (note the large colour variations).

Experimental layout

The main aim of this study was to evaluate the effect of the CO (100%) on colour stability of the yellowfin tuna. Since the reason for investigating the effect of the CO on the colour development of yellowfin tuna was as a result of muscle rapidly discolouring once frozen, two different freeze/thaw cycles were investigated as this is common practise in industry (multiple freeze thaw cycles) In the first, the whole tuna was frozen, cut and thawed as is done in industry, treated with CO, if required according to the statistical randomisation, and then subjected to a shelf-life study (the tuna underwent one freeze/thaw cycle) (Fx1). In the second, the whole tuna was frozen, cut, thawed, treated, if required according to the statistical randomisation, refrozen for 30 d, thawed and then subjected to the shelf-life trial (the tuna underwent two freeze/thaw cycles) (Fx2). The purpose of the two freeze/thaw cycles was to establish whether the CO had an effect on the colour of previously frozen tuna and to what extent, and whether the colour of treated samples would remain stable during frozen storage and subsequent thawing.

Since there is controversy surrounding the use of CO to treat tuna, other possible beneficial aspects of the treatment were also investigated with the aim of enhancing the public and scientific perception of its use. It has been postulated that the use of CO could retard/inhibit the onset of lipid and protein oxidation by stabilising the myoglobin in muscle and subsequently inhibiting its pro-oxidative effects (Kristinsson *et al.,* 2006). Thus the effect of the CO treatment on lipid and protein oxidation was also investigated. Furthermore, since the colour of muscle and lipid and protein oxidation are greatly influenced under both aerobic and anaerobic conditions (Ladikos & Lougovois, 1990) the effect of two different packaging types were also investigated; overwrap, which is oxygen permeable (aerobic) (OP) and vacuum packaging, which is oxygen impermeable (anaerobic) (OI). When all factors were included eight different treatments were established (Table 1).

Table 1 Experimental layout from which the eight treatments were established

Treatments	1	2	3	4	5	6	7	8
Packaging type	OP				OI			
Tuna number	Tuna 1-7				Tuna 8-14			
Shelf-life trial	8 d				32 d			
Sampling	24 h (T_0-T_8)				96 h (T_0-T_8)			
Gas treatment	+CO		-CO		+CO		-CO	
Freeze/thaw cycle	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2

OP – oxygen permeable; OI – oxygen impermeable; +CO – treated with 100% CO; -CO – untreated; Fx1 –one freeze/thaw cycle; Fx2 - two freeze/thaw cycles.

Shelf-life trial

The shelf-life trial was based on a partially staggered design as suggested by Gacula (1975) (Fig. 3). This design is the most commonly used approach for shelf-life testing and involves either a single batch or replicate of batches of product put on test at time zero with samples being taken for testing at intervals determined by expectations of probable shelf-life (Kilcast *et al.,* 2000). The shelf-life trial time period of 8 d for the OP treatments was established using time periods employed in a previous study (Garner, 2004) where CO treatment of fish was done with samples being taken every day (24 h). In the case of the OI samples, a 32 d shelf-life trail was conducted with samples being taken every 4 d (96 h). The 4 d intervals were based on a previous study where vacuum packaging had also been used on fish where lipid oxidation was assessed over 16 d (Khalil & Mansour, 1998). The reason for extending the shelf-life time period in this investigation to 32 d was that the same number of time periods, as used for the OP treatments, was preferred for better statistical analyses. Discussions with industry also indicated that they would like to see the shelf-life testing being conducted over a longer period. The samples for the OI treatments were taken at longer time intervals as it was assumed that, in the absence of oxygen, chemical reactions would not occur as rapidly as compared to the OP treatment samples (Ladikos & Lougovois, 1990).

Packaging

The OP samples were packed in polystyrene trays and wrapped in 10 micron thick oxygen permeable cling film (Versafilm, Crown National, Montague Gardens, Cape Town, South Africa) with a moisture vapour transfer rate of 585 $g.m^{-2}.24\ h^{-1}.1\ atm^{-1}$, O_2 permeability of 25 000 $cm^{-3}.m^{-2}.24\ h^{-1}.1\ atm^{-1}$ and a CO_2 permeability of 180 000 $cm^{-3}.m^{-2}.24\ h^{-1}.1\ atm^{-1}$.

The OI samples were packaged in 70 micron thick polyethylene bags (ESB). The samples were vacuum packed using a Multivac packaging system (Type C200; Multivac Sepp Haggenmueller GmbH & Co. KG, Wolfertschwenden, Germany) at 3 mbar pressure.

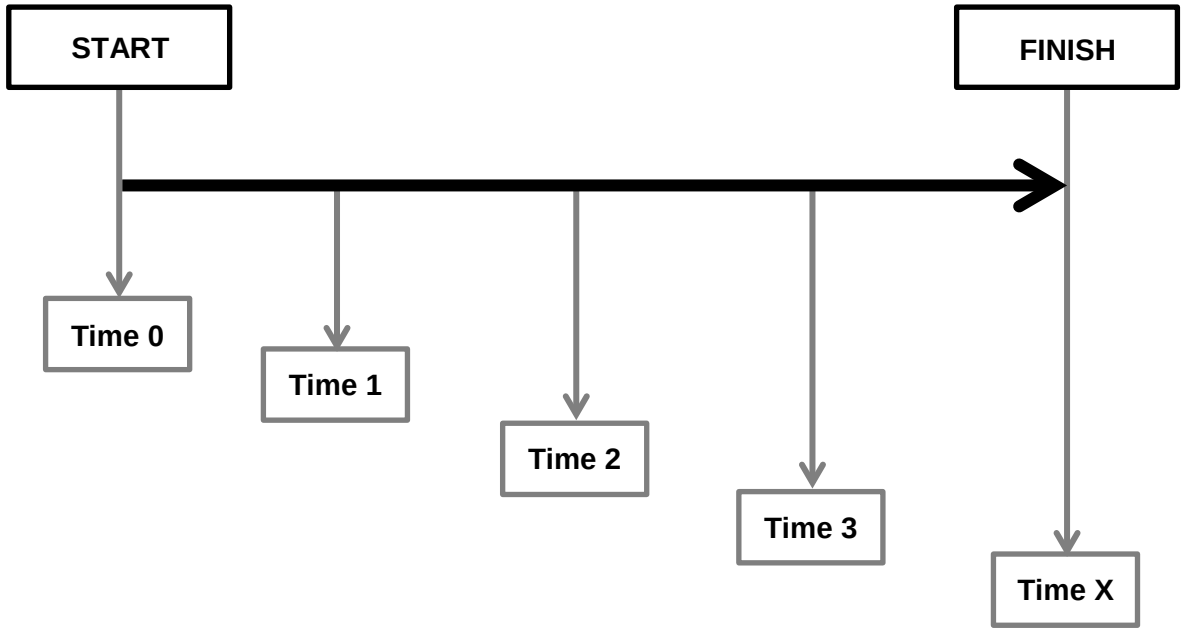

Figure 3 A partially staggered design for shelf-life testing (adapted from Kilcast *et al.,* 2000).

Tuna harvesting

Fourteen yellowfin tuna were caught off the west coast of South Africa by South Seas Safaris (owner Alan du Plessis; alan@southseasafaris.co.za) in May 2011, which is late autumn in South Africa. Tuna were caught around S 34°29'00 E17°54'00 and S 34°35'00 E17°58'00. The tuna were collected at the Gordons Bay harbour on the day of capture and exsanguinated on board, directly after being harvested. Exsanguination was done by cutting behind the pectoral fin, slitting open the gills and pithing the tuna with a stainless steel rod.

Statistical randomisation of samples

It was assumed, that since the tuna used in this study were harvested from the ocean and no control could be exercised over which tuna were selected, that the tuna used were representative of the population. It was decided that seven of the tuna harvested would be used for the OP samples and seven for the OI samples, as not enough samples could be extracted per tuna to use only seven tuna for both treatments. Furthermore, to prevent any further bias, the tuna caught were randomised across the treatments and across the days, ensuring that each tuna received each gas treatment on each time period within the packaging type (Table 1). The randomisations were done using Excel (Microsoft Excel ® 2010).

Sample preparation

The 14 yellowfin tuna used in the trial were collected from Gordons Bay harbour, the same day they were caught and transported to the Department of Animal Sciences at Stellenbosch University. The tuna had been exsanguinated on board directly after having been caught but had not been eviscerated. The tuna were weighed before (Table 2) and after evisceration (Table 3) and the head, gills, caudal fin (tail fin), second dorsal fin and anal fin were removed and discarded. The tuna were then wrapped in plastic bin bags and frozen (-20°C) until required.

Table 2 Mass frequency table for the 14 tuna prior to evisceration

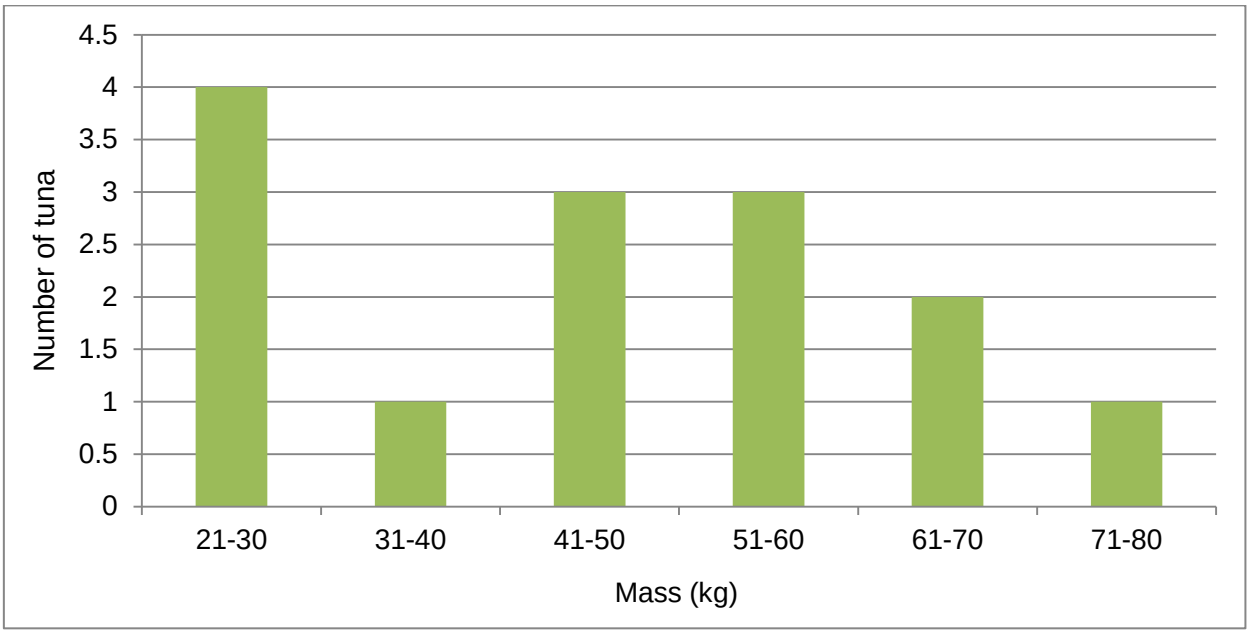

Table 3 Minimum and maximum mass of the 14 tuna, mean mass, standard deviation and average dressing percentage

	Before evisceration	**After evisceration**
Minimum mass	29	26.5
Maximum mass	75.7	71.5
Mean mass ± standard deviation (n=14)	50.8 ± 15.5	47.2 ± 14.6
Average dressing %	92.8	

The day before the trial commenced the tuna were cut into steaks while still frozen on a band saw. Seven tuna were used for the OP treatments and seven for the OI treatments as not enough steaks could be cut per tuna to allow for OP and OI samples to be taken from the same tuna. The tuna were cut from directly behind the pectoral fin, vertically, into 18, 2.5 cm thick steaks

(± industry standard) (Fig. 4). From these steaks two, 10 cm in diameter (Fig. 5), samples (called rounds) were cut from the loins (white muscle) of the tuna (Fig. 4). Since only two rounds could be extracted from each steak, and four were required (one for each treatment), the rounds from two consecutive steaks were counted as one experimental unit. These rounds were vacuum packed, marked according to their statistical randomisation and allowed to defrost overnight in the refrigerator at ~4°C.

The defrosted rounds were then removed from their vacuum packaging and either treated with CO, if required, and then repacked according to their randomisation, clearly labelled and stored according to their randomisation. The OP and OI samples which underwent one freeze/thaw cycle (Fx1) were placed in polystyrene trays and wrapped in oxygen permeable film or repacked in vacuum bags and vacuum sealed, respectively. The OP and OI samples which underwent two freeze/thaw cycles (Fx2) were repacked in vacuum bags, vacuum sealed and refrozen (-20°C) for 30 d. The samples were then defrosted (~4°C) a day before the commencement of the shelf-life trials. The OP samples were then removed from the vacuum packaging and placed in polystyrene trays, wrapped in oxygen permeable film, clearly labelled and refrigerated (~4°C). The OI samples were kept in the same refrigerator (~4°C).

Figure 5 Tool used to extract the rounds from the loins of the yellowfin tuna steaks.

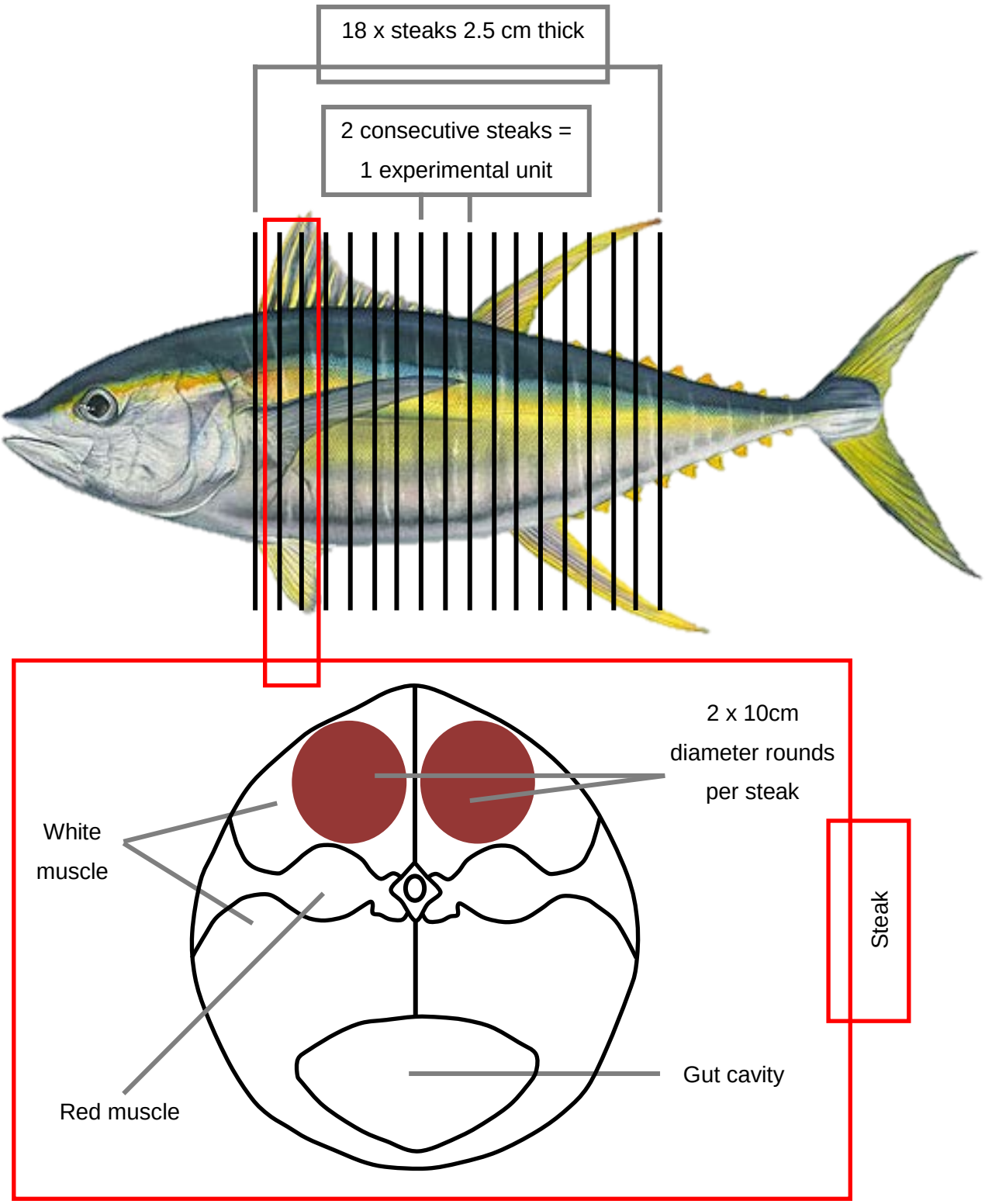

Figure 4 Tuna sample preparation and sampling area.

ESTABLISHING GAS (CO) APPLICATION PARAMETERS

Since the method of CO application used in the current study was not the same as those previously used, the exposure time/pressure combination needed to be determined. In this study,

only 100% CO (99.97% min, AFROX, Cape Town, South Africa) was used and thus the gas concentration was considered constant. The variables evaluated in this application method were the exposure time to the CO and the pressure that would be used.

The importance of exposure time has been noted (Kristinsson *et al.,* 2006), with longer exposure times leading to higher a* values. Thus higher CO concentrations require shorter exposure times for the development of high a* values. Most of the commercial application methods currently employed, expose the tuna for 24 h and longer at atmospheric pressure to allow for maximum CO binding to myoglobin (Kristinsson *et al.,* 2003; Garner, 2004; Anderson & Wu, 2005; Balaban *et al.,* 2005; Huang *et al.,* 2006; Mantilla *et al.,* 2008). Chambers, containers or bags are used, which are flushed with CO, where after the samples are left for a predetermined exposure time (24 h and longer). Although these application methods have proven effective, they are lengthy and also require large amounts of space. The aim behind the specific application method employed in the current study was not only to reduce the exposure time required, but also the amount of space required. Treating the tuna for a considerably shorter time than is currently used, and subsequently being able to package it in packaging that takes up considerably less space, such as vacuum packaging, will have financial advantages for the industry (Garner, 2004). To achieve this, the majority of the air inside the chamber is removed, by creating a vacuum, after which the tuna is exposed to the CO under pressure, in hopes that the CO will be forced into the tuna muscle, increasing the exposure of the myoglobin in the muscle to the CO. This should theoretically reduce the time required for the binding of the CO to the myoglobin and penetration of the CO into the tuna muscle. Thus effectively reducing the exposure time required for achieving a considerable increase in the surface a* (colour ordinate) value of the tuna muscle and penetration of the colour into the muscle.

Pilot studies

Several preliminary studies using exposure times of between 240 and 360 min at 3 bar pressure resulted in tuna which was too pink, creating an artificial looking product. Thus during the pilot studies, shorter time periods were assessed. Two pilot studies were done to investigate the best time/pressure combination to be used. Although, it would have been ideal to test a broad range of time/pressure combinations, time and cost constraints allowed for only two pilot studies. From these the best combination was deduced. The samples used in the pilot studies were obtained from SAMPER SA, Réunion Island (www.samper.com).

Pilot study one

In the first pilot study, four different time/pressure combinations were tested: 10 min/2 bar; 20 min/2 bar; 10min/3 bar; and 20 min/3 bar. Three yellowfin tuna loin samples were used for each combination which were approximately 2.5 cm thick. Data from this study indicated that the higher

exposure times and higher pressure lead to higher surface a* values (more pink/red) (Fig. 6). The higher pressure also resulted in better colour penetration, although not complete penetration, which was evaluated by cutting the samples in half. Although the importance of exposure time has previously been noted (Kristinsson *et al.,* 2006), the interaction between exposure time and pressure has not. Thus it was decided that a 3 bar pressure would be used for the main investigation. The a* values achieved during this pilot study resulted in acceptable colour development on the surface but not thorough penetration of the sample. Thus a longer exposure time at 3 bar pressure needed to be investigated.

Pilot study two

In the second pilot study the pressure was kept constant (3 bar) and the exposure time was increased to 60 min and 180 min. Since the idea behind the shorter exposure time was to freeze the sample immediately after treatment, both refrigerated and frozen treatment, post CO exposure, were investigated. From the results (Fig. 7) it could be seen that the a* values were higher in the frozen (-20°C) samples compared to that of the refrigerated (~4°C) samples, thus showing that colour development continued even during frozen storage. The 60 min exposure time resulted in tuna samples which had developed acceptable surface colour but again insufficient colour penetration. The 180 min samples resulted in sufficient surface colour development and sufficient colour penetration.

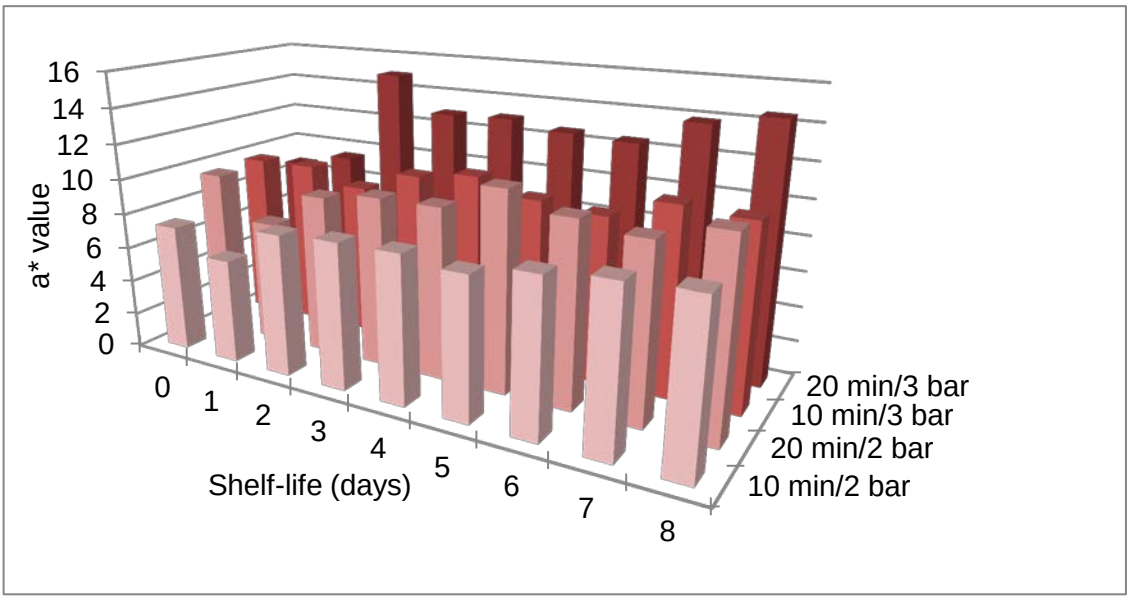

Figure 6 Pilot study conducted to investigate the effect of exposure time and pressure on the a* values of 100% CO treated yellowfin tuna loin.

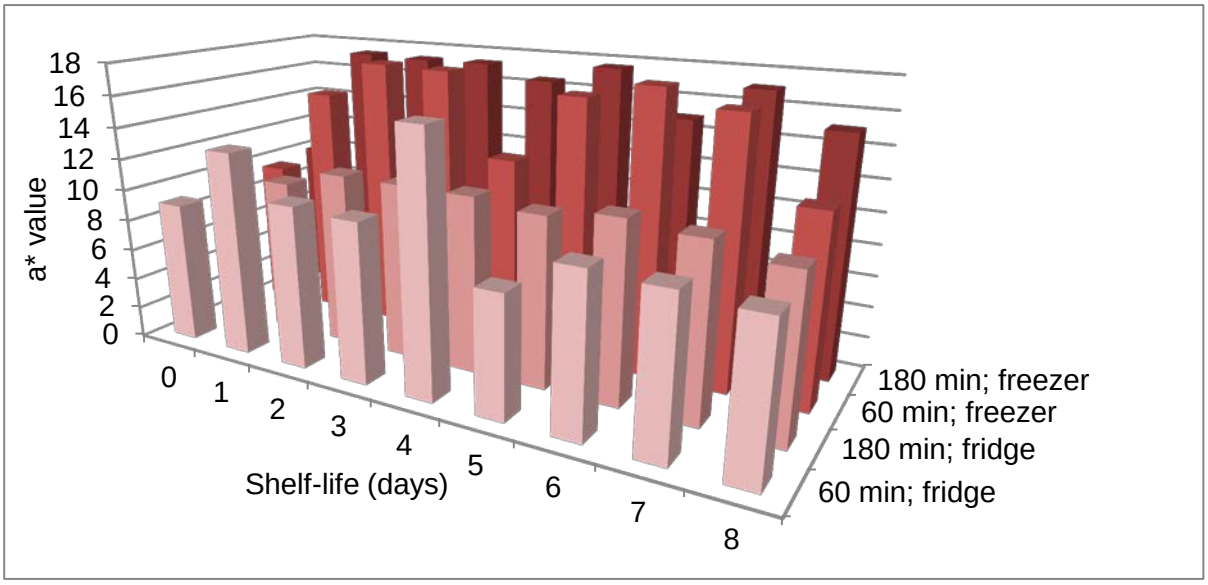

Figure 7 Pilot study investigating the effect of longer exposure times of 100% CO at a constant pressure (3 bar) under refrigerated (~4°C) and frozen storage (-20°C) conditions on yellowfin tuna loin.

In the second pilot study it was found that there was little difference in the surface a* values between the 60 min and 180 min exposure times at 3 bar pressure. It was also found that the 180 min exposure time resulted in sufficient colour penetration. Since the desired result was sufficient surface colour development and sufficient colour penetration in the shortest time, 30 min was removed from the 180 min exposure time so as to shorten the exposure time (150 min). It was found (data not shown) that 150 min resulted in sufficient colour penetration and this exposure time was used for the rest of the study.

CONCLUSION

It was found that the both the pressure and exposure time were important with regard to the amount of colour development (increase in surface a* value), speed of colour development and the depth of colour penetration. A higher pressure and longer exposure time led to the desired colour development and penetration. It was established that 150 min exposure time at 3 bar pressure was the most optimum combination and would be used in the main study as it resulted in the desired surface colour development and colour penetration.

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CHAPTER 4

EFFECT OF CARBON MONOXIDE TREATMENT ON THE COLOUR OF YELLOWFIN TUNA (*THUNNUS ALBACARES*) MUSCLE

ABSTRACT

This study was aimed at investigating the effect of a 100% carbon dioxide (CO) treatment on the surface colour of yellowfin tuna muscle, under aerobic (overwrap) and anaerobic (vacuum packed) conditions, subjected to either one or two freeze/thaw cycles. The yellowfin tuna samples were either subjected to an 8 d (overwrap) or 32 d shelf-life trial (vacuum packed), with samples taken every 24 h or 96 h, respectively. The colour was assessed using the CIE Lab colour system. The results showed that the CO treatment resulted in higher surface a* values for all the treatments, with those stored under aerobic conditions having reduced surface a* values over time (oxidation) and those stored under anaerobic conditions maintaining higher a* values over time. The number of freeze/thaw cycles did play a role in the treated and untreated samples with those which underwent two freeze thaw cycles having lower a* values. It was concluded that the vacuum packaged, treated samples which underwent one freeze/thaw cycle resulted in the best product with regards to colour development and stability over time.

KEYWORDS: Carbon monoxide; Yellowfin tuna; Carboxymyoglobin; CIE Lab

INTRODUCTION

The colour of meat is the most important factor influencing the purchasing decision of consumers (Gee & Brown, 1978; Mancini & Hunt, 2005), with consumers preferring the bright red colour associated with fresh meat and disliking the brown colour associated with older, poor quality meat (Kropf, 1980; Livingston & Brown, 1981). This is also true for dark-muscle fish species such as tuna (Garner, 2004), where the purchase intent of consumers has been linked to its bright red colour which is associated with fresh, wholesome tuna. Bright red tuna fetches higher prices than brown, discoloured tuna (Carpenter *et al.,* 2001; Otwell, 2006). The perception that “fresh” bright red tuna is of better quality than brown, discoloured tuna is often incorrect. Due to loses in market value of tuna which has discoloured, tuna can be sold as “fresh” for up to three weeks after being harvested due to the vast distances between where tuna is harvested and the end consumer. In industry, to preserve tuna, it is often frozen directly after being harvested which leads to discolouration. Thus some of the frozen tuna available can be of better quality than the “fresh” tuna as the former is frozen directly after being harvested (Olson, 2006). The problem is that tuna readily discolours when frozen and thawed (Chow *et al*., 1988; Chow *et al.,* 1989) and loses a considerable amount of market value (Kropf, 1980). Processors thus face the task of maintaining the bright red colour of tuna during processing, transportation, frozen storage and display to ensure

maximum profit while at the same time maintain the integrity and quality of the product (Kristinsson *et al.,* 2008). One method which has proved very effective is the use of carbon monoxide (CO) as either a single gas or as part of a mixture of gasses, such as filtered smoke, tasteless smoke or as part of modified atmospheric packaging (Balaban *et al.,* 2005). This research focussed only on the use of 100% CO.

Although CO treatment of tuna has several theorised benefits, the main reason for its use is to maintain the bright red colour of the muscle during frozen storage (Kristinsson *et al.,* 2006). The exposure of the muscle to CO causes a similar reaction to that of oxygen when bound to myoglobin but with the formation of a more stable (240 times more stable), bright cherry-red colour known as carboxymyoglobin (Sørheim *et al*., 1997) owing to myoglobin's high affinity for CO (Hunt *et al.,* 2004). The carboxymyoglobin complex is stable during refrigerated and frozen storage and is the one of the main reasons why it is deemed so effective in its use with tuna (Balaban *et al.,* 2005).

The main concern regarding the use of CO for colour preservation of tuna is that it maintains the bright red colour of the muscle long after it is no longer safe for human consumption due to microbial deterioration, effectively masking visible spoilage indicators (Balaban *et al.,* 2005). The process also allows for potential product abuse (Anderson & Wu, 2005) as the carboxymyoglobin formed is highly resistant to autoxidation even under abusive conditions (Balaban *et al.,* 2005). Despite these concerns there is still a growing market demand for CO treated tuna which has caused producers to branch out into a variety of new products and different methods of application (Kristinsson *et al.,* 2003; Otwell, 2006). The demand is mainly driven by convenience, appeal, lower cost, increase in revenue and the availability of both frozen and thawed products (Kristinsson *et al*., 2003; Anderson & Wu, 2005; Otwell, 2006). Producers frequently receive whole frozen tuna, which is subsequently thawed, processed and either sold as "fresh" or re-frozen for further transportation to an alternative destination where it is again thawed. Thus the influence of multiple freeze/thaw cycles on the colour stability of the tuna was investigated.

Several studies have shown that the use of CO significantly influences the red colour (a* value) of tuna muscle (Kristinsson *et al.,* 2003; Otwell *et al*., 2003; Garner, 2004; Balaban *et al.,* 2005; Mantilla *et al.,* 2008). Although some of these studies used similar experimental procedures as those used in the current study, none were identical. The current study employed the use of 100% CO under 3 bar pressure with an exposure time of 150 min, with the aim at investigating the effect of the treatment on the surface L*, a*, b*, hue angle and chroma values, under both aerobic and anaerobic conditions. The effect on the number of freeze/thaw cycles was also evaluated.

MATERIALS AND METHODS

The experimental layout can be seen in Table 1. For the full experimental design, sample preparation and packaging refer to Chapter 3.

Table 1 Experimental layout from which the eight treatments were established

Treatments	1	2	3	4	5	6	7	8
Packaging type	OP				OI			
Tuna number	Tuna 1-7				Tuna 8-14			
Shelf-life trial	8 d				32 d			
Sampling	24 h (T_0-T_8)				96 h (T_0-T_8)			
Gas treatment	+CO		-CO		+CO		-CO	
Freeze/thaw cycle	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2

OP – oxygen permeable; OI – oxygen impermeable; +CO – treated with 100% CO gas; -CO – untreated; Fx1 –one freeze/thaw cycle; Fx2 - two freeze/thaw cycles.

Surface L* a* b* measurements

The surface colour of the tuna samples was measured according to the CIE Lab colour system using a colour-guide 45°/0° colorimeter (BYK-Gardner GmbH, Geretsried, Germany). The colorimeter was calibrated using the light trap, white and high gloss standards provided (BYK-Gardner GmbH, Geretsried, Germany). The colorimeter was calibrated at least once a week and checked against the green standard daily to ensure the calibration was still correct. The samples were removed from their packaging before being measured, as previous trials had shown that the packaging influenced the results. Three L*, a* and b* measurements were taken with the colorimeter on different areas of the sample and the average of these three measurements, L*, a* and b* respectively, was used in the statistical analysis (Honikel, 1998). The hue angle ($h°_{ab}$) and the chroma (C*) were calculated using the a* and b* values:

$$h°_{ab} = \arctan (b^*/a^*)$$

$$C^* = (a^{*2} + b^{*2})^{½}$$

Statistical analysis

The colour determination involved two main effects, time and treatment, as well as the seven tuna used for each type of packaging. The data was analysed using a two way repeated measure analysis of variance (RMANOVA) using the general linear models (GLM) procedure. Each variable, L*, a*, b*, hue angle and chroma were analysed separately. The model for the RMANOVA of the data is indicated by the following equation:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{(i)k} + \delta_{(j)k} + \varepsilon_{ijk}$$

The terms within the model are defined as:

y_{ijk}= observation k in the i^{th} treatment at the j^{th} time

μ - the overall mean;

α_i - the effect of the i^{th} treatment (fixed effect);

β_j - the effect of the j^{th} time (fixed effect);

$(\alpha\beta)_{ij}$ - the interaction of the i^{th} treatment with the j^{th} time;

$\gamma_{(i)k}$ - the effect of the k^{th} tuna on the i^{th} treatment (random effect);

$\delta_{(i)k}$ - the effect of the k^{th} tuna on the i^{th} time period (variable effect); and

ε_{ijk} - the error associated with the time, treatment and tuna.

The RMANOVA is performed on the assumption that the data has compound symmetry on the time and treatment correlation. The data was also assumed to be normally distributed. The least significant interactions (LSD) were calculated at a 95% significance level to compare the treatment means i.e. results were defined as significant when $P \leq 0.05$ and not significant when $P > 0.05$. If the interaction between the main effects, time and treatment, was not significant ($P > 0.05$) then the main effects could be interpreted separately. In the case where the interaction is found to be significant a Bonferroni pair wise comparisons was done to identify the specific interactions. STATISTICA version 10 (StatSoft South Africa Pty (Ltd)) was used to analyse the data collected for each treatment.

RESULTS

For the results of the L*, a*, b*, hue angle and chroma values, where no significant interaction ($P > 0.05$) was found between the treatments, only the combined data were reported.

Surface L* a* b* measurements

Oxygen permeable (OP) treatments

Fx1 treatments - The RMANOVA showed no significant interactions ($P > 0.05$) between the main effects tested for the L*, a*, b*, hue angle or chroma values for the Fx1 treatments over time (Fig. 2). There was an overall increase in the L*, b* and hue angle values over time and an overall decrease in the a* values, for both treatments. The chroma values showed an initial increase (T_0 to T_1), after which the values decreased (T_3), and then remained relatively constant until T_8.

Fx2 treatments - The RMANOVA showed no significant interactions ($P > 0.05$) between the main effects tested for the L* and b* values for the Fx2 treatments over time (Figs. 3a and c); however, significant interactions ($P \leq 0.05$) were found between the a*, hue angle and chroma values over time (Figs. 3b, d and e). There was an overall increase in the L* and b* values over time. The a*

values showed and overall decrease over time for both the treated and untreated samples, with the treated samples having higher a* values over time but resulting in similar a* values as the untreated samples by T_8. The chroma values for the treated samples showed an initial increase, then a decrease, levelling out after T_6 and having the same/similar values as the untreated samples. The chroma values for the untreated samples showed an overall increase over time, resulting in similar values as the treated samples after T_6. The hue angle values for the treated and untreated sample increase over time, with the untreated sample having overall higher hue angle values over time but resulting in similar values as the treated samples by T_8.

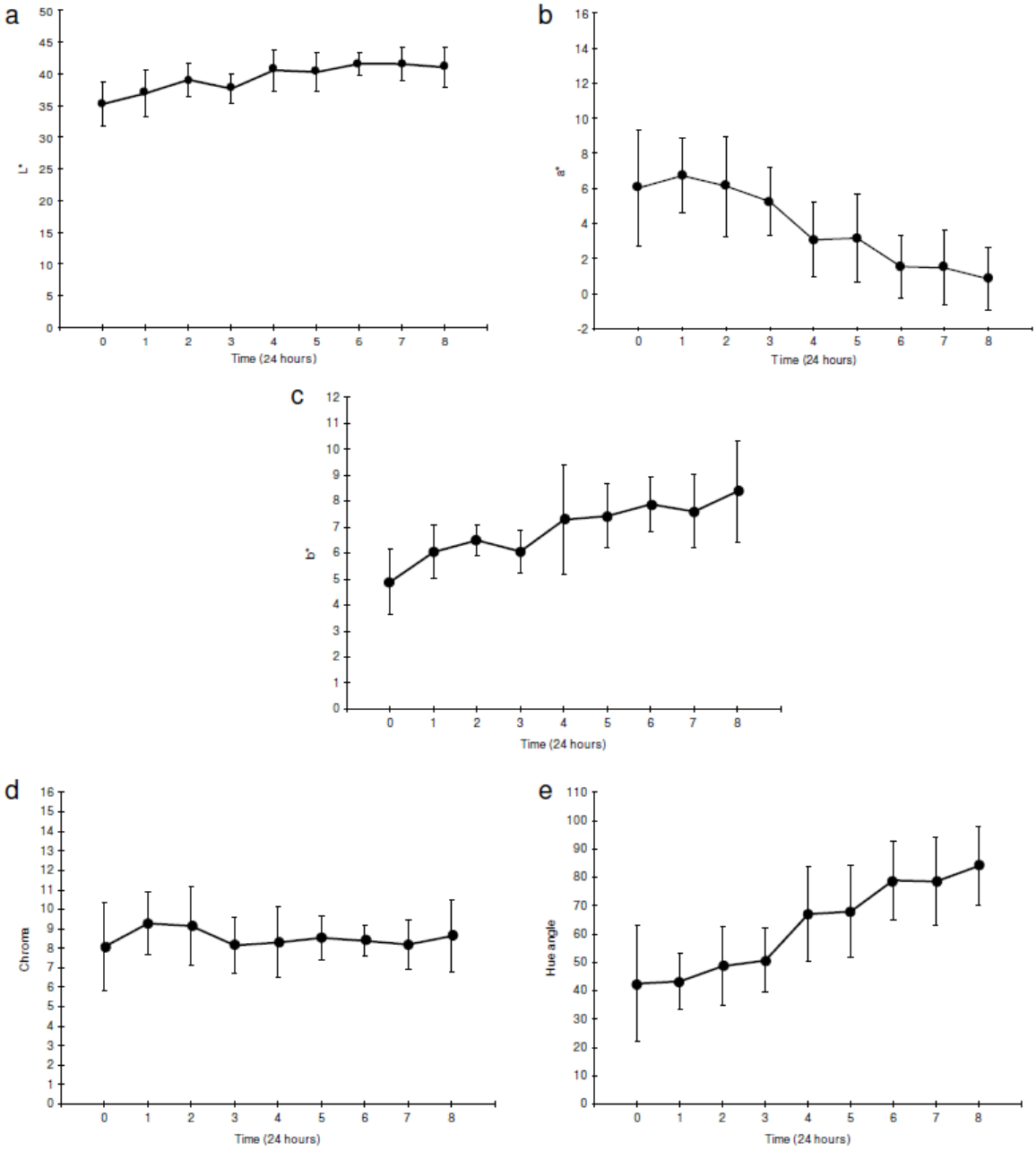

Figure 2 Surface L*, a* and b* values as well as the chroma and hue angle values for the OP Fx1 treatments: a) combined L* values; b) combined a* values; c) combined b* values; d) combined chroma values; and e) combined hue angle values.

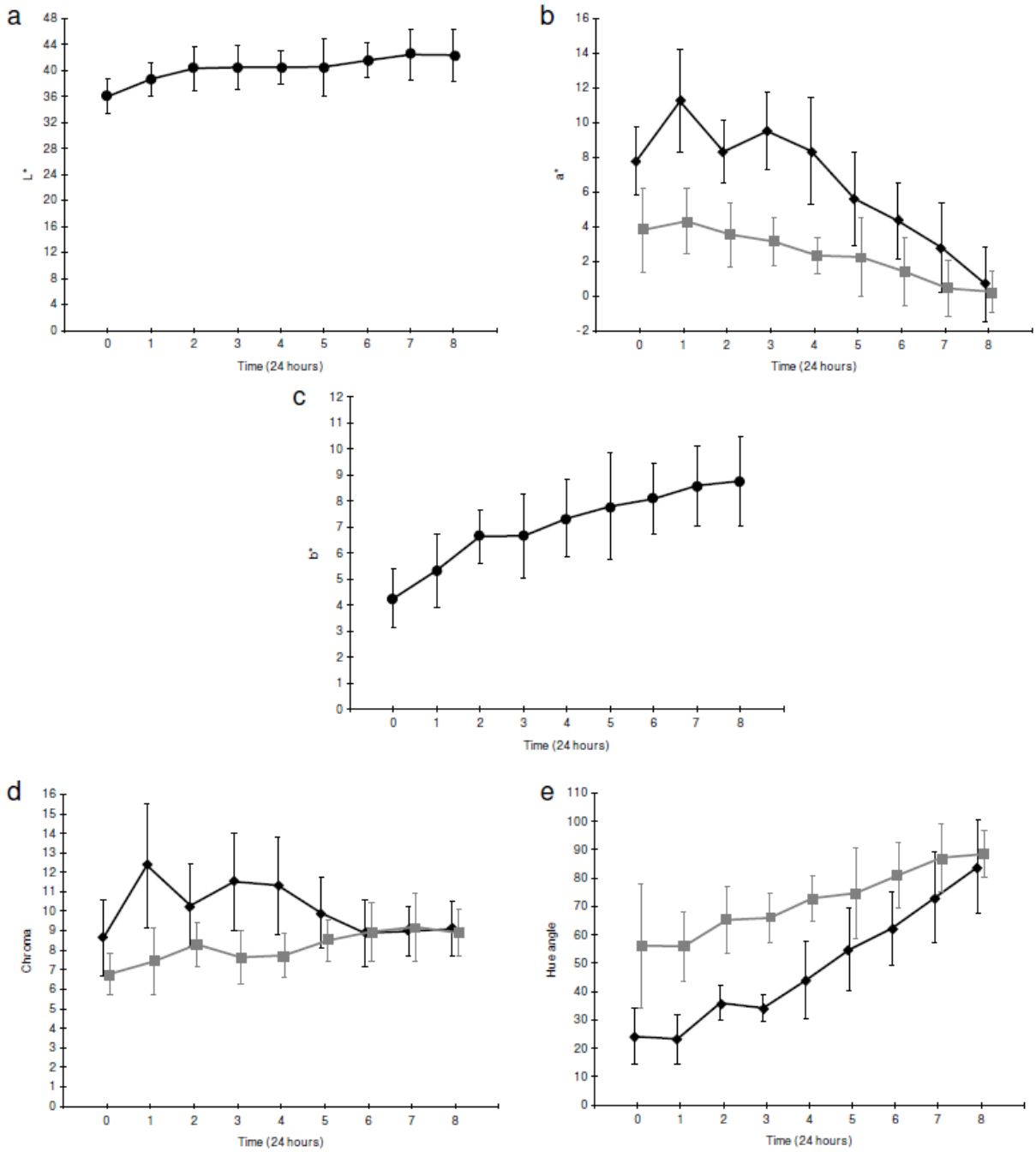

Figure 3 Surface L*, a* and b* values as well as the chroma and hue angle values for the OP Fx2 treatments: a) combined L* values; b) a* values - (◆) +CO and (■) -CO; c) combined b* values; d) chroma values - (◆) +CO and (■) -CO; and e) hue angle values - (◆) +CO and (■) -CO.

Combined Fx1 and Fx2 treatments - The RMANOVA for the combined OP treatments showed no significant interaction (P>0.05) between the main effects tested for the L*, b* and hue angle values (Figs. 4a, c and e) and significant interactions between the a* and chroma values (Figs. 4b and d). The L*, b* and hue angle values showed an increase over time. All the treatments showed a decrease in a* values over time, with the Fx2 +CO treatment having the highest overall a* values and the Fx2 -CO treatments having the lowest, with all the treatments resulting in similar a* values at T_8. The chroma values show a varying trend (as discussed above) with all treatments resulting

in similar chroma values by T_8. The Fx2 +CO samples had the highest initial chroma value and the Fx2 -CO values the lowest.

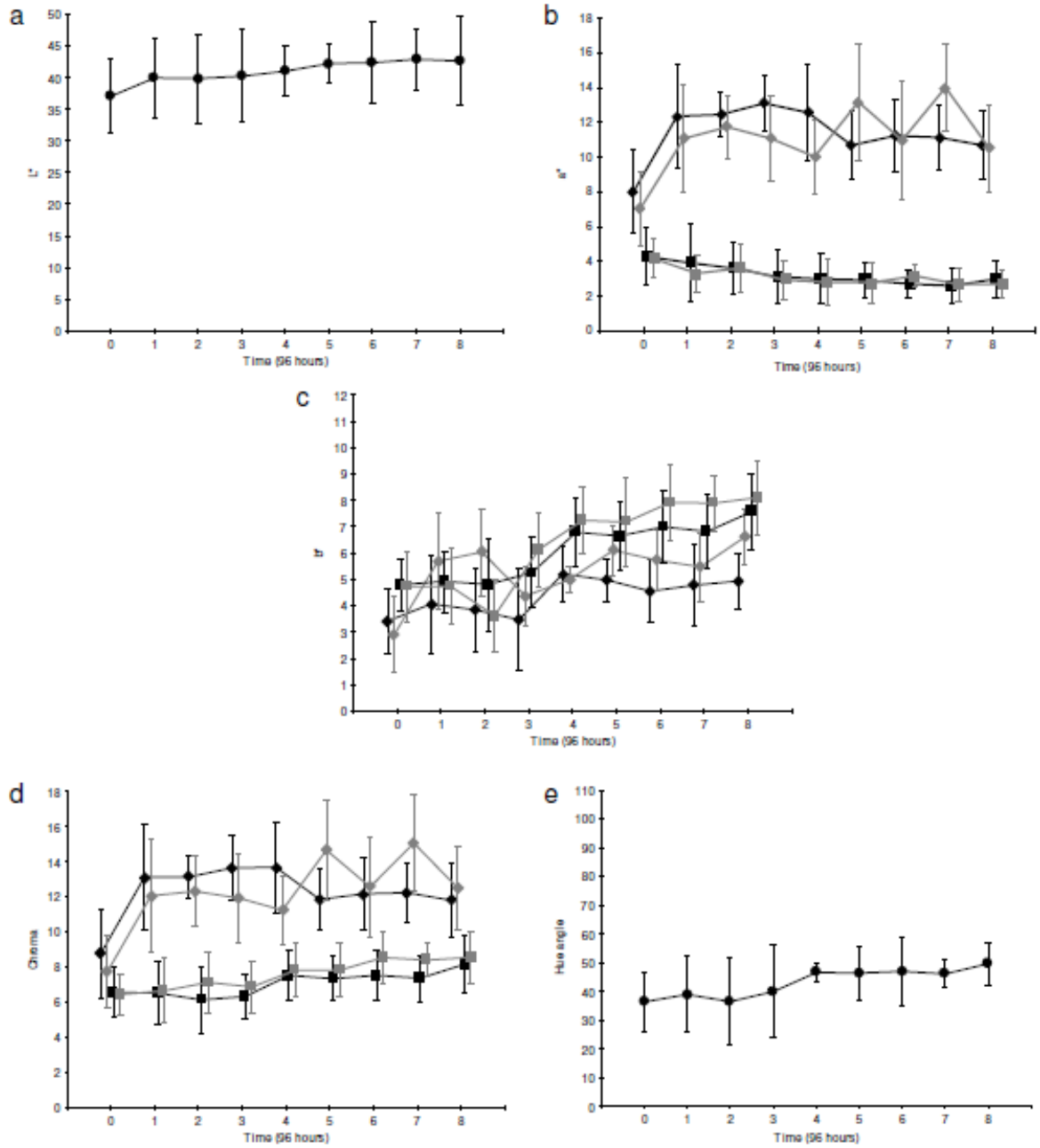

Figure 4 Surface L*, a* and b* values as well as the chroma and hue angle values for all the OP treatments: a) combined L* values; b) a* values - (◆) +CO Fx1, (■) -CO Fx1, (◆) +CO Fx2 and (■) -CO Fx2; c) combined b* values; d) chroma values - (◆) +CO Fx1, (■) -CO Fx1, (◆) +CO Fx2 and (■) -CO Fx2; and e) combined hue angle values.

Oxygen Impermeable (OI) treatments

Fx1 treatments - The RMANOVA showed no significant interactions (P>0.05) between the main effects tested for the L*, b* and hue angle values (Figs. 5a, c and e) and significant (P≤0.05)

interactions between the a* and chroma values (Figs. 5b and d). The L*, b* and hue angle values all showed an increase over time. The a* values for the treated samples were considerably higher than those of the untreated samples, with the a* values of the untreated samples decreasing over time. The a* values for the treated samples showed a sharp increase from T_0 to T_1, after which the a* values decrease from T_3 to T_4, with the a* values levelling off/decreasing slightly from T_5 to T_8. Overall, the chroma values for the treated samples were higher than those of the untreated samples. The treated samples show a sharp increase in the chroma values from T_0 to T_1, after which the values level off/increase slightly over time, then decrease from T_4 to T_5 and finally level off/decrease slightly.

Fx2 treatments - The RMANOVA showed no significant interactions ($P>0.05$) between the main effects tested for the L*, b* and hue angle values (Figs. 6a, c and d) and significant interactions ($P\leq0.05$) between the a* and chroma values (Figs. 6b and d). The L*, b* and hue angle values increased over time. The a* values for the treated samples were considerably higher than those of the untreated samples, with the a* values for the untreated samples decreasing over time. There was also a sharp increase in the a* values from T_0 to T_1. The treated samples showed an increase in the a* values over time. The chroma values for the treated and the untreated samples showed an increase over time, with the treated samples having higher chroma values than the untreated samples.

Combined Fx1 and Fx2 treatments - The RMANOVA for all the OI treatments showed no significant ($P>0.05$) interactions between the main effects tested for the L* and hue angle values (Figs. 7a and e) and significant ($P\leq0.05$) interactions between the a*, b* and chroma values (Figs. 7b, c and d). The L* and hue angle values showed an overall increase over time. The a* values for the treated samples were considerably higher than those of the untreated samples, with the a* values for the untreated samples decreasing over time. There was also a sharp increase in the a* values from T_0 to T_1. The a* values for the treated samples follow a similar trend, with the Fx2 treated samples having slightly lower initial a* values compared to the Fx1 samples but resulting in similar a* values over time. The untreated samples for both treatments follow a similar trend and have similar values for the entire shelf-life trial. The b* values for all the treatments showed an increase over time, with the Fx2 -CO samples having the highest b* values and the Fx1 +CO samples having the lowest. The chroma values for the treated and untreated samples showed an increase over time, with the treated samples having higher chroma values than the untreated samples over time.

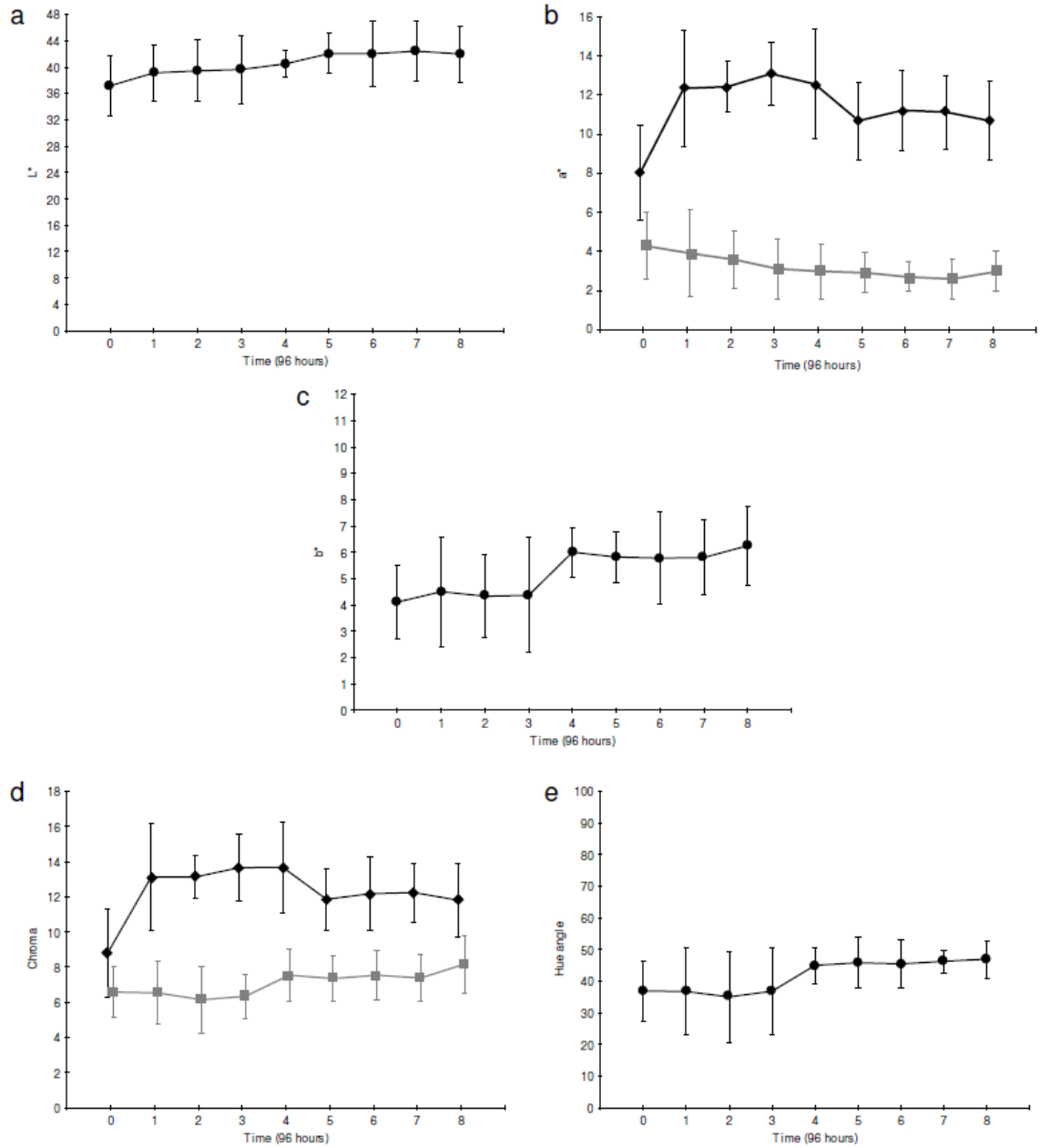

Figure 5 Surface L*, a* and b* values as well as the chroma and hue angle values for the OI Fx1 treatments: a) combined L* values; b) a* values - (◆) +CO Fx1 and (■) -CO Fx2; c) combined b* values; d) chroma values - (◆) +CO Fx1 and (■) -CO Fx2; and e) combined hue angle values.

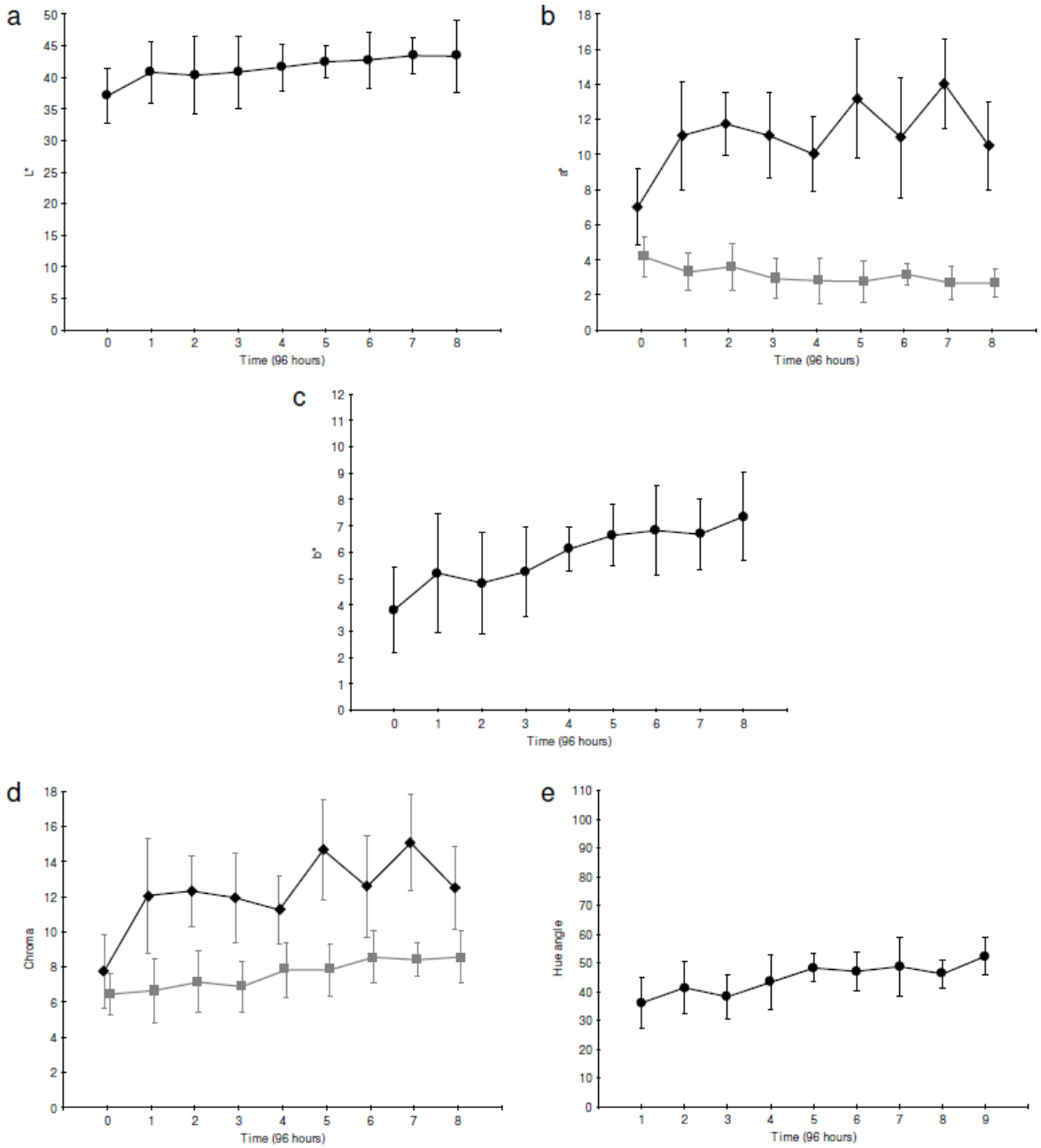

Figure 6 Surface L*, a* and b* values as well as the chroma and hue angle values for the OI Fx2 treatments: a) combined L* values; b) a* values - (◆) +CO Fx1 and (■) -CO Fx2; c) combined b* values; d) chroma values - (◆) +CO Fx1 and (■) -CO Fx2; and e) combined hue angle values.

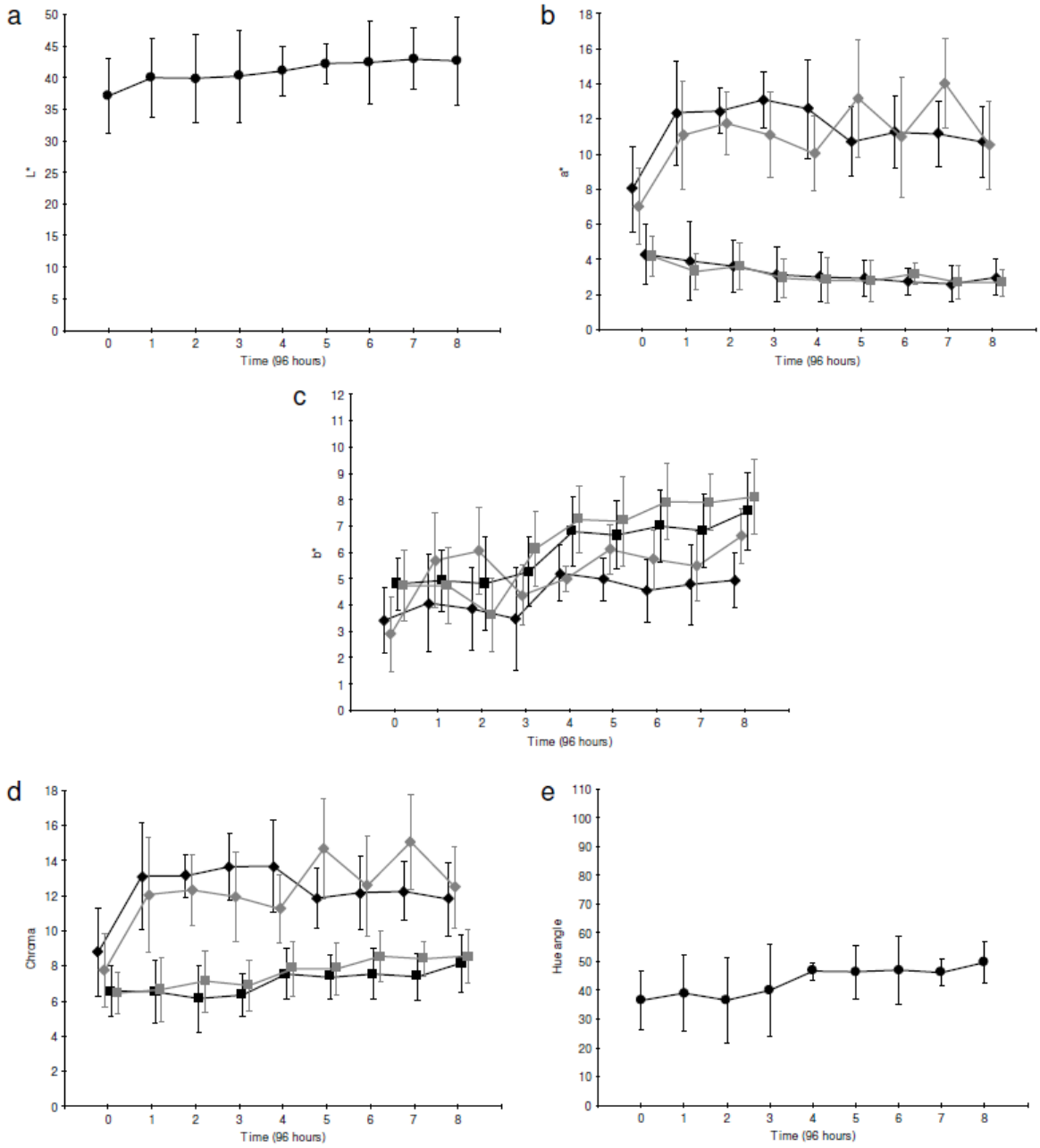

Figure 7 Surface L*, a*and b* values as well as the chroma and hue angle values for all the OI treatments: a) combined L* values; b) a* values - (◆) +CO Fx1, (■) -CO Fx1, (◆) +CO Fx2 and (■) -CO Fx2; c) b* values - ◆) +CO Fx1, (■) -CO Fx1, (◆) +CO Fx2 and (■) -CO Fx2; d) chroma values - (◆) +CO Fx1, (■) -CO Fx1, (◆) +CO Fx2 and (■) -CO Fx2; and e) combined hue angle values.

DISCUSSION

Although the L* values were included in the results for thoroughness, previous studies have shown that the CO treatment of meat has little effect on the L* values (Garner, 2004; Balaban *et al.,* 2005; Kristinsson *et al.,* 2006). The results from this study reiterate this, as none of the treatments had a significant on the L* values. Thus the L* values are not indicative of any colour changes. The a* value gives an indication of redness in meat, which is correlated with the presences of

oxymyoglobin (Mancini & Hunt, 2005) and/or carboxymyoglobin (Garner, 2004; Kristinsson *et al.,* 2006) in meat. The b* value is an indication of yellowness, which in meat has been correlated with the browning of meat (formation of metmyoglobin) (Mancini & Hunt, 2005).

The hue angle and chroma values are calculated using the a* and b* values (Anon., 1998) and thus give a better indication of the overall colour of the muscle. The hue angle values indicate where in the colour spectrum the colour of the meat lies (red, yellow, orange, green, blue or violet). In this case it will give an indication of whether the samples lie more towards the red (0°) or yellow area (90°) of the spectrum. The chroma value gives an indication of the colour saturation, with higher values indicating a more saturated colour (Anon., 1998).

Surface L* a* b* measurements

Oxygen permeable (OP) treatments

Fx1 treatments - The Fx1 treatments resulted in an overall decrease in a* values over time with a concurrent increase in the b* values (Figs. 2b and c). This is to be expected since, over time, in the presence of oxygen, the oxymyoglobin and carboxymyoglobin in the tuna muscle (measured by the a* values) will oxidise to form brown metmyoglobin (measured by the b* value) (Livingston & Brown, 1981; Krause *et al.,* 2003; Anderson & Wu., 2005). This is further reiterated by the hue angle (Fig. 2e) which increased over time, showing that both the treated and untreated samples move from the red area (0°) of the colour spectrum toward the yellow (90°) over time. The chroma values (Fig. 2d) initially increase slightly, which could be explained by the increase in CO binding to myoglobin and oxygenation of myoglobin leading to the formation of oxy- and carboxymyoglobin, respectively, which will lead to a more saturated red colour (Mancini & Hunt, 2005; Kristinsson *et al.,* 2006). Overall however, there is little to no change in the chroma value, indicating that there was not much change in colour saturation over time. These results are similar to results seen in other studies on yellowfin tuna (Kristinsson *et al.,* 2006) and other dark meat fish species treated with CO (Garner, 2004). Furthermore, the rapid discolouration can be explained by the tuna being frozen and thawed prior to being treated. Freezing and thawing of tuna causes myoglobin to oxidise more readily to metmyoglobin resulting in a brown coloured muscle (Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012). This is thought to be caused by denaturation of the globin moiety at some point during the freeze/thaw process resulting in the myoglobin being more susceptible to discolouration (Leygonie *et al.,* 2012). This hypothesis has been proven in several studies (Marriott *et al.,* 1980; Lanari *et al.,* 1990; Lanari & Zaritzky, 1991; Farouk & Swan, 1998; Abdallah *et al.,* 1999; Otremba *et al.,* 1999; Leygonie *et al*., 2011). The change in colour is also partly due to the loss of myoglobin in the exudate once the meat is thawed (Añón & Cavelo, 1980). The difference between the initial colour (T_0) and the final colour (T_8) is illustrated in Fig. 8.

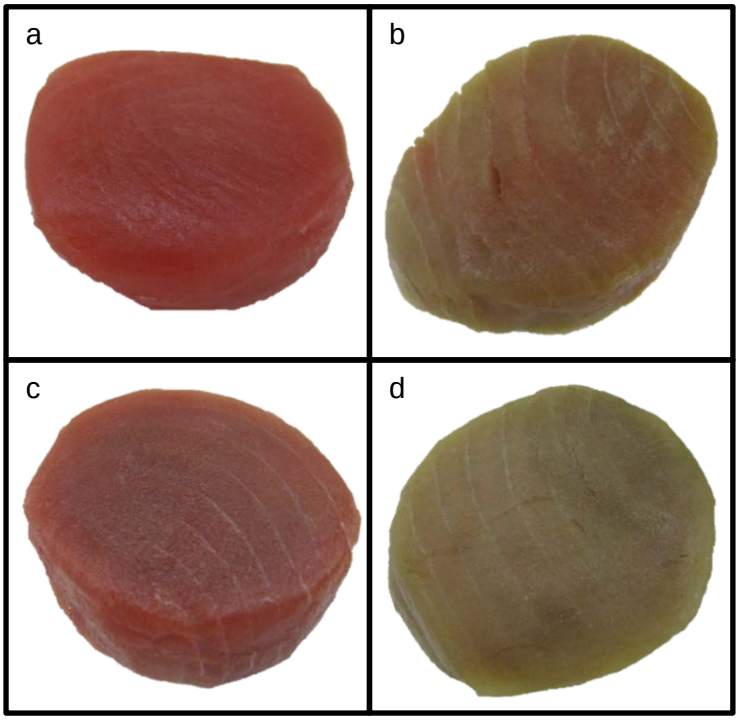

Figure 8 The colour difference between the treated and untreated tuna rounds of T_0 and T_8: a) OP Fx1 +CO T_0; b) OP Fx1 +CO T_8; c) OP Fx1 -CO T_0; and d) OP Fx1 –CO T_8.

Fx2 treatments - The Fx2 treatments resulted in a decrease in the a* values for both the treated and untreated samples over time (Fig. 3b), with a concurrent increase in the b* values (Fig. 3c). This is to be expected since, over time, in the presence of oxygen, the oxymyoglobin and carboxymyoglobin in the tuna muscle (measured by the a* values) will oxidise to form brown metmyoglobin (measured by the b* value) (Livingston & Brown, 1981; Krause *et al.,* 2003; Anderson & Wu., 2005). This is similar to the results of the Fx1 treatments, except that there is a significant interaction ($P \leq 0.05$) between the a* values for the treated and untreated samples, with the treated samples having higher a* values than the untreated samples for most of the shelf-life trial. The higher a* values of the treated samples is due to the fact that the samples were vacuum packed immediately after being treated, allowing for more CO to bind to myoglobin before being frozen. In the absence of oxygen the CO will not dissociate from the myoglobin and residual CO will bind to the myoglobin (Kristinsson *et al*., 2006). It has also been postulated that residual CO will bind to the unbound myoglobin during thawing (Kristinsson *et al.,* 2006). The increased binding before freezing and during thawing will result in a higher concentration of carboxymyoglobin and thus increased a* values. The rapid decrease and low a* values of the untreated samples is due to the damage caused to the tuna muscle during freezing and thawing which results in accelerated oxidation of the myoglobin to metmyoglobin (Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012) as well as a loss of myoglobin in the exudate when the meat is thawed (Añón & Cavelo, 1980). The negative effects of freezing on the tuna muscle are amplified by the tuna having gone through two freeze/thaw cycles. The chroma and hue angle results

reiterate the above findings, with the results showing that the treated samples initially have a more saturated, more red colour than the untreated samples, which have a less saturated more brown/tan colour, with the samples eventually developing the same/similar surface colour over time. The difference in colour between the initial colour (T_0) and the final colour (T_8) can be seen in Fig. 9.

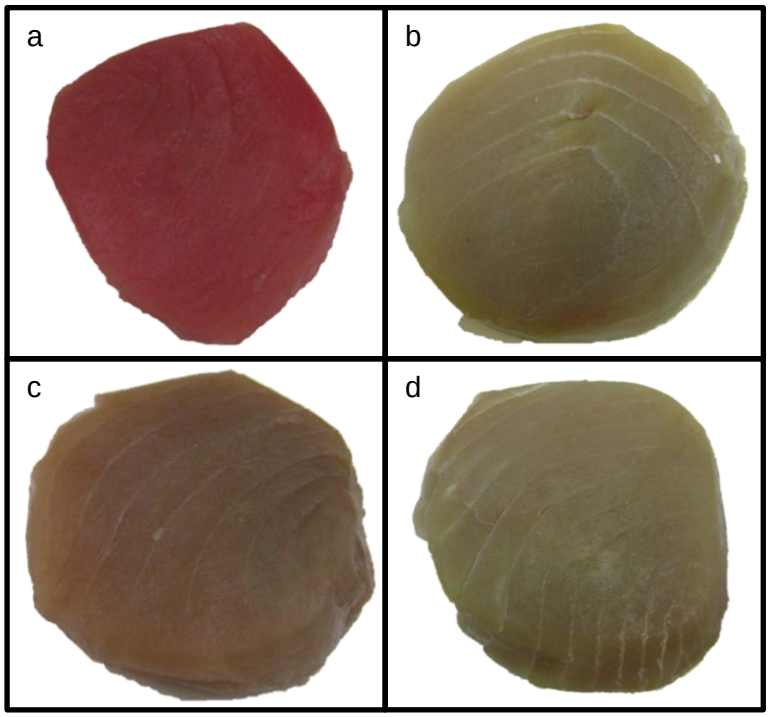

Figure 9 The colour difference between the treated and untreated tuna rounds of T_0 and T_8: a) OP Fx2 +CO T_0; b) OP Fx2 +CO T_8; c) OP Fx2 -CO T_0; and d) OP Fx2 -CO T_8.

Combined Fx1 and Fx2 treatments - Combined Fx1 and Fx2 treatment data revealed a decrease in a* values over time and a concurrent increase in b* values i.e. all the samples lost redness and became more brown over time. The chroma and hue angle indicated that both Fx1 and Fx2 treatments resulted in a similar meat colour by T_8 which was confirmed by visual observations of meat colour (Figs. 8 and 9). The results illustrated that the Fx2 +CO samples were initially the darkest red (chroma and a* values) and the Fx2 -CO samples the least red. The darker red colour is attributed to the higher percentage of carboxymyoglobin which formed as a result of the tuna being vacuum packed directly after having been treated with CO. In the absence of oxygen, the residual CO bound to the unbound myoglobin before the samples were refrozen. Furthermore during thawing further binding would occur between residual CO and any unbound myoglobin resulting in a darker red colour (Kristinsson *et al.,* 2006). Effectively the tuna muscle had a longer exposure time to the CO than the Fx1 +CO samples. The lack of redness in the Fx2 -CO samples can be attributed to the loss of myoglobin in the exudate during thawing (Añón & Cavelo, 1980) and the increased oxidation of the myoglobin in frozen/thawed tuna muscle (Chow *et al.,* 1988;

Chow *et al.,* 1989; Leygonie *et al.,* 2012), both of which were more pronounced due to the sample having undergone two freeze/thaw cycles.

Oxygen impermeable (OI) treatments

Fx1 treatments – Large differences were observed between the treated and untreated OI samples with regards to the a* and chroma values. Similar trends are seen in the b* and hue angle values, which increased over time. The b* and hue angle values indicate that both the treated and untreated samples browned to some extent over time. The a* and chroma values indicated that the untreated samples decreased in redness over time and become more saturated in colour i.e. a darker brown/tan colour. On the other hand, the treated samples displayed a rather dark (saturated) red colour initially and then become less red and lighter (less saturated) over time, most probably due to microbial growth (Mancini & Hunt, 2005). These results are consistent with those of Mantilla *et al*. (2008) who also observed a decrease in colour saturation over time. The results observed in this study are to be expected since, in the absence of oxygen, any residual CO will bind to the unbound myoglobin resulting in a higher concentration of carboxymyoglobin (Kristinsson *et al.,* 2006), leading to a more saturated red colour. The low a* and chroma values of the untreated samples would have been due to the increased myoglobin oxidation as a result of the freeze/thaw cycle (Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012) it underwent prior to vacuum packaging. The results are consistent to what was visually observed (Fig. 10).

Fx2 treatments - The Fx2 results mirrored the Fx1 displaying large interactions between the treated and untreated samples with regards to the a* and chroma values, and similar trends for the b* and hue angle values, which increase over time. The b* and hue angle values indicate that both the treated and untreated samples brown to some extent over time. The a* and chroma values indicated that the untreated samples decrease in redness over time and become more saturated in colour i.e. a darker brown/tan colour. On the other hand, the treated samples displayed quite a dark (saturated) red colour initially, then became less red and lighter (less saturated) and yielded erratic colour changes near the end, most probably due to microbial growth (Mancini & Hunt, 2005). These results were expected since, in the absence of oxygen, the residual CO will bind to the unbound myoglobin resulting in a higher concentration of carboxymyoglobin (Kristinsson *et al.,* 2006), leading to a more saturated red colour. The low a* and chroma values of the untreated samples are ascribed to the increased myoglobin oxidation and loss in myoglobin in the tuna muscle as a result of the freeze/thaw cycle (Añón & Cavelo, 1980; Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012) that was applied prior to vacuum packaging. The results are consistent to what was visually observed (Fig. 11).

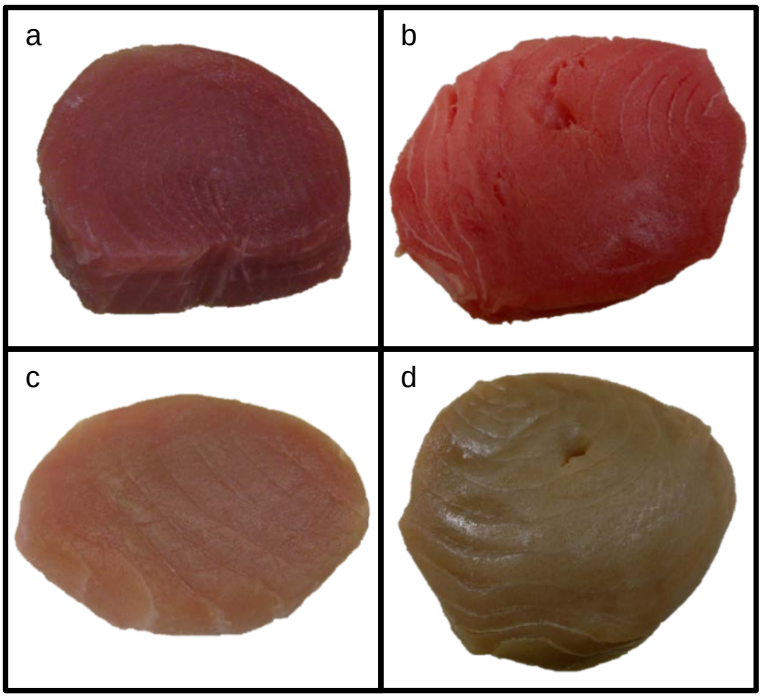

Figure 10 The colour difference between the treated and untreated tuna rounds of T_0 and T_8: a) OI Fx1 +CO T_0; b) OI Fx1 +CO T_8; c) OI Fx1 -CO T_0; and d) OI Fx1 -CO T_8.

Combined Fx1 and Fx2 treatments - Combined Fx1 and Fx2 treatment data revealed that the hue angle indicates that all the treatments results in tuna muscle that browns to some extent (increase in hue angle over time). This is reiterated by the b* values which all follow a similar upward trend over time, with the Fx2 -CO samples having the highest b* values (more brown) and the Fx1 +CO samples having the lowest (less brown). This was expected since the two freeze/thaw cycles that the Fx2 -CO samples underwent would have exaggerated the browning and colour loss caused by freezing and thawing (Añón & Cavelo, 1980; Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012). The Fx1 +CO samples having the least damage to the muscle as they were only frozen and thawed once and were also treated with CO giving them the lowest b* values (least amount of browning). This is reiterated by the a* values where the Fx1 +CO treatment has higher a* values than the Fx2 +CO treatment and both the untreated treatments. The untreated samples yielded similar a* and chroma values, which revealed a decrease in redness and an increase in saturation i.e. the untreated samples became more brown/tan over time. For the treated samples the Fx1 treatment has higher initial a* and chroma values than the Fx2 treatments, indicating that the Fx1 treatments samples were a darker (more saturated) red initially than the Fx2 samples. This could have been due to the increased in damage to the proteins (myoglobin) (Chow *et al.,* 1988; Chow *et al.,* 1989; Leygonie *et al.,* 2012) and an increased loss of myoglobin (Añón & Cavelo, 1980) in the exudate caused by the second freeze/thaw cycle which resulted in less CO binding to the myoglobin and subsequently a lower carboxymyoglobin percentage and thus lower a* values. This is consistent to what was observed (Figs. 10 and 11). The decrease in a* and chroma values over

time and the erratic pattern of the Fx2 samples could be explained by microbial growth (Mancini & Hunt, 2005).

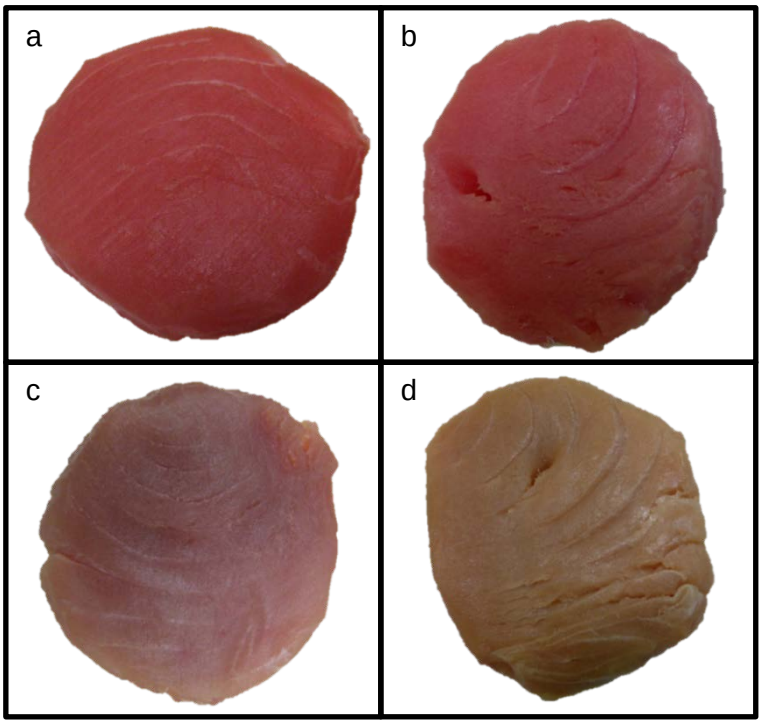

Figure 11 The colour difference between the treated and untreated tuna rounds of T_0 and T_8: a) OI Fx2 +CO T_0; b) OI Fx2 +CO T_8; c) OI Fx2 -CO T_0; and d) OI Fx2 –CO T_8.

CONCLUSION

It was clear from the results that the treatment of yellowfin tuna muscle with CO had a positive effect on the red colour. In all the treatments, exposure to CO led to an increase in the surface a* values (redness) of the tuna muscle. With regard to the OP samples, although the surface a* values were increased by the CO exposure, the effect was short lived, as the exposure to oxygen rapidly lead to oxidation of the carboxymyoglobin to metmyoglobin and a concurrent increase in b* values (browning). The OI samples, however, maintained the increased a* values over time. The number of freeze/thaw cycles also had an effect on the overall colour development. In the OP treatments the pronounced damage and the resulting discolouration caused by the two freeze/thaw cycles, was only apparent in the untreated samples with the effect being overshadowed in the treated samples by the longer CO exposure time and consequent higher carboxymyoglobin concentration, resulting in the OP Fx2 +CO samples having the highest initial surface a* values. The effect of the longer exposure time was cancelled out in the OI samples as all the samples were vacuum packed. The results showed that the second freeze/thaw cycle led to lower values in the treated samples, with the OI Fx1 +CO samples having the highest initial surface a* values. It

could thus be concluded that the OI Fx1 +CO treatment resulted in the best product with regard to surface colour development and stability.

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CHAPTER 5

EFFECT OF CARBON MONOXIDE ON THE LIPID AND PROTEIN OXIDATION OF YELLOWFIN TUNA (*THUNNUS ALBACARES*) MUSCLE

ABSTRACT

This study investigated the effect of carbon monoxide (CO) treatment of yellowfin tuna muscle on the lipid and protein oxidation, stored under aerobic (overwrap) and anaerobic (vacuum packed) conditions. The tuna had been subjected to either one or two freeze/thaw cycles. The CO treatment and the number of freeze/thaw cycles had no effect ($P>0.05$) on the lipid and protein oxidation of the yellowfin tuna. The vacuum packaged (anaerobic) samples showed lower thiobarbituric acid reactive substances (TBARS) values than those covered in overwrap (aerobic) and also retarded the onset of protein oxidation. The number of freeze/thaw cycles had an effect on the carbonyl concentration of both the vacuum packaged and overwrapped samples, with the twice frozen samples indicating accelerated protein oxidation in the yellowfin tuna muscle. It was recommended that vacuum packaging be used with the yellowfin tuna (both treated and untreated) and that the number of freeze/thaw cycles be kept to a minimum as this resulted in better quality tuna.

KEYWORDS: Yellowfin tuna; Carbon monoxide; Carboxymyoglobin; Lipid oxidation; Protein oxidation

INTRODUCTION

Oxidation of muscle foods, including seafood, is the leading cause of quality deterioration during processing and storage (Xiong, 2000). This is especially true for fish which are high in polyunsaturated fatty acids (PUFAs) (Strasburg *et al.,* 2007). PUFAs are particularly liable to oxidation (Gray, 1978; Apgar & Hultin, 1982; Gordon, 2003; Munasinghe *et al.,* 2005; Kristinsson *et al*., 2006a). There are also several pro-oxidants which naturally occur in fish muscle including the haem proteins (myoglobin and haemoglobin), copper and iron which will catalyse oxidative processes (Kristinsson *et al*., 2006a). The haem proteins are believed to be the two main pro-oxidants in meat (Richards *et al.,* 1998; Undeland *et al.,* 2004). It can also be assumed that if exsanguination was performed correctly, only myoglobin will play a significant role in muscle oxidation (Kristinsson *et al.,* 2006b).

Myoglobin has the ability to cause oxidation when oxygen is released from oxymyoglobin to form ferric (Fe^{3+}) metmyoglobin and super oxide anion radicals. Metmyoglobin can oxidise further to ferryl (Fe^{4+}) myoglobin which is highly reactive. It is thought that ferryl (Fe^{4+}) myoglobin is the main catalyst of oxidation (Richards & Hultin, 2002). Antioxidants have been found to successfully

retard oxidation (Richards *et al.,* 1998) by inhibiting the activity of the pro-oxidants, such as myoglobin (Richards *et al.,* 1998; Kristinsson, 2002).

Myoglobin remains in the reduced state when bound to CO and this reduced state does not readily oxidise (Kristinsson *et al.,* 2005). Thus, it is expected that the stabilisation of myoglobin with CO will reduce oxidation. It can further be surmised that fish muscle treated with CO may be less prone to oxidation, with several studies supporting this theory (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011).

Frozen storage of meat is widely accepted as a good method to lengthen the shelf-life of fish and other meat products (Kjærsgård *et al.,* 2006). However, during frozen storage various biochemical reactions can still occur. Fish are particularly susceptible to oxidation during frozen storage due to the high concentration of pro-oxidants (Soyer *et al.,* .2010). On the other hand, vacuum packaging of meat has been shown to reduce oxidation by limiting the amount of oxygen available for oxidative reactions (Min & Ahn, 2005). This however may not be the case since the oxidation of both lipids and proteins include oxidative and non-oxidative reactions (Gray, 1978; Gray & Monohan, 1992; Dean *et al.,* 1997).

The objective of this study was to investigate the effect that 100% CO treatment had on the lipid and protein oxidation of yellowfin tuna muscle under aerobic and anaerobic conditions subjected to a different freeze/thaw cycles. The oxidative state was determined by measuring the rate of oxidation of the lipids and proteins using the TBARS (Lynch & Frei, 1993) and DNPH (Oliver *et al*., 1987) methods, respectively.

MATERIALS AND METHODS

The experimental layout can be seen in Table 1. For the full experimental design, sample preparation and packaging refer to Chapter 3.

Table 1 Experimental layout from which the eight treatments were established

Treatments	1	2	3	4	5	6	7	8
Packaging type	OP				OI			
Tuna number	Tuna 1-7				Tuna 8-14			
Shelf-life trial	8 d				32 d			
Sampling	24 h (T_0-T_8)				96 h (T_0-T_8)			
Gas treatment	+CO		-CO		+CO		-CO	
Freeze/thaw cycle	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2

OP – oxygen permeable; OI – oxygen impermeable; +CO – treated with 100% CO gas; -CO – untreated; Fx1 –one freeze/thaw cycle; Fx2 - two freeze/thaw cycles.

Lipid oxidation – Quantification of MDA using the TBARS method

Sample preparation

The level of lipid oxidation over time was assessed by the 2-thiobarbituric acid (TBARS) extraction method (Lynch & Frei, 1993). Each tuna sample was cut into blocks (±1 cm x 1 cm) and a sub-sample (≈5 g) were wrapped in aluminium foil. This sub-sample was snap frozen in liquid nitrogen, to prevent any further oxidation occurring, and subsequently stored at -80°C until required for analysis (< one month).

As described by the method of Lynch and Frei (1993), a 1 g sample was cut from the snap frozen tuna and placed in 10 ml of 0.15 M KCl with 0.1 mM BHT solution, and homogenised (P-8, Kinematica AG Littau, Switzerland) for 30 sec. From this sample, 0.5 ml was pipetted into a separate test tube containing 0.25 ml of 50 mM NaOH with 1% (w/v) 2-thiobarbituric acid and 0.25 ml of 2.8% (w/v) trichloroacetic acid. The test tube was then incubated in a boiling waterbath for 10 min, removed and cooled in a bath of cold tap water. At this point the mixture in the test tube had turned varying shades of pink depending on the amount of lipid oxidation which had occurred. To extract the pink chromagen from the solution, 2 ml of *n*-butanol was pipetted into the test tube which was then vortexed for 30 sec. The sample was centrifuged (Allegra X22R, Beckman Coulter, Germany) at 2 240 x*g* for 25 min to clear all debris from the *n*-butanol extraction. The pink *n*-butanol extracted was then removed from the test tube using a Pasteur pipette and placed in a cuvette and the absorbance measured spectrophotometrically (CE 2021, Cecil, Cambridge, England) at 532 nm. The TBARS concentrations were calculated from a standard curve and expressed as mg malondialdehyde (MDA).kg^{-1} of meat (Shahidi & Zhong, 2005).

Standard curve

A standard curve was drawn up using known concentrations of 1,1,3,3-tetramethoxypropane (TMP) (0-68 µM) (Lynch & Frei, 1993). The various TMP concentrations were made by diluting varying amounts of TMP in distilled water. The standard solutions were prepared in the same way as the tuna samples above, except using the dilutions in lieu of the tuna i.e. 0.5 ml of each dilution was added to the test tubes containing 0.25 ml of 50 mM NaOH with 1% (w/v) 2-thiobarbituric acid and 0.25 ml of 2.8% (w/v) trichloroacetic acid. The incubation, extraction and absorbances were conducted as described for the samples above. The values obtained were used to draw up a standard curve of known TMP concentrations that could be used to calculate the amount of TBARS in each sample.

Protein oxidation – Quantification of carbonyls using DNPH

The amount of protein oxidation over time was determined using the protein carbonyl concentration with the derivation of DNPH as described by Oliver *et al*. (1987). Each tuna sample was cut into

blocks (±1 cm x 1 cm) and a sub-sample (≈5 g) were wrapped in aluminium foil, snap frozen and stored at -80°C until required for analysis (< one month).

As described by the method of Oliver *et al.* (1987) 1 g sample was cut from the snap frozen tuna and placed in 10 ml of a 0.15 M KCl with 0.1 mM BHT solution and homogenised (P-8, Kinematica AG Littau, Switzerland) for 30 sec. Two equal 0.1 ml aliquots were taken from the homogenised solution and pipetted into two separate vials. Each aliquot was incubated with 10% trichloroacetic acid (TCA) and centrifuged for 5 min at 2 240 x*g* (Allegra X22R, Beckman Coulter, Germany). The supernatant on the surface was discarded. One pellet was treated with 1 ml 2 N HCl and the other with equal volumes (0.5 ml) of 2 N HCl and 0.2% (w/v) dinitrophenylhydrazine (DNPH). Both samples were incubated at room temperature for 60 min and then precipitated with 10% TCA (w/v) and centrifuged for 5 min at 2 240 x*g.* The supernatant of both was again discarded and the pellets were washed twice with ethanol:ethyl acetate (1:1) to eliminate any residual lipids. The pellets were then dissolved in 2 ml 8 M urea and 20 mM sodium phosphate buffer (pH 6.5). The samples were again centrifuged for 2 min at 2 240 x*g*. The supernatant from each pellet was transferred into two separate cuvettes and the absorbance of each was measured using a spectrophotometer (CE 2021, Cecil, Cambridge, England) at 320 nm. The carbonyl concentration was calculated by subtracting the absorbance of the HCl control sample from the DNPH sample, using 21.0 $mM.cm^{-1}$ as the absorption coefficient. The protein concentration was determined on the HCl control sample using a bicinchoninic acid (BCA) protein assay kit and measuring the absorbance spectrophotometrically (CE 2021, Cecil, Cambridge, England) at 562 nm. The two absorbance values obtained were used to determine the concentration of protein oxidation which was expressed as nM $DNPH.mg^{-1}$ protein.

Statistical analysis

The protein and lipid oxidation determination involved two main effects, time and treatment, as well as the seven tuna used for each type of packaging. The data was analysed with a two-way repeated measure analysis of variance (RMANOVA) using the general linear models (GLM) procedure. The model for the RMANOVA of the data is indicated by the following equation:

$$y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \gamma_{(i)k} + \delta_{(j)k} + \varepsilon_{ijk}$$

The terms within the model are defined as:

y_{ijk}= observation k in the i^{th} treatment at the j^{th} time

μ - the overall mean;

α_i - the effect of the i^{th} treatment (fixed effect);

β_j - the effect of the j^{th} time (fixed effect);

$(\alpha\beta)_{ij}$ - the interaction of the i^{th} treatment with the j^{th} time;

$\gamma_{(i)k}$ - the effect of the k^{th} tuna on the i^{th} treatment group (variable effect);

$\delta_{(i)k}$ - the effect of the k^{th} tuna on the i^{th} time period (variable effect); and

ε_{ijk} - the error associated with the time, treatment and tuna.

The RMANOVA is performed on the assumption that the data has compound symmetry on the time and treatment correlation. The data was also assumed to be normally distributed. The least significant interactions (LSD) were calculated at a 95% significance level to compare the treatment means i.e. results were defined as significant when P≤0.05 and not significant when P>0.05. If the interaction between the main effects, time and treatment, was not significant (P>0.05) then the main effects could be interpreted separately. In the case where the interaction is found to be significant, a Bonferroni pair wise comparison was done to identify the specific interactions. STATISTICA version 10 (StatSoft South Africa Pty (Ltd)) was used to analyse the data collected for each treatment.

RESULTS

Lipid oxidation

Oxygen permeable (OP) treatments

Fx1 treatments - The RMANOVA for the OP Fx1 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 1a). The data could thus be combined and then interpreted (Fig. 1b) allowing for trends in the data to be seen. From the combined data (Fig. 1b) it can be seen that there is an overall increase in the TBARS values over time. Initially the values decrease, followed by a rapid increase, with the values levelling-off/decreasing slightly after T_5.

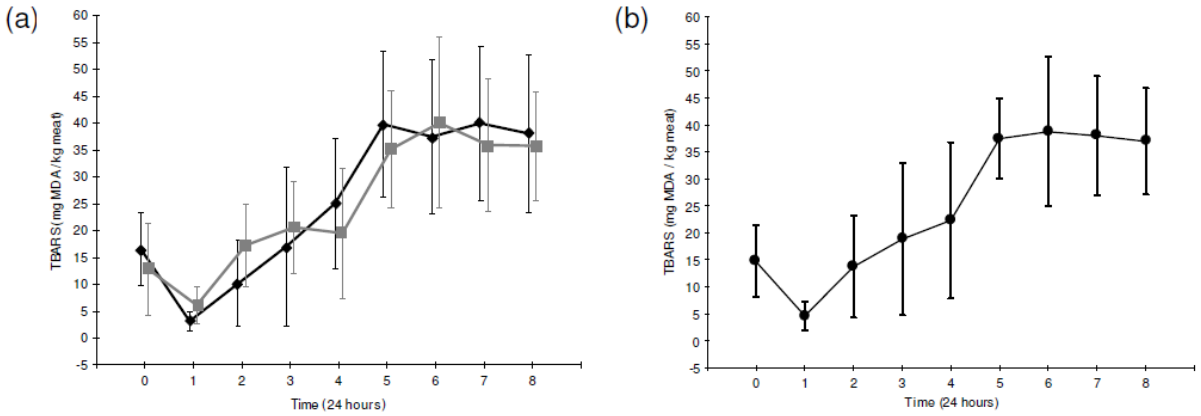

Figure 1 (a) The mean TBARS values (with confidence intervals) for the (◆) OP Fx1 +CO and (■) OP Fx1 -CO treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for the OP Fx1 treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

Fx2 treatments - The RMANOVA for the OP Fx2 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 2a). The data could thus be combined and then interpreted (Fig. 2b) allowing for trends in the data to be seen. The combined data (Fig. 2b) shows an overall increase in the TBARS values over time. The TBARS values initially decrease slightly and then increase rapidly, finally level-off/decrease at T_6.

Combined Fx1 and Fx2 treatments - The combined data for the OP treatments allows for the comparison of the effect of the number of freeze/thaw cycles on the lipid oxidation (TBARS values) of the tuna samples. The RMANOVA for the combined OP treatments (Fx1 and Fx2) showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 3a). The data could thus be combined and then interpreted (Fig. 3b) allowing for trends in the data to be seen. The combined data of all the OP treatments (Fig. 3b) shows that initially there is a slight decrease in the TBARS values, followed by a rapid increase and then a levelling-off/slight decrease in the TBARS values at T_6.

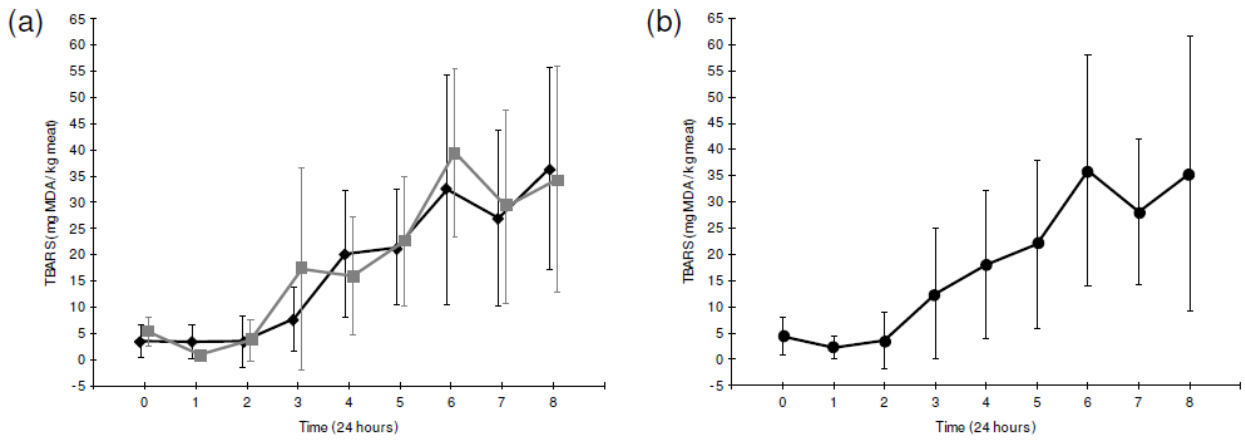

Figure 2 (a) The mean TBARS values (with confidence intervals) for the (◆) OP Fx2 +CO and (■) OP Fx2 -CO treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for the OP Fx2 treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

Oxygen impermeable (OI) treatments

Fx1 treatments - The RMANOVA for the OI Fx1 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 4a). The data could thus be combined and then interpreted (Fig. 4b) allowing for trends in the data to be seen. The combined data shows an increase in the TBARS values until T_5 after which the TBARS values decrease, resulting in a slight overall increase in TBARS values over time.

Fx2 treatments - The RMANOVA for the OI Fx2 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 5a). The data could thus be combined and then interpreted (Fig. 5b) allowing for trends in the data to be seen. The combined data shows a very slight overall increase in the TBARS values over time. Initially there is little change in the TBARS values, the TBARS values then begin to increase (T_3) until T_5, after which the values decrease until T_7.

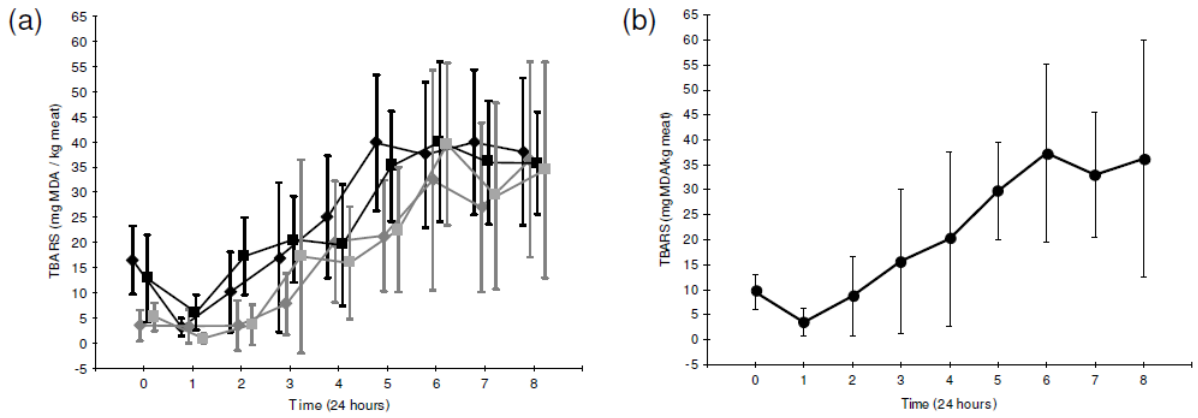

Figure 3 (a) The mean TBARS values (with confidence intervals) for all the OP treatments, (◆) OP Fx1 +CO, (■) OP Fx1 -CO, (◆) OP Fx2 +CO and (■) OP Fx2 -CO, measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for all the OP treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

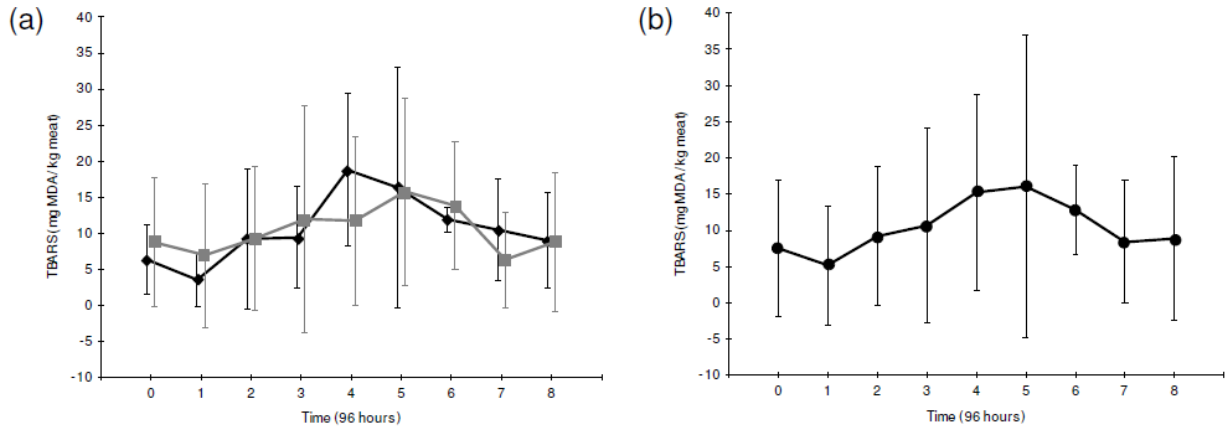

Figure 4 The mean TBARS values (with confidence intervals) for the (◆) OI Fx1 +CO and (■) OI Fx1 -CO treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for the OI Fx1 treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins.

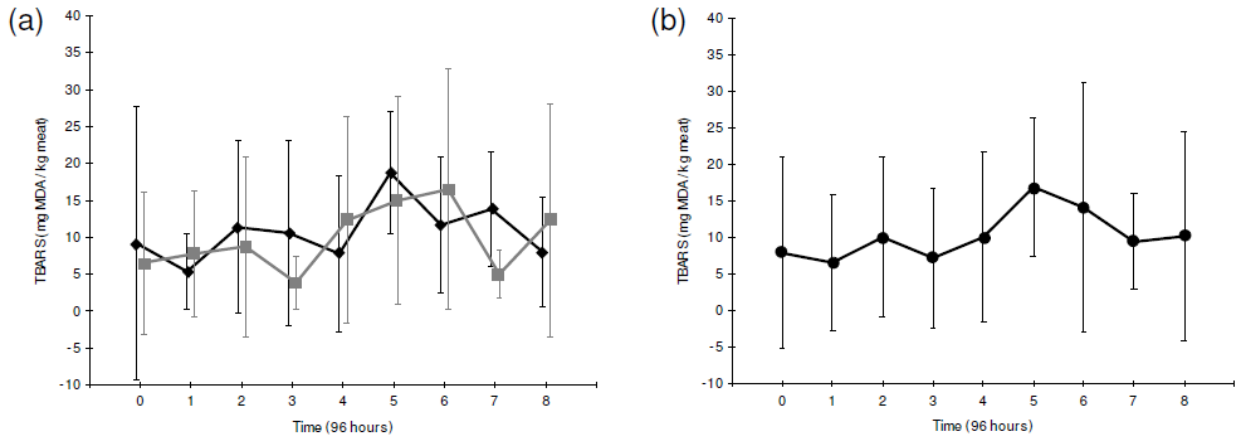

Figure 5 (a) The mean TBARS values (with confidence intervals) for the (◆) OI Fx2 +CO and (■) OI Fx2 -CO treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for the OI Fx2 treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins.

Combined Fx1 and Fx2 treatments - The combined data for the OP treatments allows for the comparison of the effect of the number of freeze/thaw cycles on the lipid oxidation (TBARS values) of the tuna samples. The RMANOVA for the combined OI treatments (Fx1 and Fx2) showed that there was no significant interaction ($P>0.05$) between the main effects (time and treatment) (Fig. 6a). The data could thus be combined and then interpreted (Fig. 6b) allowing for trends in the data to be seen. The combined data for all the OI treatments shows a very slight overall increase in the TBARS values over time.

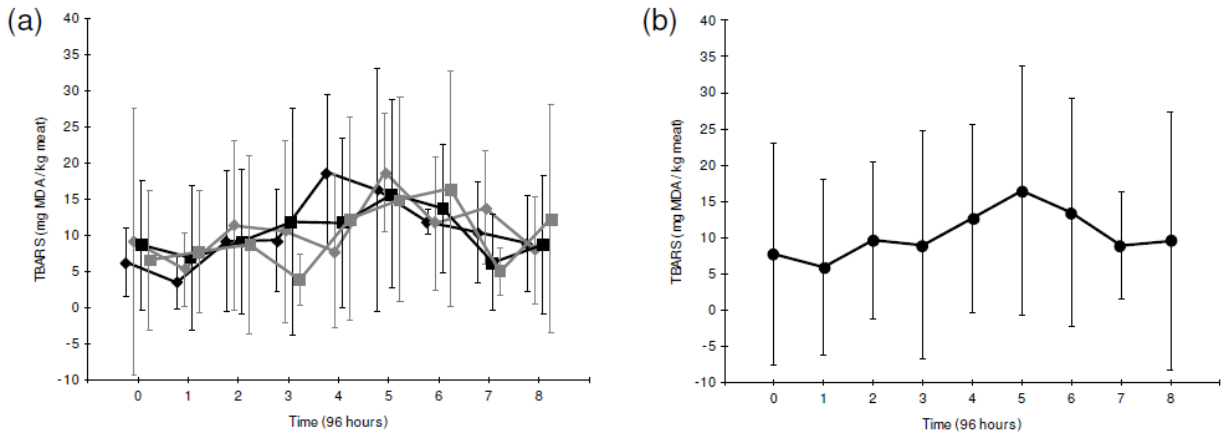

Figure 6 (a) The mean TBARS values (with confidence intervals) for all the OI treatments, (◆) OI Fx1 +CO, (■) OI Fx1 -CO, (◆) OI Fx2 +CO and (■) OI Fx2 -CO, measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins; (b) the combined TBARS values (with confidence intervals) for all the OI treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins.

Comparison between OP and OI treatments - The OP and OI samples could not be compared statistically as different yellowfin tuna were used in each case and because the shelf-life time periods differed (8 d vs. 32 d). However, the data could still be combined to highlight interactions between the packaging types (Fig. 7). Since there was no significant interaction found between the four treatments of both the OP and OI samples, the average of all four treatments in each case was taken and plotted on the same graph (Fig. 7). It can clearly be seen that the TBARS values for the final OP samples are considerably higher than those for the OI final samples, even though the OI samples were subjected to considerably longer shelf-life studies.

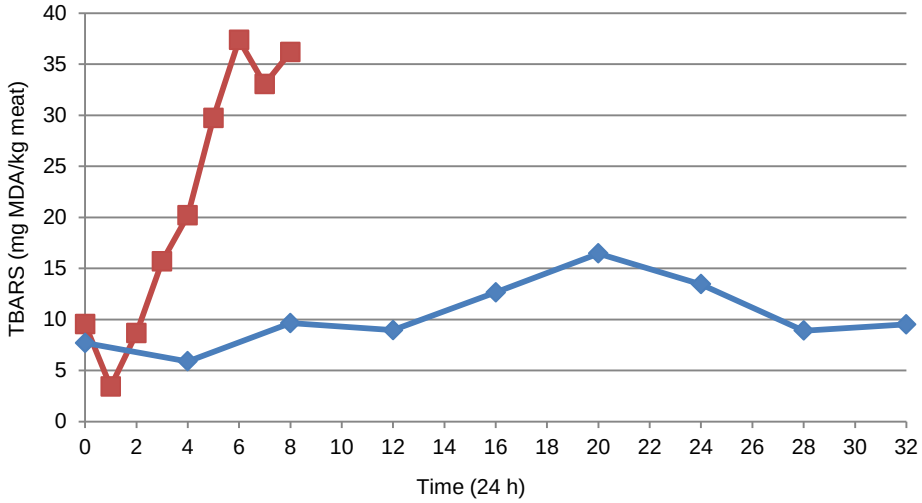

Figure 7 Comparison of (■) OP and (◆) OI treatments.

Protein oxidation

Oxygen permeable (OP) treatments

Fx1 treatments - The RMANOVA for the OP Fx1 treatments showed that there was no significant interaction ($P>0.05$) between the main effects (time and treatment) (Fig. 8a). The data could thus be combined and interpreted together (Fig. 8b), allowing for trends in the data to be seen. The combined data shows an initial increase in the carbonyl values, followed by a decrease at T_2 and finally a levelling-off (T_5) of the values. Overall there was a slight increase in the carbonyl values.

Fx2 treatments - The RMANOVA for the OP Fx2 treatments showed that there was no significant interaction ($P>0.05$) between the main effects (time and treatment) (Fig. 9a). The data could thus be combined and interpreted together (Fig. 9b). This allows for trends in the data to be seen. The combined values showed little to no change in the carbonyl concentration over time.

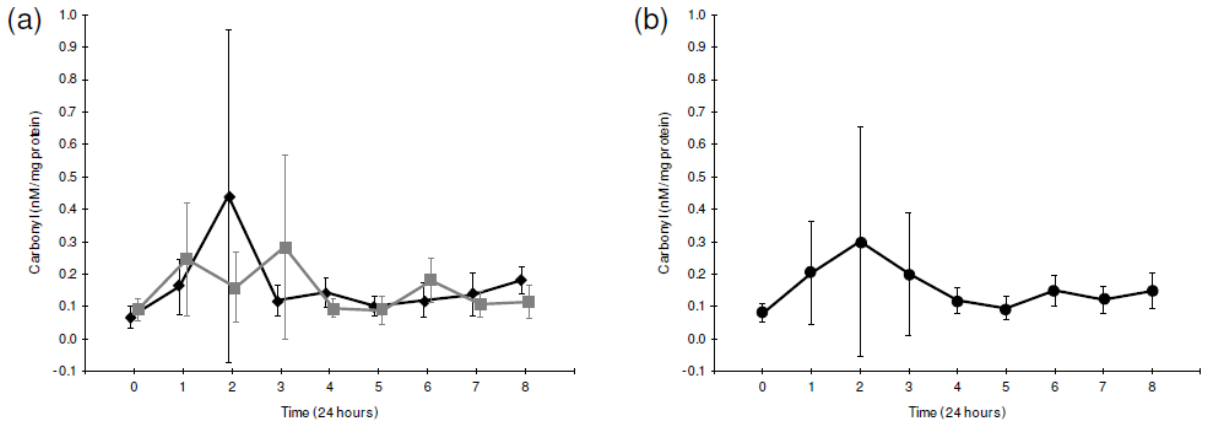

Figure 8 (a) The mean carbonyl values (with confidence intervals) for the (◆) OP Fx1 +CO and (■) OP Fx1 -CO treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins; (b) the combined carbonyl values (with confidence intervals) for the OP Fx1 treatments measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

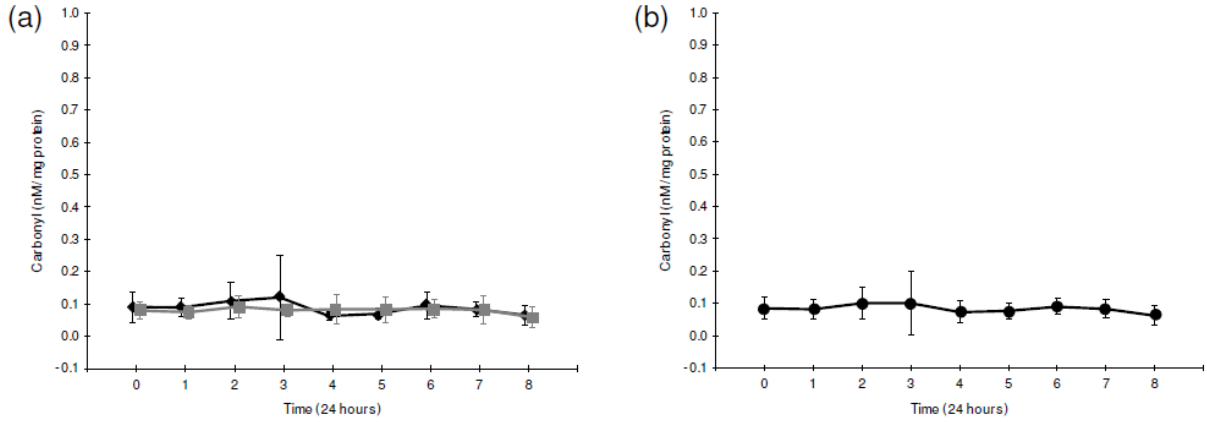

Figure 9 (a) The mean carbonyl values (with confidence intervals) for the (◆) OP Fx2 +CO samples and (■) OP Fx2 -CO samples measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins; (b) the combined carbonyl values (with confidence intervals) for the OP Fx2 samples measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

Combined Fx1 and Fx2 treatments - The combined data for the OP treatments allows for the comparison of the effect of the number of freeze/thaw cycles on the protein oxidation (carbonyl concentration) of the tuna samples. The RMANOVA for the combined OP treatments (Fx1 and Fx2) showed that there was a significant interaction ($P \leq 0.05$) between the main effects (time and treatment) (Fig. 10). The combined data shows a similar trend and similar values for all the OP treatments over time, except at time points T_1, T_2 and T_3. The Bonferroni pairwise analysis shows that only the peak of OP Fx1 +CO values at T_2 differed significantly ($P \leq 0.05$) from all the rest and that the peaks at T_1 and T_3 did not differ significantly ($P > 0.05$). Overall there was little to no overall increase in the carbonyl values over time for all treatments.

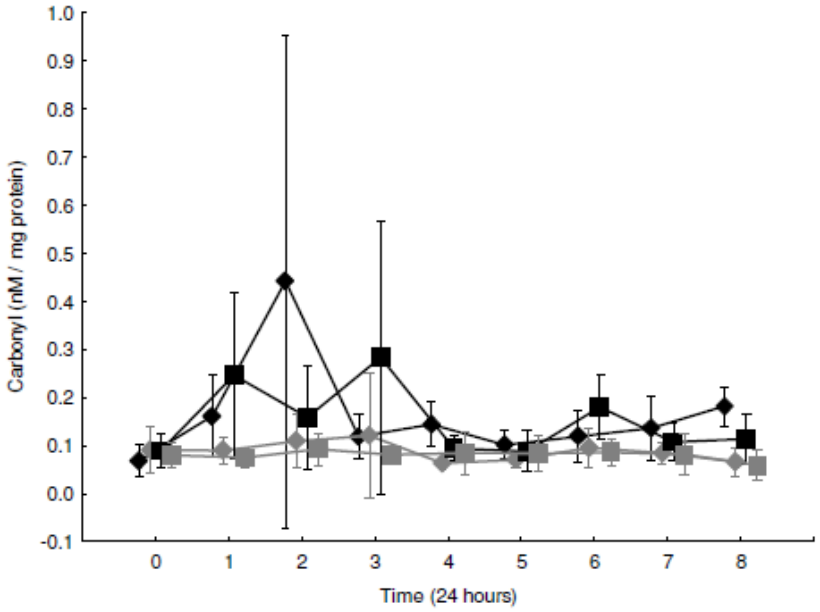

Figure 10 The mean carbonyl values (with confidence intervals) for all OP treatments, (◆) OP Fx1 +CO, (■) OP Fx1 -CO, (◆) OP Fx2 +CO and (■) OP Fx2 -CO measured every 24 h for 8 d (T_0-T_8) on yellowfin tuna loins.

Oxygen impermeable (OI) treatments

Fx1 treatments - The RMANOVA for the OI Fx1 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 11a). The data could thus be combined and then interpreted (Fig. 11b), allowing for trends in the data to be seen. The combined data showed and overall decrease in the carbonyl values over time with a peak at T_3.

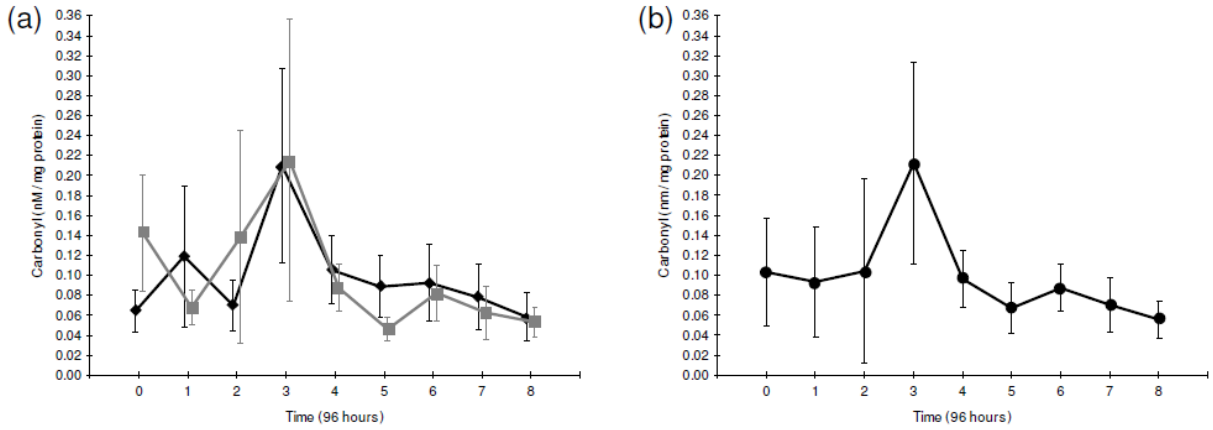

Figure 11 (a) The mean carbonyl values (with confidence intervals) for the (◆) OI Fx1 +CO and (■) OI Fx1 -CO treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins; (b) the combined carbonyl values (with confidence intervals) for the OI Fx1 treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins.

Fx2 treatments - The RMANOVA for the OI Fx2 treatments showed that there was no significant interaction (P>0.05) between the main effects (time and treatment) (Fig. 12a). The data could thus be combined and then interpreted (Fig. 12b) allowing for trends in the data to be seen. The combined data shows an overall increase in the carbonyl values over time with a peak in the carbonyl concentration at T_1.

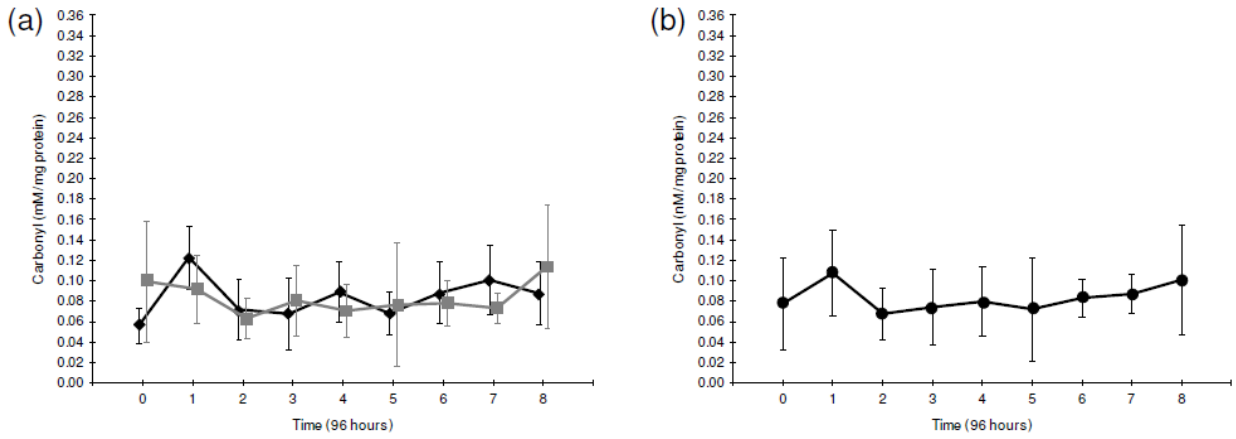

Figure 12 (a) The mean carbonyl values (with confidence intervals) for the (◆) OI Fx2 +CO and (■) OI Fx2 -CO treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins; (b) the combined carbonyl values (with confidence intervals) for the OI Fx2 treatments measured every 96 h for 32 d (T_0-T_8) on yellowfin tuna loins.

Combined Fx1 and Fx2 treatments - The RMANOVA for the OI treatments showed that there was a significant interaction (P≤0.05) between the main effects (time and treatment) (Fig. 13). The Fx1 and Fx2 treatments have a similar trend over time except for the Fx1 samples showing a sharp increase in carbonyl concentration at T_3. The Bonferroni analysis shows that the peaks of the Fx1 treatments at T_3 differs significantly (P>0.05) from the Fx2 treatments.

Comparison between OP and OI treatments

The OP and OI samples could not be compared statistically as different yellowfin tuna were used in each case and due to the shelf-life time periods differing (8 d vs. 32 d). However, the data could still be combined to highlight differences between the packaging types (Fig. 14). The treatments were compared using their combined data. The Fx2 samples for both the OP and OI treatments show little to no increase in carbonyl values whereas the Fx1 treatments show increases in carbonyl values.

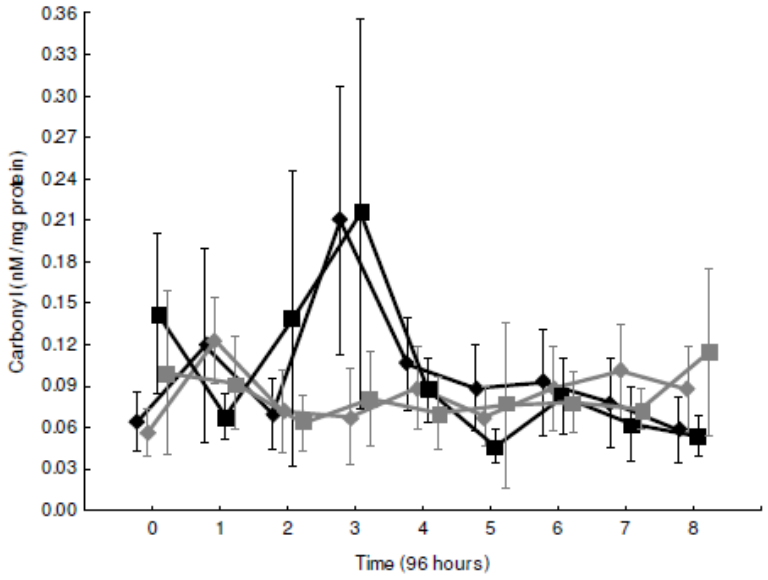

Figure 13 The mean carbonyl values (with confidence intervals) for all OI treatments, (◆) OI Fx1 +CO, (■) OI Fx1 -CO, (◆) OI Fx2 +CO and (■) OI Fx2 -CO measured every 96 h for 8 d (T_0-T_8) on yellowfin tuna loins.

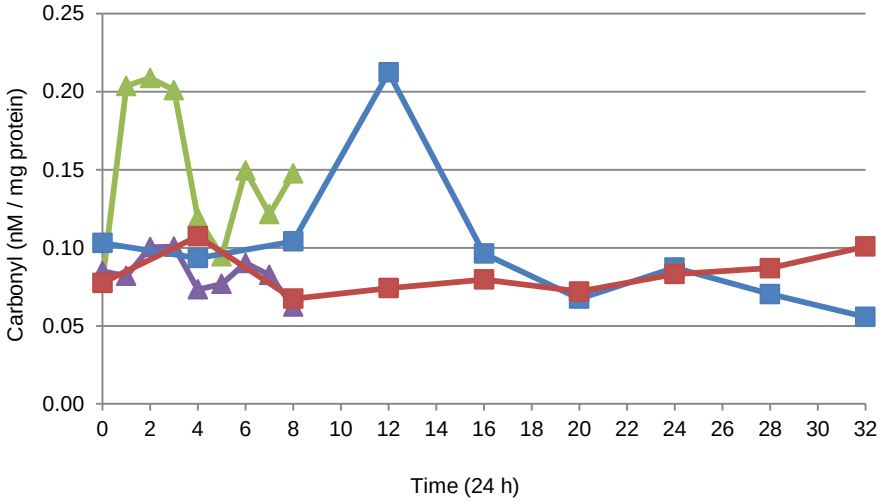

Figure 14 Comparison of OP and OI treatments: (▲) OP Fx1, (▲) OP Fx2, (■) OI Fx1, (■) OI Fx2.

DISCUSSION

Lipid oxidation

The TBARS method for assessing secondary lipid oxidation is frequently employed to test the extent of lipid oxidation in muscle foods even though it lacks specificity and sensitivity (Raharjo & Sofo, 1993; Shahidi & Zhong, 2005). It has also been noted that due to interference, the TBARS method should only be used to assess the extent of lipid oxidation in general (Gray & Monohan, 1992). The method was thus ideal for this study as only a general overview of the results was

required i.e. the overall amount of lipid oxidation over time. The data from this study showed a large amount of variation in the lipid oxidation results which could be attributed to the large amount of biological variation in chemical composition between the tuna (Baron *et al.,* 2007). This variation was also seen in other studies where the TBARS method was used to assess the lipid oxidation in muscle foods (Richards & Hultin, 2002; Richards *et al*., 2002; Garner, 2004; Soyer *et al.,* 2012).

The results showed that there were no significant interactions (P>0.05) between either the OP or OI treatments (Figs. 1b, 2b, 4b and 5b), nor was there any significant interaction found when the four OP and the four OI treatments were analysed together (Figs. 3b and 6b, respectively). The number of freeze/thaw cycles did thus not have a statistically significant effect on the TBARS values of the yellowfin tuna samples, regardless of whether they were treated or not. The overall trend of all the combined data of the four OP treatments and four OI treatments (Figs. 3b and 6b, respectively) will thus be discussed.

The overall increase observed in the TBARS values over time for the OP treatments (Fig. 3b) was expected since the plastic overwrap film used for the packaging of the samples was oxygen permeable. Lipid oxidation primarily involves autoxidative reactions (Gray, 1978) and thus, in the presence of oxygen, lipid oxidation proceeds spontaneously and will lead to increased TBARS values (Gordon, 2003). This is especially true for fish which are high in PUFAs (Strasburg *et al.,* 2007), PUFAs being particularly liable to oxidation (Gray, 1978; Apgar & Hultin, 1982; Gordon, 2003; Munasinghe *et al.,* 2005; Kristinsson *et al*., 2006a). Fish muscle also has a high concentration of pro-oxidants which enhance lipid oxidation (Kristinsson *et al*., 2006a). This, coupled with the PUFAs, explains the sudden sharp increase in lipid oxidation early in the shelf-life trial. The effect of the pro-oxidants was also most likely exaggerated by the damaged cause to the cell membranes by ice crystal formation during freezing (Leygonie *et al.,* 2012). This damage results in the release of pro-oxidants, especially myoglobin, when the meat is thawed, resulting in accelerated lipid oxidation and subsequently, higher TBARS values (Benjakul & Bauer, 2001). It has also been postulated that chemical reactions can continue during frozen storage. These reactions could initiate primary lipid oxidation which subsequently leads to accelerated secondary oxidation, the products of which are measured by the TBARS method, upon thawing as seen in these results (Owen & Gray, 1975; Leygonie *et al.,* 2012). This concurs with findings in other studies where accelerated lipid oxidation was observed in frozen and thawed meat which was also subsequently subjected to refrigerated shelf-life studies (Akamittath *et al.*, 1990; Hansen *et al.,* (2004).

With regards to the effect of the 100% CO treatment on the OP samples, the results from this study are similar to those of Garner (2004) who found that there was an overall increase in the mean TBARS of Spanish mackerel fillets of the treated (100% CO) and untreated fillets when sampled over an 8 d shelf-life trial. However, in that study it was found that the 100% CO treatment did have a significant effect on the oxidation of the lipids. The treated samples had lower overall TBARS values after 8 d compared to the untreated samples (Garner, 2004). The

differences seen between these results and those of this study may be attributed to several factors being different between the two studies. Garner (2004) used a different method of application and a longer application time employed, the fillets were not frozen prior to being used and a different fish species (thus containing different levels of pro-oxidants) was used. Garner (2004) treated the samples by placing them in bags flushed with 100% CO and treating the fillets for 24 h. This is considerably longer than the 150 min used in the current study. The longer exposure time may have allowed a higher quantity of CO to bind to the myoglobin, resulting in better stabilisation of the lipids. However, it is more likely that the frozen storage prior to the shelf-life study had the greatest effect on the difference in the results as these results are similar to those in studies were the fish had been frozen prior to treatment (Akamittath *et al.*, 1990; Hansen *et al.,* 2004).

The overall increase in the TBARS values of the OI treatments is consistent with results found in vacuum packaged beef samples where an overall increase in TBARS values over time was also observed (Lynch *et al.,* 1999). It is however expected that in the absence of oxygen, lipid oxidation would not occur. There are two possible reasons why, even though the samples were vacuum packed in oxygen impermeable packaging, lipid oxidation still occurred. The first is that it was found by Lynch *et al.* (1999) that that vacuum packaging does not remove all the oxygen from the packaging. Thus any residual oxygen in the packaging could have led to the lipid oxidation which was seen in the results of this study. The second is that the TBARS method measures secondary lipid oxidation products and not primary lipid oxidation products (Draper & Hadley, 1990; Gray & Monohan, 1992; Leygonie *et al.,* 2012). These secondary reactions involve both oxidative and non-oxidative reactions (Gray, 1978; Gray & Monohan, 1992). Thus in the absence of oxygen, secondary lipid oxidation will still occur. Furthermore, as mentioned for the OP treatments, there is some evidence that suggests primary lipid oxidation could occur during frozen storage (initial frozen storage of the whole tuna). This leads to accelerated secondary oxidation during thawing (Owen & Gray, 1975; Leygonie *et al.,* 2012), the products of which, as mentioned, are measured by the TBARS method (Draper & Hadley, 1990; Gray & Monohan, 1992; Leygonie *et al.,* 2012).

Overall comparison between OP and OI treatments

The overall comparison of the OP and OI treatments (Fig. 7) clearly shows considerably higher TBARS values for the OP treatments compared to those of the OI treatments, even though the OP treatments were subjected to a considerably shorter shelf-life study. It has been noted that the most obvious precaution to take against lipid oxidation is the removal of oxygen (Ladikos & Lougovois, 1990). The high TBARS values seen in the OP samples is most likely due to the cumulative effect caused by both the oxidative and non-oxidative secondary reactions of lipid oxidation (Gray, 1978; Gray & Monohan, 1992), resulting in higher TBARS values. This is in agreement with the results seen by Hwang and Regenstein (1988) and Khalil and Mansour (1998) who showed that vacuum packaging retarded lipid oxidation.

Protein oxidation

The DNPH method is widely employed as a general overall measure of protein oxidation in food systems (Estévez *et al.,* 2009; Estévez, 2011), including fish and fish products (Lund *et al.,* 2011). The method was ideal for this study as only a general overview of the results was required i.e. the overall amount of protein oxidation over time. The method has been described as being robust and accurate for the measurement of protein oxidation (Estévez *et al*., 2008). The variation in the data is consistent with results where the DNPH method was also employed (Lund *et al.,* 2007). The large amount of variation seen in the results may also be due to the amount of biological variation between the tuna (Baron *et al.,* 2007).

The results showed that there were no significant interactions ($P>0.05$) between the OP and OI treatments (Figs. 8b, 9b, 11b and 12b) and thus it could be assumed that the 100% CO treatment has no effect on the protein oxidation of yellowfin tuna muscle regardless of the number of freeze/thaw cycle. The combined data showed that the OP (Fig. 8b) and OI (Fig. 11b) Fx1 treatments showed an initial increase in the carbonyl concentration over time, indicating that protein oxidation occurred. The combined data for the OP (Fig. 9b) and OI (Fig. 12b) Fx2 treatments showed that there was little to no change in the carbonyl concentration over time, with the peak at T_1 being explained by variation caused by the method used (Lund *et al.,* 2011).

In the case of the OP Fx1 treatments, the increase in the carbonyl concentration is to be expected since, in the presence of oxygen, protein oxidation proceeds spontaneously with the overall reaction of protein oxidation being oxygen dependant (Dean *et al.,* 1997). After the initial increase in the carbonyl concentration, it begins to decrease over time (T_2). This decrease in the concentration may be due to the protein carbonyls (that which is measured by the DNPH method) formed, reacting further with other cellular constituents and could thus not be detected (Baron *et al.,* 2007). The involvement of protein carbonyls in other biologically significant interactions has also been noted (Estévez, 2011). However, in the case of the OI Fx1 treatments the increase in the carbonyl concentration is contradictory to what is expected. In the absence of oxygen, protein oxidation would not be expected to occur. As with lipid oxidation, this could be due to not all the oxygen being removed during vacuum packaging (Lynch *et al.,* 1999) and it may be that residual oxygen inside the packaging lead to the protein oxidation. Another possible reason is that although protein oxidation overall is oxidative, the process involves both aerobic and anaerobic processes (Dean *et al.,* 1997). It may be that the carbonyls resulting from anaerobic reactions reacted with other cellular constituents (Baron *et al.,* 2007) and could thus result in the decrease in the concentration.

In the case of the OP Fx2 treatments, the absence of protein oxidation is contradictory to what is expected since, as mentioned, in the presence of oxygen, protein oxidation proceeds spontaneously with the overall reaction of protein oxidation being oxygen dependant (Dean *et al.,* 1997). A possible reason for the lack of observed protein oxidation is that the two freeze/thaw

cycles emphasised the effect that freezing and thawing has on increasing protein oxidation (Leygonie *et al.,* 2011). Thus the protein oxidation reactions have proceeded further and the protein carbonyls which formed have been used in other reactions and are no longer detectable (Baron *et al.,* 2007). However, in the case of the OI Fx2 treatments, the lack of protein oxidation is to be expected since in the absence of oxygen, protein oxidation is not expected to occur (Lynch *et al.,* 1999). However, in light of the above results, the more likely explanation is that the lack of observed protein oxidation is due to, as mentioned, the two freeze/thaw cycles emphasising the effect that freezing and thawing have on increasing protein oxidation. The protein oxidation reactions had thus proceeded further and the carbonyls (measured by the DNPH method) were no longer detectable.

The combined results for the OP treatments showed that there was a significant interaction ($P>0.05$) between the treatments. The Bonferroni pairwise analysis showed that only the peak at T_2 (Fig. 10), differed significantly from the other treatments. This would indicate that only the Fx1 +CO treatment differed significantly from the other treatments. However, if you were to combine the Fx1 and Fx2 values, respectively (as done in Figs. 8b & 9b) and then compare the Fx1 and Fx2 treatments, there would probably be a significant interaction between the two. The dip (negative peak) at T_2 resulted in the Fx1 -CO treatment not being significantly different from the Fx2 treatments. This dip in the result is most likely caused by the DNPH method used and this phenomenon has been noted by Lund *et al*. (2011). Thus it will be assumed that the Fx1 and Fx2 treatments differed significantly. Leygonie *et al.* (2011) made similar conclusions when using the DNPH method. The combined results for the OI treatments (Fig. 13) showed that there was a significant interaction ($P>0.05$) between the treatments.

Since the results of the Fx1 and Fx2 treatments for both the OP and OI treatments differed significantly, it showed that the number of freeze/thaw cycles did have an effect on the protein oxidation of yellowfin tuna muscle. The results showed an initial increase in the carbonyl concentration for the Fx1 treatments and little to no change in the carbonyl concentrations over time for the Fx2 treatments. Although it would be assumed that two freeze/thaw cycles inhibited protein oxidation, the more likely explanation is that the two freeze/thaw cycles accentuated the effect that freezing and thawing have on increasing protein oxidation (Leygonie *et al.,* 2012).

Comparison between OP and OI treatments

The comparison of the OP and OI treatments indicate that the Fx1 treatments both show increased carbonyl concentrations whereas the Fx2 treatments show little to no increase. As mentioned above, the lack of increase in carbonyl concentration, may be misleading and is most likely not due to a lack of protein oxidation. Although an increase in carbonyl values was seen in both the OP and OI Fx1 treatments, the onset of lipid oxidation took longer in the OI treatments. Vacuum

packaging would thus be the better choice of the two types of packaging used when considering the inhibition/retardation of protein oxidation.

CONCLUSIONS

The result of the lipid oxidation of all the treatments showed that neither the CO treatment nor the number of freeze/thaw cycles (either Fx1 or Fx2) had a significant effect on the TBARS values over time. Although this was contradictory to results found in other studies, none used the same combination of method of application, application time or species, which could explain the differences in the results. The packaging did play a substantial role in the amount of lipid oxidation which occurred. The result clearly showed that the OI treatments resulted in much lower overall TBARS values than that of the OP treatments.

The results for the protein oxidation showed that the CO treatment had no significant ($P>0.05$) effect on the carbonyl concentration over time. The results did however show a difference with regards to the number of freeze/thaw cycles and the packaging used. In both the OP and OI Fx1 treatments protein oxidation was observed but the onset of oxidation was retarded in the OI treatments. It was expected that the two freeze/thaw cycles would increase the protein oxidation and lead to higher carbonyl values. This was however not the case, with no protein oxidation being observed for the OP and OI Fx2 treatments. It was postulated that this was due to the two freeze/thaw cycles emphasising the effect that freezing and thawing has on increasing the rate of protein oxidation. The protein oxidation reactions had thus proceeded further and the carbonyls produced (measured by the DNPH method) had reacted with other biological constituents and were thus no longer detectable.

From the above results it is recommended that vacuum packaging conditions be used when packaging yellowfin tuna as significantly lower lipid oxidation was observed and the onset of protein oxidation was also retarded in comparison to the overwrap. It is also recommended that the number of freeze/thaw cycles be kept to a minimum when dealing with yellowfin tuna, especially with regards to protein oxidation. Furthermore, it is suggested that a more sensitive and specific method be used for protein oxidation determination. A method which could detect protein biomarkers such as α-aminoadipic and γ-glutamic semialdehydes (Daneshvar *et al.,* 1997) would be advisable. Although the CO treatment as determined in this study had no effect on the amount of lipid or protein oxidation which occurred, it was found in Chapter 4 that it improved the colour of the yellowfin tuna. The use of CO on yellowfin tuna can thus still be advocated with regards to colour development and stability but not with regards to inhibiting lipid and protein oxidation.

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CHAPTER 6

RELATIONSHIP BETWEEN MYOGLOBIN OXIDATION AND LIPID OXIDATION IN CARBON MONOXIDE TREATED YELLOWFIN TUNA (*THUNNUS ALBACARES*) MUSCLE

ABSTRACT

An investigation of the relationship between surface a* and b* colour ordinate values and TBARS values was done to determine the link between myoglobin oxidation and lipid oxidation in 100% carbon monoxide (CO) treated yellowfin tuna. The tuna was also subjected to aerobic and anaerobic storage conditions as well as to one or two freeze/thaw cycles. The a* and b* values were used to assess the oxidation of oxy- and carboxymyoglobin to metmyoglobin. In the majority of the treatments, there was a negative correlation between the a* values and a positive correlation between the b* values with the TBARS values, indicating that the oxidation of the myoglobin (decrease in a* values and increase in b* values) was concurrent with an increase in lipid oxidation (increase in TBARS values). In those treatments where both the a* and b* values showed a positive correlation to the TBARS values, it was concluded that the combination of vacuum packaging and CO treatment masked the visual indicator (browning) of myoglobin oxidation but that lipid oxidation and myoglobin oxidation still occurred simultaneously. It was thus surmised that there is a link between lipid oxidation and myoglobin oxidation in yellowfin tuna muscle.

KEYWORDS: Yellowfin tuna; Carbon monoxide; Carboxymyoglobin; Myoglobin oxidation; Lipid oxidation; CIE Lab; Shelf-life; Colour stability

INTRODUCTION

Correlations have been found between myoglobin oxidation and lipid oxidation in meat (Renerre, 2000). Research on tuna muscle has also shown that there is an interaction between myoglobin oxidation and lipid oxidation (Pivarnik *et al.,* 2011). Thus there is a relationship between pigment oxidation and lipid oxidation (Andersen *et al.,* 1990; Fraustman *et al*., 1992), with metmyoglobin formation being positively correlated to lipid oxidation (Chaijan *et al.,* 2006).

Fish is particularly liable to oxidation due to the high concentration of polyunsaturated fatty acids (Gray, 1978; Apgar & Hultin, 1982; Gordon, 2003; Munasinghe *et al.,* 2005; Kristinsson *et al*., 2006a; Strasburg *et al.,* 2007; Chaijan, 2008) and pro-oxidants found in the muscle (Kristinsson *et al*., 2006a). It is thought that the main pro-oxidant in fish muscle is myoglobin (Kristinsson *et al*., 2006a). Myoglobin has the ability to cause oxidation when oxygen is released from oxymyoglobin to form ferric (Fe^{3+}) metmyoglobin and super oxide anion radicals. Metmyoglobin can further oxidise to ferryl (Fe^{4+}) myoglobin which is highly reactive. This oxidised form of myoglobin is thought to be the main catalyst of oxidation (Richards & Hultin, 2002). When carbon monoxide

(CO) binds to myoglobin the resulting carboxymyoglobin is more stable than oxymyoglobin (>240 times more) (Sørheim *et al.,* 1997) and is thus more resistant to autoxidation (Sørheim *et al.,* 1997; Kristinsson *et al.,* 2005). It can therefore be postulated that the increased stability of the carboxymyoglobin to autoxidation would also inhibit lipid oxidation by impeding the pro-oxidative effect of myoglobin (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011).

The formation of carboxymyoglobin and oxymyoglobin are related to the a* values and metmyoglobin to the b* values of the meat when measured spectrophotometrically (Mancini & Hunt, 2005; Kristinsson *et al.,* 2006b). Thus the correlation between a* and b* values of the yellowfin tuna and the TBARS values can be used to determine whether there is a relationship between myoglobin oxidation and lipid oxidation.

The aim of this study was to investigate whether there is any evidence of a correlation between lipid oxidation and myoglobin oxidation in CO treated yellowfin tuna muscle. To ascertain this, the surface colour of the tuna muscle was measured using the CIE Lab colour system (Mancini & Hunt, 2005) and the lipid oxidation using the TBARS method (Lynch & Frei, 1993).

MATERIALS AND METHODS

The experimental layout can be seen in Table 1. For the full experimental design, sample preparation and packaging refer to Chapter 3.

Table 1 Experimental layout from which the eight treatments were established

Treatments	1	2	3	4	5	6	7	8
Packaging type	OP				OI			
Tuna number	Tuna 1-7				Tuna 8-14			
Shelf-life trial	8 d				32 d			
Sampling	24 h (T_0-T_8)				96 h (T_0-T_8)			
Gas treatment	+CO		-CO		+CO		-CO	
Freeze/thaw cycle	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2	Fx1	Fx2

OP – oxygen permeable; OI – oxygen impermeable; +CO – treated with 100% CO gas; -CO – untreated; Fx1 –one freeze/thaw cycle; Fx2 - two freeze/thaw cycles.

Surface L*, a*, b* measurements

The surface L*, a*, b* measurements were done using a colorimeter as per the method described in Chapter 4.

Lipid oxidation – Quantification of MDA using the TBARS method

Lipid oxidation was measured using the TBARS methods as described in Chapter 5.

Correlations

The correlations (r-value) between the a* and b* values and TBARS values and their associated p-values were calculated using SAS Enterprise Guide (SAS Institute Inc., SAS Online Documentation 9.2 Copyright © 2002-2010 by SAS Institute Inc., Cary, NC, USA).

RESULTS

OP treatments

Fx1 treatments

Fx1 +CO treatment - A strong negative correlation (r=-0.86; p=0.00) and strong positive correlation (r=0.73; p=0.03) was observed between the a* and b* values and TBARS values, respectively (Fig. 1).

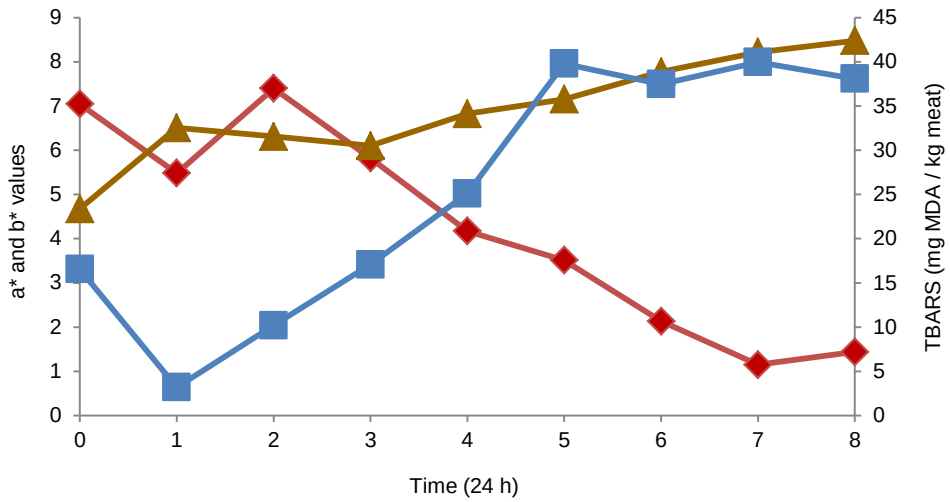


Figure 1 The (◆) a*, (▲) b* and (■) TBARS values for the OP Fx1 +CO samples of yellowfin tuna loins taken every 24 h for 8 d (T_0-T_8).

Fx1 -CO treatment - A strong negative correlation (r=-0.88; p=0.00) and strong positive correlation (r=0.80; p=0.01) was observed between the a* and b* values and TBARS values, respectively (Fig. 2).

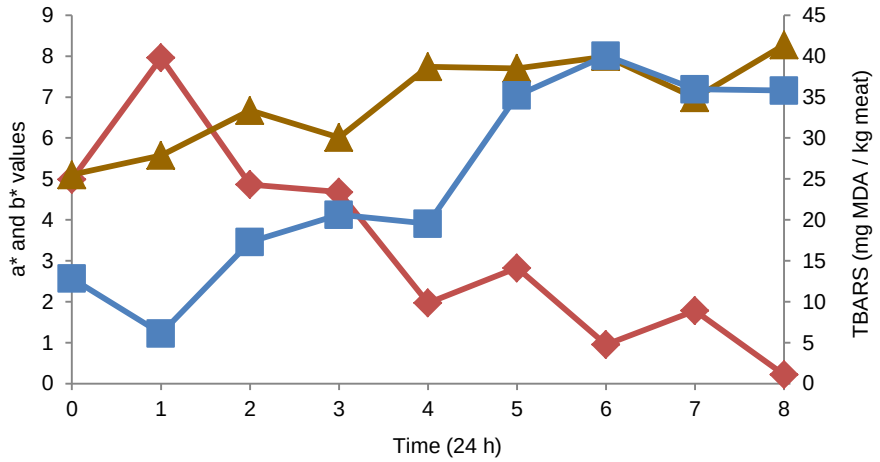


Figure 2 The (◆) a*, (▲) b* and (■) TBARS values for the OP Fx1 -CO samples of yellowfin tuna loins taken every 24 h for 8 d (T_0-T_8).

Fx2 treatments

Fx2 +CO treatment - A strong negative correlation (r=-0.88; p=0.00) and strong positive correlation (r=0.87; p=0.00) was observed between the a* and b* values and TBARS values, respectively (Fig. 3).

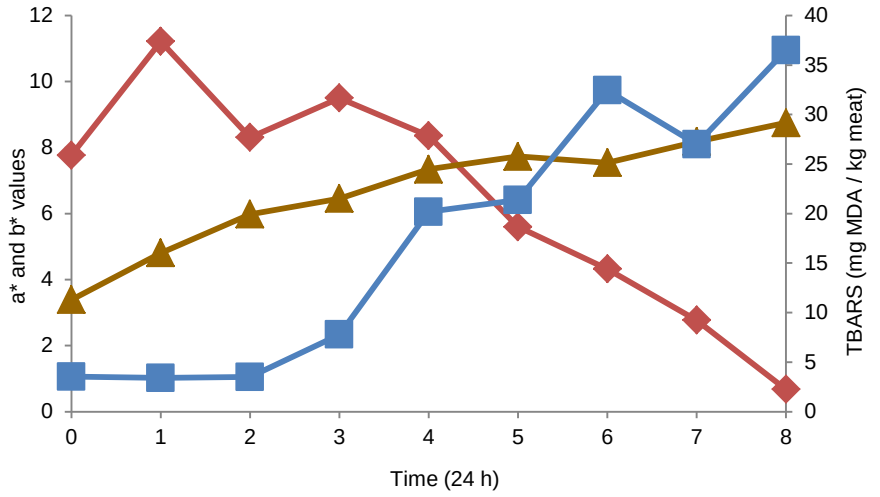


Figure 3 The (◆) a*, (▲) b* and (■) TBARS values for the OP Fx2 +CO samples of yellowfin tuna loins taken every 24 h for 8 d (T_0-T_8).

Fx2 -CO treatment - A strong negative correlation (r=-0.91; p=0.00) and strong positive correlation (r=0.86; p=0.00) was observed between the a* and b* values and TBARS values, respectively (Fig. 4).

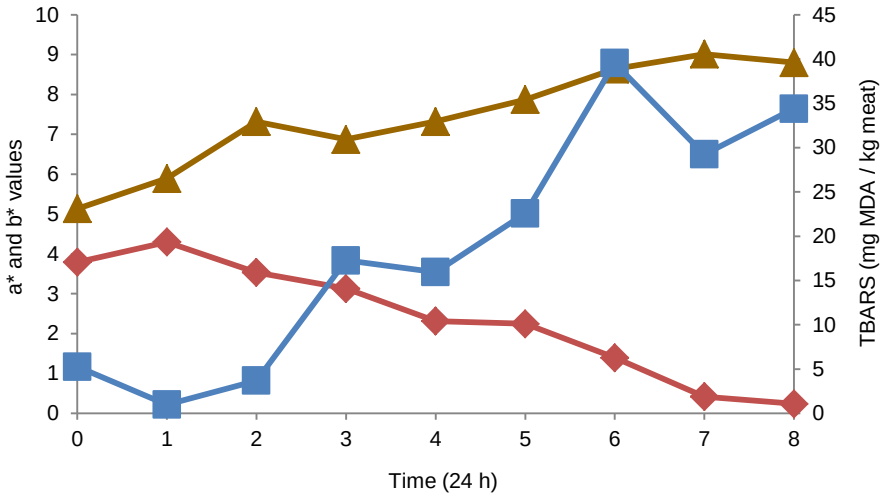
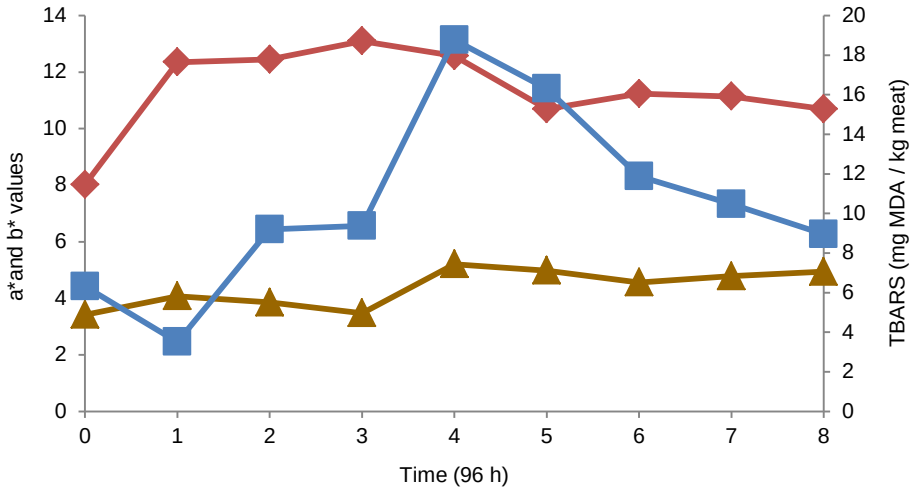


Figure 4 The (◆) a*, (▲) b* and (■) TBARS values for the OP Fx2 -CO samples of yellowfin tuna loins taken every 24 h for 8 d (T_0-T_8).

OI treatments

Fx1 treatments

Fx1 +CO treatment - A weak positive correlation (r=0.19; p=0.63) and strong positive correlation (r=0.69; p=0.04) was observed between the a* and b* values and TBARS values, respectively (Fig.



5).

Figure 5 The (◆) a*, (▲) b* and (■) TBARS values for the OI Fx1 +CO samples of yellowfin tuna loins taken every 96 h for 32 d (T_0-T_8).

Fx1 -CO treatment - A weak negative correlation (r=-0.38; p=0.32) and positive correlation (r=0.29; p=0.45) was observed between the a* and b* values and TBARS values, respectively (Fig. 6).

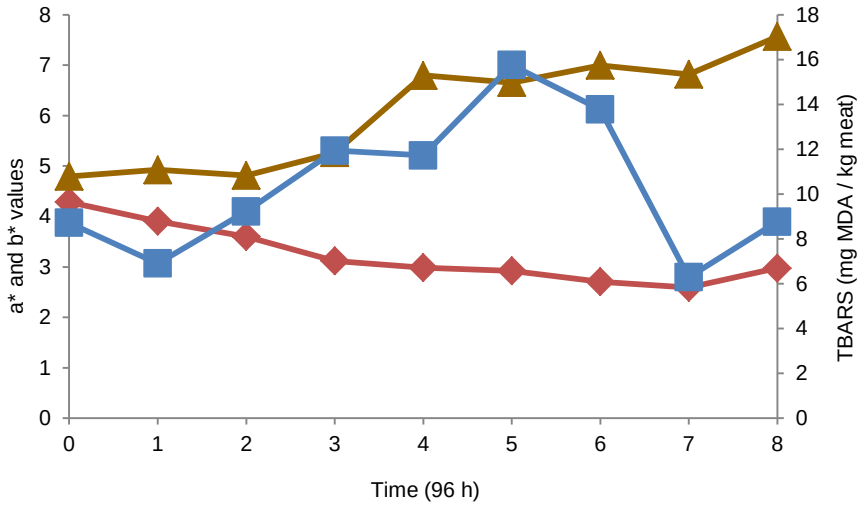


Figure 6 The (◆) a*, (▲) b* and (■) TBARS values for the OI Fx1 -CO samples of yellowfin tuna loins taken every 96 h for 32 d (T_0-T_8).

Fx2 treatments

Fx2 +CO treatment - A weak positive correlation (r=0.32; p=0.39) and weak positive correlation (r=0.34; p=0.36) was observed between the a* and b* values and TBARS values, respectively (Fig. 7).

Fx2 -CO treatment - A weak negative correlation (r=-0.12; p=0.75) and weak positive correlation (r=0.44; p=0.23) was observed between the a* and b* values and TBARS values, respectively (Fig. 8).

Comparison of all the correlation values for all the treatments

The *r* values for the correlations between the a* and b* values with the TBARS values, respectively, are given in Table 1. It can be seen that, for the OP treatments, when there is a strong negative correlation between the a* and TBARS values, there is a concurrent strong positive correlation between the b* and TBARS values. With the OI +CO samples for both the Fx1 and Fx2 treatments, both the a* and b* value correlations are positive, whereas the OI -CO treatments follow a similar trend to the OP treatments with a negative a* value correlation being concurrent to a positive b* value correlation. Overall the OP treatments displayed higher correlation values than the OI treatments.

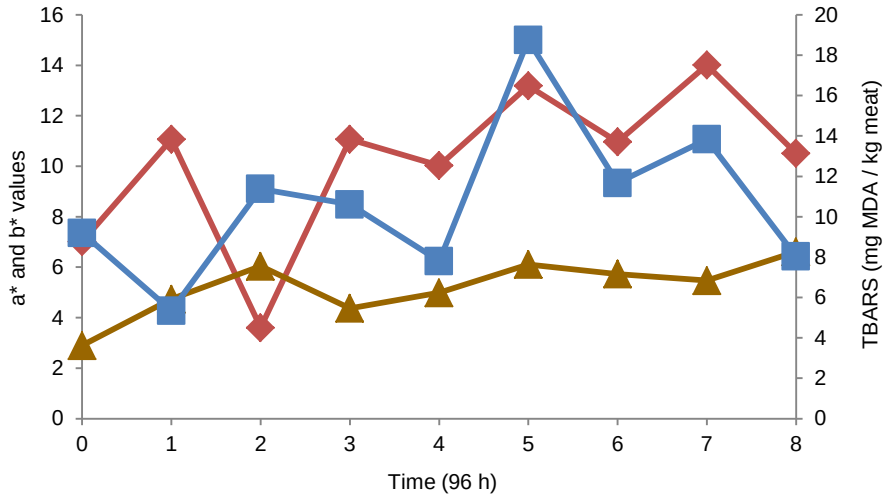


Figure 7 The (◆) a*, (▲) b* and (■) TBARS values for the OI Fx2 +CO samples of yellowfin tuna loins taken every 96 h for 32 d (T_0-T_8).

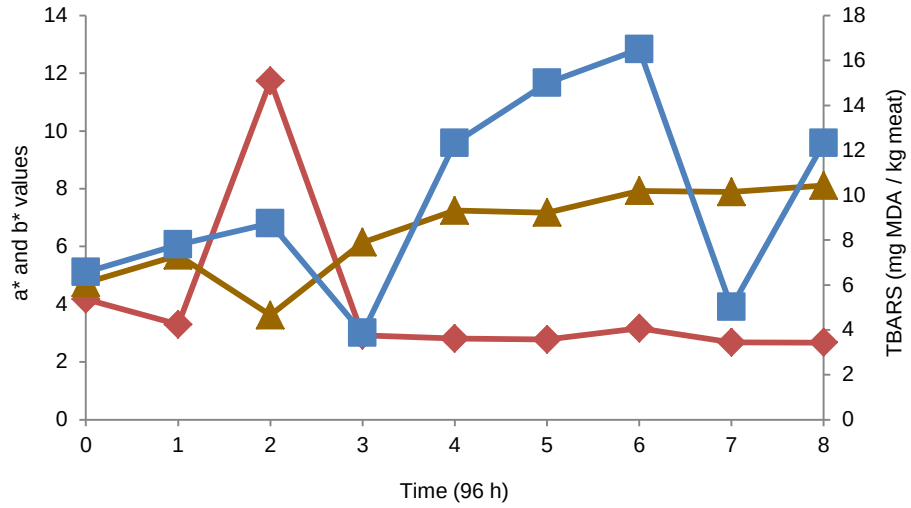


Figure 8 The (◆) a*, (▲) b* and (■) TBARS values for the OI Fx2 -CO samples of yellowfin tuna loins taken every 96 h for 32 d (T_0-T_8).

Table 1 The correlation values between the a* values and TBARS values and the b* values and TBARS values for the eight treatments

Treatments	Correlation between TBARS and a* values	P value	Correlation between TBARS and b* values	P value
OP Fx1 +CO	-0.86	0.00	0.73	0.03
OP Fx1 -CO	-0.88	0.00	0.80	0.01
OP Fx2 +CO	-0.88	0.00	0.87	0.00
OP Fx2 -CO	-0.91	0.00	0.86	0.00
OI Fx1 +CO	0.19	0.63	0.69	0.04
OI Fx1 -CO	-0.38	0.32	0.29	0.45
OI Fx2 +CO	0.32	0.39	0.34	0.36
OI Fx2 -CO	-0.12	0.75	0.44	0.23

DISCUSSION

OP treatments

The results for the Fx1 treatments showed a strong negative correlation and strong positive correlation between the a* and b* values and the TBARS values, respectively. The correlations were related to a decrease in the a* values and an increase in the b* values with a concurrent increase in the TBARS values (Figs. 1 and 2). The decrease in the a* values (redness) and increase in b* values (brownness) is consistent with the oxidation of oxy- and carboxymyoglobin (red/pink) and the formation of metmyoglobin (brown) (Mancini & Hunt, 2005; Kristinsson *et al.,* 2006b). This was expected, as the samples were packaged in oxygen permeable plastic overwrap. In the presence of oxygen the lipids will readily oxidise (autoxidation), resulting in an increase in TBARS values (Gray, 1978; Gordon, 2003). Carboxymyoglobin, although more stable than oxymyoglobin, will oxidise to metmyoglobin in the presence of oxygen over time, as will any oxymyoglobin present in the tuna muscle (Livingston & Brown, 1981; Krause *et al.,* 2003; Anderson & Wu, 2005), thus resulting in decreased a* values and increased b* values. The oxidation of oxymyoglobin to metmyoglobin has the ability to initiate lipid oxidation (Chaijan, 2008).

It could be surmised that, since the increase in TBARS values is concurrent with the oxidation of oxy- and metmyoglobin (decrease in a* and increase in b* values), that lipid oxidation is initiated/promoted by the oxidation of myoglobin. With regards to the untreated samples, this is similar to other studies that concluded that lipid oxidation and myoglobin oxidation are linked

(Andersen *et al.,* 1990; Fraustman *et al.,* 1992). For the treated samples however, this is contradictory to other studies which found evidence that treatment of muscle with CO and the subsequent formation of carboxymyoglobin led to a reduction in lipid oxidation (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011). There are several possible reasons for this. In some of the other studies, different species were used. Different species, and different muscles, have different myoglobin concentrations and thus different oxidative abilities (Chaijan, 2008). Fish muscle, in general, is also more liable to autoxidation than other species (Balaban *et al.,* 2005; Chaijan, 2008). It is more likely that the results differ as, in most of the studies, the exposure time to the CO was considerably longer (24 h and longer) than those used in this investigation. The longer exposure times led to the formation of higher concentrations of carboxymyoglobin (more CO bound to the myoglobin) (Kristinsson *et al.,* 2006b). Carboxymyoglobin is more resistive to oxidation than oxymyoglobin (Sørheim *et al.,* 1997) and thus the higher concentration of carboxymyoglobin in the muscle would have led to a reduction in lipid oxidation or the retardation of its onset. The lack of difference between the correlations of the treated and untreated samples and the fact that the correlations were both linked to an increase in TBARS and a decrease in a* and increase in b* values, respectively, reiterates the results found in Chapter 5, that the CO treatment did not retard the lipid oxidation of the yellowfin tuna muscle. It also suggests a link between myoglobin oxidation and lipid oxidation.

As with the Fx1 treatments, both the treated and untreated samples for the Fx2 treatments show strong negative and positive correlations between the TBARS values and the a* and b* values, respectively (Figs. 3 and 4), thus further reiterating that myoglobin oxidation and lipid oxidation are linked and that the CO treatment had no effect on the lipid oxidation of the yellowfin tuna muscle.

OI treatments

The Fx1 results showed a weak positive correlation and strong positive correlation between the TBARS values and the a* and b* values respectively, for the treated samples. The untreated samples showed a weak negative and weak positive correlation between the TBARS values and the a* and b* values, respectively. The a* and b* values for the untreated samples reiterate what was found in the OP treatments, that an increase in the TBARS values leads to an increase in the b* values and a decrease in the a* values, again suggesting a link between myoglobin oxidation and lipid oxidation in tuna muscle. The treated samples however, show a dissimilar pattern to all the previous treatments, with the increase in the TBARS values having a positive correlation with both the a* and b* values, with an overall increase in both the a* and b* values over time (Figs. 5 and 6). This would suggest not only an increase in the carboxymyoglobin but also metmyoglobin concentrations with a concurrent increase in lipid oxidation. This dissimilar pattern is due to the combination of the vacuum packaging with the CO treatment. In the absence of oxygen, the

residual CO will bind to any unbound/undamaged myoglobin to form carboxymyoglobin which increases the redness of the tuna (a* value) (Kristinsson *et al.,* 2006a). In theory, the increased carboxymyoglobin concentration (stabilisation of myoglobin) should result in a decrease/retardation of the lipid oxidation (Pivarnik *et al.,* 2011) and, in fact, other studies support this theory (Luno *et al.,* 2000; Garner, 2004; Kristinsson *et al.,* 2005; Pivarnik *et al.,* 2011). However, these studies allowed for considerably longer exposure times (24 h and longer), which would have allowed for the development of much higher carboxymyoglobin concentrations and the samples used had not been previously frozen. In this treatment although the a* values increased, it is most likely that the exposure time and residual binding did not create a high enough concentration of carboxymyoglobin to stabilise enough of the myoglobin to prevent lipid oxidation. The tuna was also frozen prior to being treated. Lipid oxidation can occur during frozen storage (Owen & Gray, 1975; Leygonie *et al.,* 2012) and thus, since TBARS measure secondary lipid oxidation products (Draper & Hadley, 1990; Gray & Monahan, 1992; Leygonie *et al.,* 2012), it can be assumed that primary oxidation occurred during frozen storage. It has also been found that accelerated lipid oxidation occurs during thawing (Benjakul & Bauer, 2001). This, coupled to the damage caused to the proteins during freezing (Leygonie *et al.,* 2012), explains why there is a positive correlation between the a* and b* values and the TBARS values. The a* values increased (CO binding to myoglobin in the absence of oxygen) even though lipid and myoglobin oxidation (due to freeze/thaw cycle) still occurred (increase in b* values). Thus, the CO treatment masked the effects of lipid oxidation (decrease in a*) to some extent and a link between lipid oxidation and myoglobin oxidation is still evident.

The untreated samples follow the same pattern as the OP treatments and the OI Fx1 -CO treatment (Figs. 7 and 8), thus further reiterating that myoglobin oxidation and lipid oxidation are linked and that the CO treatment had no effect on the lipid oxidation of the yellowfin tuna muscle. The a* and b* values for the untreated samples reiterate what was found in the OP treatments, that an increase in the TBARS values leads to an increase in the b* values and a decrease in the a* values, suggesting a link between myoglobin oxidation and lipid oxidation in tuna muscle.

Comparison of all the correlation values for all the treatments

The OP treatment have higher correlations with the TBARS values for both the a* and b* values compared to the OI treatments. This is ascribed to the effect that the aerobic environment, created in the oxygen permeable packaged samples, has on oxidative processes, with the presence of oxygen increasing their rate (Ladikos & Lougovois, 1990).

CONCLUSION

In the majority of the treatments there was an increase in TBARS values with a concurrent decrease in a* values and increase in b* values, indicating a decrease in oxy- and

carboxymyoglobin respectively, with a concomitant increase in metmyoglobin. This indicates the oxidation of myoglobin is concurrent with the oxidation of lipids. In the treatments where both the a* and b* values increased with a concurrent increase in TBARS values, it was established that the CO treatment of the tuna muscle combined with vacuum packing resulted in the occurrence of lipid oxidation being masked.

Thus from the above results it could be surmised that myoglobin oxidation is linked to lipid oxidation. It also further reiterates the concerns of the use of CO treatment of meat as some of the treatments were able to mask the visible indicator (browning) of lipid oxidation and thus could also mask other underlying quality and safety issues.

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CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Fishing is of great economic importance to many countries (Garner, 2004), including South Africa. In 2007 396 660 tons were harvested for direct human consumption, of which 144 005 tons were exported (FAO, 2010). In 2008 the revenue generated from fishery exports was over 5 million US dollars (FAO, 2010). Among the fish harvested and exported was yellowfin tuna (*Thannus albacares*) (FAO, 2010). However, over-fishing has resulted in this species currently being listed as "*Lower Risk/near threatened"* (LR/nt) on the International Union for Conservation of Nature (IUCN) red list of threatened species (IUCN, 2012).

As with all fish species, tuna muscle is particularly liable to oxidative changes with tuna rapidly perishing. Proper processing and storage is crucial in ensuring maximum shelf-life (Garner, 2004), with muscle quality rapidly deteriorating after it has been harvested and this continues during processing, during transportation, storage and retail display. The main factors affecting the quality deterioration of the tuna muscle are microorganisms, oxygen, lipid and protein oxidation (particularly oxidation of the haem proteins) and enzymes (Garner, 2004). The most effective and widely accepted method to lengthen the shelf-life of tuna is freezing (Balaban *et al.,* 2005). Unfortunately freezing causes tuna muscle to discolour from bright red to brown (Chow *et al.,* 1988; Chow *et al.,* 1989). Consumer acceptance of tuna is based on its bright red colour which is associated with fresh tuna and as such, the market value of tuna is based in its colour (Garner, 2004). Once tuna has discoloured, it is perceived as being older and of poorer quality and subsequently loses market value (Carpenter *et al.,* 2001; Otwell, 2006). This problem is exasperated by the fact that the tuna is harvested in areas far from the markets and thus shelf-life time is lost during the transport of the fish to the markets. In an attempt to maintain the market value, tuna is frequently sold as "fresh" for up to three weeks after being harvested (Kristinsson *et al.,* 2008). Tuna processors thus need to find ways of increasing the shelf-life of tuna while maintaining the colour of tuna muscle, thereby maintaining the optimum market value, without compromising product quality (Kristinsson *et al.,* 2008).

Rapidly freezing tuna to very low temperatures (-56°C) and maintaining these low temperature has proved effective in preventing discolouration until the tuna is thawed (Balaban *et al.,* 2005). Vacuum packaging has also been used but the resulting purple colour of deoxymyoglobin is not desirable to consumers (Kristinsson *et al.,* 2008). Another way that proved effective was the use of carbon monoxide (CO). Treating tuna with CO results in a stable cherry-red myoglobin derivative, known as carboxymyoglobin (Sørheim *et al.,* 1997). Carboxymyoglobin is stable during frozen storage and thawing and under low oxygen conditions such as vacuum packaging (Balaban *et al.,* 2005; Kristinsson *et al.,* 2008).

Several studies have been conducted on the use of CO with fish (Kristinsson *et al.,* 2003; Garner, 2004; Anderson & Wu, 2005; Mantilla *et al.,* 2008), including yellowfin tuna (Balaban *et al.,* 2005; Huang *et al.,* 2006). In these studies various concentrations of CO as well as various applications were employed. Each combination of CO concentration and application method requires the establishment of parameters to ensure that the desired results are achieved. In this study, a purpose built chamber was used, which allowed for the tuna to be treated under pressure. It was thought that treating the tuna under pressure would reduce the time required for surface colour development and colour penetration. In this study, 100% CO was used and thus only the exposure time and pressure needed to be established. Both the pressure and exposure time used were found to influence the surface colour and colour penetration. It was established that 150 min exposure at 3 bar pressure resulted in the desired surface colour development and colour penetration. Untreated samples were used as a control. The effect of aerobic (overwrap) (OP) and anaerobic (vacuum packaging) (OI) conditions was also investigated as the samples are often removed from vacuum packaging and placed in polystyrene trays and overwrapped for retail display. The effect of one (Fx1) and two freeze/thaw (Fx2) cycles was also investigated as this correlated to industry practices.

It was found that the treatment of the yellowfin tuna muscle with CO did have an effect on the surface colour, resulting in an increase in the surface a* values (redness). Although the treated OP samples showed an increase in the surface a* values, the increase was short lived. The exposure to oxygen resulted in oxidation of the carboxymyoglobin to metmyoglobin (increase in b* values). On the other hand, the OI samples maintained the surface a* values resulting from the CO treatment. The effect of the second freeze/thaw (Fx2) cycle on the treated samples was not as apparent in the OP samples as the OI samples, as the longer exposure time in the OP Fx2 +CO samples overshadowed the effects. In the OI samples the effect was clearly evident with the OI Fx1 +CO samples having the highest initial surface a* values. Thus the OI Fx1 +CO treatment resulted in the best product with regard to surface colour development and stability.

Since the use of CO on meat and fish products is highly controversial due to its ability to mask underlying safety issues, other benefits of its use were also investigated. It has been postulated that lipid and protein oxidation may be retarded by the formation of carboxymyoglobin (Kristinsson *et al.,* 2006). It was however found in this study that neither the CO treatment nor the number of freeze/thaw cycles had an effect on the lipid oxidation. The CO treatment also had no effect on the protein oxidation. The number of freeze/thaw cycles did however have an effect of the protein oxidation. Protein oxidation was observed for the Fx1 treatments (both the OI and OP treatments), with the onset being retarded for the OI treatments. Although it was expected that the Fx2 treatments would result in higher carbonyl values this was not the case, with none being observed. It was postulated that this was due to the protein oxidation having progressed so far that the carbonyls being measured had reacted with other biological constituents and were thus no longer detectable. In both lipid and protein oxidation the packaging was found to have an effect.

Considerably lower levels of lipid oxidation were observed as well as retardation of the protein oxidation in the OI treatments compared to the OP treatments.

A correlation between myoglobin oxidation and lipid oxidation has been observed in tuna (Pivarnik *et al.,* 2011). As mentioned the carboxymyoglobin formed from the CO treatment is more stable than oxymyoglobin and will thus not oxidise as readily (Sørheim *et al.,* 1997). It was thus investigated whether the same correlation was observed with carboxymyoglobin. The results showed a correlation between myoglobin oxidation and lipid oxidation for all of the treatments. However, the OI +CO samples resulted in an increase in the a*, b* and TBARS values i.e. the surface colour remained bright cherry-red even though lipid oxidation was occurring. Thus the visible indicator (browning) of lipid oxidation was masked.

From the above results it can be concluded that the treatment of yellowfin tuna with 100% CO for 150 min under 3 bar pressure is effective in producing a product that has a desirable surface colour and sufficient colour penetration. However, the CO treatment did not have an effect on the rates and levels of protein and lipid oxidation i.e. neither increased nor decreased the rate and level of lipid and protein oxidation. The OI +CO Fx1 sample resulted in the best quality product overall with regards to colour stability and maintenance, and lipid and protein oxidation. The question does however arise that since the visible indicators of lipid and protein oxidation are masked by this method, at what point does the lipid and protein oxidation result in a product that is undesirable to consumers.

Nonetheless, the results reiterated the concerns regarding the use of CO on tuna meat and its ability to mask underlying safety issues. Thus it is strongly recommended that the use of CO on tuna and any other meat or fish should be considered carefully and should be subjected to strict hygiene, safety and labelling regulations.

Future research should be done to assess whether longer exposure times and varying CO concentrations, using the same application method, will give acceptable colour development, stability and penetration as well as what effect the longer exposure time and varying CO concentrations have on lipid and protein oxidation. It would also be recommended that more specific methods be used for the determination of the protein and lipid oxidation. Myoglobin extraction and the assessment of the composition of the moisture lost may also give a better understanding of the colour changes which occurred. Furthermore a sensory study should be done regarding the levels of protein and lipid oxidation in CO treated tuna that result in a product that is undesirable to consumers'.

From the knowledge gained from the research done and in light of the finding from this study there are clearly both arguments for and against the use of CO in the treatment of yellowfin tuna. On the one hand there is the question of whether the use of CO on tuna is an ethical industry practise due to the possibility of defrauding consumers being so great. Since the colour of tuna is used by consumers as an indicator of wholesomeness, the potential to manipulate the colour could have dire consequences with regard to product quality and safety. Consumers could,

unknowingly, potentially purchase inferior quality tuna or tuna which is no longer safe to consume which appears to be fresh and wholesome. On the other had, in a world where the demand for perfection is so high and where good quality tuna is downgraded solely based on discolouration, it seems that the use of CO is the ideal solution to maintain market value of tuna and prevent wastage and monetary losses to the industry. If this method was advocated with regard to its use on tuna solely for colour maintenance, it is recommended that it only be done under strict regulations and labelling requirements such as those applicable in the USA. It would also have to be classified as a preservative in the Foodstuffs, Cosmetics and Disinfectants Act (DOH, 1997) and as such would thus have to be labelled. Labelling could be similar to that used in the USA: *Tuna, Carbon Monoxide (as colour preservative).* Tuna which is treated and then frozen would also have to be labelled as previously frozen in accordance with the labelling legislation is South Africa (DOH, 1997).

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