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FISH WELFARE AND QUALITY ASSESSMENT BY CONVENTIONAL AND INNOVATIVE METHODS

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ABSTRACT

This thesis is based on 3 main trials and a further study. The overall aim of my research was on one side, to investigate the possibility of using rapid and non-destructive methods for the determination of fish fillets quality and their classification, on the other side, to find out the stunning/slaughtering method able to guarantee a minimal or to completely avoid stress condition at the moment immediately prior of the slaughtering process, by assessing the effects on fillets quality by conventional and innovative methods, from two different farmed species [Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Onchorynchus mykiss*)]. Percussion, asphyxia in the air, electroshock and asphyxia by carbon monoxide (CO) were tested; behaviour (on Atlantic salmon), stress indicators, shelf-life evolution during cold storage (raw fillets) and sensory analysis (cooked fillets) have been investigated, depending on stunning/slaughtering method and, in the case of rainbow trout, also on water rearing temperature.

The first study on rainbow trout investigated the possibility of using near infrared spectroscopy (NIRS) for the authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) fillets. Latent variable models applied on the spectral data were developed and used to estimate proximate composition, fatty acid profile, fillet yield and cooking loss, and to classify the available dataset by the rearing farm and genetic strain of each sample. Results showed that NIR spectra can be used both to accurately estimate several chemical properties and to classify samples by rearing farm. In order to classify samples by genetic strain instead, a data fusion approach in which colour and mechanical information were combined with spectral data was used. No major differences were observed between the results obtained from raw freeze-dried fillets and those obtained from cooked freeze-dried fillets, except for the estimation of certain chemical constituents of interest such as C22:6n-3 and polyunsaturated fatty acid content, both of which were better estimated from cooked freeze-dried fillets.

In the second study on Atlantic salmon, behaviour analysis showed that CO gas used for stunning/slaughtering is not sensed in the first 8-10 min., and then fish respond with aversive behaviour, probably elicited by loosing buoyancy or a biological

response to hypoxia, before becoming fully sedated. Exposition to CO seems to increase catecholamine's level, resulted in an earlier onset of *rigor mortis*, lower final *post mortem* muscle pH, higher drip loss after filleting and slighted increase of L* and b* values. This study also evaluate and compared sensory analysis, NIRS, Electronic nose (EN) and Electronic tongue's (ET) ability in discriminating Atlantic salmon (*Salmo salar L.*) fillets according to the considered stunning methods (percussion: Control; Carbon Monoxide: CO), Storage time (T1; T2) and different preparation (raw and thawed; freeze-dried; ethanol storage) of the specimens. Samples were NIRS analysed by three different Research Units (RU): Hungary (H), Padova (PD) and Torino (TO). As a general pattern, the Storage factor was the main source of effects for the instrumental discernment when compared to the Stunning effect. According to the two considered factors and their combinations for the seven instruments and preparations, the maximum efficiency was performed by the freeze-dried samples scanned by NIRS devices from the RU of TO, PD and H, also NIRS on thawed samples performed by the H RU was efficient; EN, ET and NIRS of ethanol specimens resulted to be the worse preparations and analysis methodologies. The correlation of maximum *rigor* time with the spectra resulted to be greater for freeze-dried and thawed samples according to all the different considered NIRS devices and for ethanol specimens. A general accordance between the spectral signature and the appreciation expressed by the panel for some sensory traits was observed, indicating that rheological but also taste and flavour properties are involved in this vibrational characterization. As general result freeze-dried preparation and NIRS devices resulted to be the best combination in samples discernment according to Storage Time and Stunning factors, but also to maximum *rigor mortis* time and sensory scores.

In the third study performed on rainbow trout reared at two different temperature conditions (8 °C and 12 °C), the effects of stunning/slaughtering methods (carbon monoxide asphyxia, CO; electroshock, E; asphyxia in the air, A) on *pre rigor mortis* [blood parameters, *rigormortis* development, fillets contraction changes, ATP depletion and Adenylate Energy Charge (AEC) in muscle] and *post rigor mortis* (K-value, texture, lipid oxidation and sensory analysis) changes have been investigated.
Concerning *pre rigor mortis* changes, it resulted that electroshock was the most suitable slaughtering method, able of limit stress in rainbow trout; asphyxia in air

seemed to be the most stressing, as confirmed also by *rigor mortis* and pH evolution. Fillets from asphyxiated fish had the strongest area and perimeter contractions, followed by CO and E on one side, and the most rapid length shrinkage and height increase, followed by E and CO, on the other. The CO treated fish, reared both at 8 °C and 12 °C, were able to preserve the higher amount of muscle's ATP immediately after death.

Post rigor mortis evaluation showed that at the end of the shelf-life (7 days *postrigor mortis* resolution),considering both K and K_1-values, freshness results well preserved irrespective of the stunning/slaughtering method applied, and water temperature. No significant drip losses were detected at any considered time. At *rigor* resolution ($T_{RR}0$)CO fillets showed higher pH than A fillets ($P<0.01$), whereas seven days after rigor resolution ($T_{RR}7$) also E fillets' pH resulted significantly higher than A fillets.CO treatment ensured higher a* and C* colour values, and intermediate b* value, whereas electroshock provided the lowest fillets colour values. Texture profile analysis revealed an effect of the stunning/slaughtering method and of the temperature for the cohesiveness parameter. Fish slaughtered by CO presented significantly lower ($P<0.001$) Malondialdehyde content in fillets when compared to the other two groups at $T_{RR}0$, whereas at $T_{RR}7$ no differences were detected. Canonical Discriminant Analysis of sensory attributes, instrumental texture and physicochemical measurementsresulted as an accurate tool in discriminating and classifying the three groups of treatments at the two considered rearing water temperatures.

RIASSUNTO

L'obiettivo generale di questo studio è stato, da un lato, quello di approfondire la possibilità di utilizzare metodi di valutazione rapidi e non distruttivi per la determinazione della qualità di filetti e per la loro classificazione in relazione ai fattori di influenza, dall'altro quello di identificare i metodi di stordimento/macellazione in grado di garantire una minima o di evitare completamente la condizione di stress nel momento della macellazione. A tale scopo sono stati valutati gli effetti prodotti da metodi di macellazione tradizionali e innovativi sulla qualità dei filetti di due specie diverse di salmonidi, il salmone Atlantico (*Salmo salar* L.) e la trota iridea (*Onchorynchus mykiss*). Percussione, asfissia in aria, elettroshock e asfissia tramite CO sono stati testati; comportamento (sul salmone Atlantico), indicatori di stress, evoluzione della shelf-life durante la conservazione in condizioni refrigerate (filetti crudi) e analisi sensoriale (fletti cotti) sono stati studiati in relazione al metodo di macellazione e alla temperatura di allevamento (solo sulla trota iridea) applicati.

Un primo studio sulle trote iridee ha esaminato la possibilità di usare la spettroscopia del vicino infrarosso (NIRS) per l'autenticazione di filetti liofilizzati di trota iridea (*Oncorhynchus mykiss*) crudi e cotti, derivanti da ceppi genetici diversi, allevati in aziende diverse. Modelli di variabili latenti sono stati sviluppati sui dati spettrali e utilizzati per stimare la composizione centesimale, il profilo acidico, la resa in filetto e le perdite di cottura, e per classificare il data set disponibile a seconda dell'azienda e del ceppo genetico di appartenenza di ogni campione. I risultati hanno mostrato che gli spettri NIR possono essere utilizzati sia per stimare in modo accurato diverse proprietà chimiche che per classificare i campioni a seconda dell'azienda di appartenenza. Allo scopo di classificare i campioni in base al ceppo genetico invece, è stato considerato un approccio di fusione dei dati nel quale colore e informazioni relative alle caratteristiche fisiche sono stati combinati con i dati spettrali. Nessuna differenza importante è stata riscontrata tra filetti crudi e cotti liofilizzati, se non nella stima di qualche costituente chimico d'interesse, come il tenore in C22:6n-3 e il contenuto di acidi grassi polinsaturi, entrambe i quali sono stati meglio stimati nei filetti cotti liofilizzati.

Nel secondo studio, l'analisi comportamentale sul salmone Atlantico macellato con il monossido di carbonio ha mostrato che il CO non è percepito nei primi 8-10 minuti, poi il pesce risponde con comportamento avverso, forse provocato dalla perdita di equilibrio o da una risposta biologica all'ipossia, prima di essere completamente sedato. L'esposizione al CO sembra aumentare il livello di catecolamine, con un anticipato inizio del *rigor mortis*, pH finale *post mortem* più basso, maggiori perdite di gocciolamento dei filetti e un leggero aumento dei valori L* e b*.
Questo studio ha anche valutato e confrontato l'abilità dell'analisi sensoriale, del NIRS, naso elettronico (NE) e lingua elettronica (LE) nel discriminare filetti di Salmone atlantico (*Salmo salar L.*) a seconda dei metodi di stordimento applicati (percussione: Controllo; Monossido di Carbonio: CO), del tempo di stoccaggio (T1; T2), e diverse preparazioni (crudi scongelati; liofilizzati; conservati in etanolo) dei campioni. I campioni sono stati scansionati al NIRS da tre diverse Unità di Ricerca (UR): Ungheria (H), Padova (PD) e Torino (TO). In generale, il fattore Tempo di Stoccaggio è risultato la principale fonte di effetti per la discriminazione strumentale rispetto al fattore Stordimento. Considerando i due fattori principali e le loro combinazioni per i sette strumenti e preparazioni, la massima efficienza è stata mostrata dai campioni liofilizzati e scansionati con le strumentazioni NIRS dalle UR di TO, PD e H, anche il NIRS sui campioni scongelati realizzato in Ungheria è risultato efficiente; NE, LE e il NIRS sui campioni conservati in etanolo sono risultate essere le peggiori preparazioni e metodologie di analisi. La correlazione del tempo di massimo *rigor* con gli spettri è risultata maggiore per i campioni liofilizzati e quelli scongelati per tutti gli strumenti NIRS considerati e per i campioni in etanolo. Una relazione generale tra l'impronta spettrale e gli apprezzamenti espressi dai pannellisti per alcuni tratti sensoriali considerati è stata osservata, indicando che le proprietà reologiche ma anche quelle del gusto e del sapore sono coinvolte nella caratterizzazione vibrazionale. In generale, i campioni liofilizzati e la strumentazione NIRS sono risultati essere la migliore combinazione per discriminare i campioni a seconda dei fattori Tempo di Stoccaggio e Stordimento, ma anche del massimo tempo di *rigor mortis* e punteggio sensoriale.

Nel terzo studio, realizzato su trote iridee allevate a due diverse temperature (8 e 12 °C), sono stati valutati gli effetti di diversi metodi di stordimento/macellazione (asfissia con monossido di carbonio, CO; elettroshock, E; asfissia in aria, A) sulle

caratteristiche *pre rigor mortis* [parametri ematici, evoluzione del *rigor mortis*, contrazione dei filetti, consumo di ATP e la Carica Energetica Adenilica (AEC) nel muscolo] e *post rigor mortis* (indice di freschezza K, texture del filetto, ossidazione lipidica e analisi sensoriale), durante la shelf-life.

Per quanto riguarda i cambiamenti durante la fase di *pre rigor mortis*, l'elettroshock è risultato essere il metodo di macellazione più adatto, in grado di limitare lo stress nella trota, mentre l'asfissia in aria è sembrato essere il metodo più stressante, come confermato anche dall'evoluzione del *rigor mortis* e del pH, mentre il CO ha prodotto effetti intermedi tra i due metodi di macellazione precedenti. I filetti ottenuti dai pesci sottoposti ad asfissia hanno presentato da un lato la più intensa contrazione dell'area e del perimetro del filetto, seguiti dai filetti ottenuti dai pesci macellati con CO e con elettroshock, ma anche la più rapida contrazione in lunghezza e aumento in altezza, seguiti dai filetti dei pesci trattati con elettroshock e CO. I pesci macellati con l'impiego del CO, indipendentemente dalla temperatura di allevamento, 8 °C o 12 °C, hanno presentato il più alto contenuto di ATP nel muscolo subito dopo la morte.

Relativamente alle caratteristiche riscontrate nelle fasi successive del*rigor mortis*, è emerso che a 7 giorni dalla risoluzione dello stesso, considerando sia il K-value che il K_1-value, la freschezza risulta ben preservata indipendentemente dal metodo di stordimento/macellazione applicato e dalla temperatura. Non sono state determinate rilevanti perdite di gocciolamento dei filetti per nessuno dei tempi considerati. Alla risoluzione del *rigor* mortis ($T_{RR}0$) i filetti CO hanno mostrato un pH più alto rispetto ai filetti A ($P<0.01$), mentre 7 giorni dopo la risoluzione del *rigor* mortis ($T_{RR}7$) anche il pH dei filetti E è risultato significativamente più alto rispetto a quello dei filetti A. Il trattamento con CO ha prodotto una colorazione rossa più intensa del filetto, alta saturazione e b* intermedio, mentre l'elettroshock ha riportato i valori più bassi. L'analisi della texture del filetto ha mostrato un effetto significativo dei metodi di stordimento/macellazione e della temperatura, limitatamente alla coesività. I pesci macellati con CO hanno presentato un contenuto di malondialdeide (MDA) significativamente più basso ($P<0.001$) rispetto agli altri due gruppi a $T_{RR}0$, mentre a $T_{RR}7$ nessuna differenza è stata riscontrata. L'Analisi Discriminante Canonica (CDA) degli attributi sensoriali, dei parametri della texture e delle misurazioni fisico-chimicheè risultata essere uno strumento accurato nel discriminare e classificare i tre trattamenti alle due temperature.

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PART I

1. INTRODUCTION

1.1 Animal welfare and food quality

Food quality is perceived as a global concept, which is unavoidable from animal's welfare. Conditions of anxiety, pain, suffering or fear above all have ethical implications, since the human being is considered to be responsible for the effective respect of the rights and welfare of other living animals, as stated in the declaration of UNESCO in 1978. The respect for animal's welfare, strongly affects consumer attitudes towards the product, influencing the choice to those products derived from animals that have not been subject to ill treatment. Operations relating the stunning and killing, in the slaughtering processes, as well as the operations immediately prior stages, can cause particular stress and disturbances that may affect meat quality. Humane slaughter procedures, therefore, can improve *post mortem* quality of fish, as reported for warm-blooded animals by many authors (Brown et al., 1998;Geesink et al., 2001). To maintain the best original quality, fish would be stunned until death and killed without any avoidable stress.

Fresh fish quality is the major concern to industry and consumers, as fish is considered an extremely perishable food commodity. Freshness is the single most important attribute when assessing fish quality. Microbiological, biochemical and sensory changes are associated with deterioration of fish quality during handling and storage (Ehira and Uchiyama, 1986; Gregory, 1994). It is fundamental to reduce muscle activity during transport and netting and to ensure stunning/slaughtering methods able to minimize *pre mortem* stress of fish since it may affect *rigor mortis* and pH evolution (Robb, 2001; Robb et al., 2000a; Thomas et al., 1999;), texture pattern (Nakayama et al., 1996), fillet colour perception (Jittinandana et al., 2003; Robb et al., 2000a; Robb and Warris, 1997), shelf-life (Lowe et al., 1993) and K-value evolution, defined as a later indicator of fish freshness as reported by Izquierdo-Pulido et al. (1992). Stress can provide greater muscle contractile tensions and shortening than observed in unstressed stated fish (Nakayama et al., 1999).

Animal welfare and product quality are linked aspects of the total quality of fish; therefore any conflict between the requirements of fish welfare and efficient aquaculture should be avoidable

1.2 Fish welfare-suffering and quality indicators

Animal welfare is by no means a straightforward concept. The major issues are the meaning, the definition of animal welfare and how best objectively measure it (Broom, 1991a;1991b;Dawkins, 1998;Mendl and Paul, 2004). Freedom from hunger and thirst, injury, disease, discomfort, fear and distress, pain, as well as the freedom to express normal behaviour provides a logical framework with which to assess welfare issues (FAWC, 1996). Physical health is certainly a necessary requirement for good welfare. It is important to remember that poor health can be both a cause and a result of poor welfare. However, for many, good animal welfare goes beyond just physical health, and also involves a lack of mental suffering. This aspect of welfare therefore seeks to understand subjective experiences of non-human animals and proposes the conscious experience of suffering in these animals (Broom, 1991b). This is a controversial issue, when it comes to fish. Concepts of animal welfare have been applied to those which are considered to have the ability to experience pain, fear and suffering and as much have been associated with species with a higher level of cognition when compared to fish. However, there is a scientific debate regarding the ability of fish to experience pain and fear.

Some have argued that fish lack of essential brain regions or any functional equivalent, making it impossible that they can experience pain and fear (Rose, 2002); others suggest that there are similarities with mammals in the basic structure of neurons and neuro-hormonal biochemistry, instress responsesand behaviour,which seem to indicatethatthe fish arecapableof experiencing pain andsuffering, especially in the last momentsof their life,when they arecatch and slaughtered (Ashley and Sneddon, 2008;Braithwaite and Huntingford, 2004;Chandroo et al., 2004a; Kestin et al., 1995;Sneddon, 2003; Sneddon et al., 2003a; 2003b;Verhejien and Flight, 1997).

There is no single way to measure welfare, and even if a wide range of behavioural, physiological and biochemical parameters are used to assess welfare, none of these are considered reliable when individually considered, thus a multidisciplinary approach needs to be done.

1.2.1 Behavioural indicators

Altered behaviour is an earlier and easily observed response to adverse conditions, specific responses to natural stressors can be used as an indicator of impaired welfare although this cannot be a sufficient approach.

Anxiety and fear as behavioural responses can be expressed by freezing (in presence of predators), or, by struggle, active avoidance, muscular spasms, pupil dilatation, aggression, exploration, risk-taking, active attempt to counteract the stressful stimulus. At slaughter, fish behaviour gives clear indications about presence or absence of consciousness; the most frequent observations indicating behaviour changes with different intensity and persistence are

- Swimming motility: changes in speed, directions and space use
- Gill movement: regulation of the ventilatory activity, that is the flow of water ventilated over the gills per unit of time. It is fundamental to maintaining homeostasis in terms of O_2 status, blood and tissues acid-base balance (pH) at precise set points. In acclimatised and unstressed fish, the respiratory frequency is adjusted close to a minimum possible value. Therefore, increased ventilatory activity is commonly used as a sign of stress and poor welfare in fish (Martins et al., 2012).

  Fish also respond to external stimuli (Marx et al., 1997;Tobiassen and Sørensen, 1999;Van De Vis et al., 2001)such as
- Capability to maintain the equilibrium when the fish is turned upside down
- Movement of eye following the changes in body postures in the longitudinal axis
- Reaction to the needle puncture on the tail or head (pricking) (Lambooij et al., 2002a; 2002b; 2002c)
- Handling along the lateral line
- Application of low voltage electricity.

  However, observation of behaviour only may not be sufficient for unconsciousness assessment. For example, electrical stunning can be very painful and paralysis may occur without unconsciousness when not properly applied (Croft, 1952; Robb et al., 2002). Therefore, some methodologies able to indicate brain function, thus the presence and the duration of a sensitive state of consciousness are recommended even if difficult to perform, such as
- Electroencephalogram (EEG): necessary to determine whether an electric current has been sufficient to induce a general epileptiform insult indicating unconsciousness and insensibility (Wageneder and Schuy, 1967). Required the positioning in the fish of 4 electrodes
- Visual Evoked Responses (VER): is the brain response to flashes of light directed toward the eyes. The absence of an average VER indicates brain dysfunction (Kestin et al., 1991;Van de Vis et al., 2001; 2003)

- Electrocardiogram (ECG): required the positioning in fish of 2 electrodes plus a ground electrode to test heart rate
- Somatosensory Evoked Responses (SER): responses in the brain to pain stimuli are registered. The pain stimulus consisted of scratching the tail by using a needle
- Vestibule-Ocular Reflex (VORs): movements of the eye when the fish is rocked side to side (in a stunned/dead fish the eye does not move).
  Their absence indicates unconscious and insensible fish.

1.2.2 Blood indicators

At slaughter and during rearing, physiological reactions to the stressors in fish are related to primary response to stress, which involves an immediate release in the blood stream of catecholamines (CAs) from the chromaffin cells. This is followed by the activation of the hypothalamic-pituitary-interrenal (HPI) axis: corticotrophin releasing factor from the hypothalamus acts on the pituitary to synthesise and release corticotrophic hormone, which in turn stimulates the synthesis and mobilisation of glucocorticoid hormones (cortisol in teleosts) from the interrenal cells (Schreck, 1981;Wendelaar-Bonga, 1997). HPI activation results in energy mobilisation, depletion of glycogen stores, increase in glucose plasma level, along with high muscle activity, anaerobic glycolysis and an increase in plasma lactate. Thus, both levels of glucose and lactate in the plasma are often use in conjunction with cortisol to assess stress levels (Arends et al., 1999;Acerete et al., 2004). Cortisol has been widely utilised both as a short and a long term stress condition index (Pickering et al., 1982;Pickering and Pottinger, 1985).

Catecholamines are not frequently considered as stress indicators, because they are not easy to detect and quickly disappear from the blood stream (Wendelaar-Bonga, 1997). Despite the use of anaesthetics, handling during blood sampling induces acute stress in fish. Therefore, a part of the research in fish welfare is directed to finding non-invasive methods for gathering needed data, such as measuring levels of fish metabolites in the water (Ruane and Komen, 2003).

1.2.2.1 Cortisol

In stressful situations to the animals, secretion of cortisol in the blood plasma increases to high levels. Cortisol is the major stress hormone in fish (Ellis et al., 2007), and is regarded as an important primary stress response (Mommsen et al.,

1999). This hormone regulates the metabolism of proteins, carbohydrates and fats; it's furthermore involved in the regulatory functions of the immune system, heart, growth and reproduction (Pottinger, 2008). When cortisol level is high in the blood, the supply of blood and gills' activity are increased, leading to different physical responses such as uneasy movements. Cortisol has the function to increase blood glucose levels, and promote the liberation of glycogen in liver. Cortisol also influence the hyperosmotic effect, by increasing haematocrit values, but also ions levels, especially sodium (Guyton and Hall, 2006). Moreover, stress can give a rapid anaerobic metabolism, resulting in reduction of glycogen which in turn gives a rapid decrease of pH and faster onset of rigor (Van Laack et al., 2000). When the stress event becomes chronic, the cortisol levels can be maintain at high value for days or weeks, even if they are gradually decreasing (Wendelaar-Bonga, 1997). Multiple stress condition seems to amplify the cortisol response (Mazur and Iwama, 1993; Ortuño et al., 2002). Blood sampling procedures inevitably involve introducing stressors that, to different extents, affect the levels of blood chemistry constituents; thus it could be helpful to test cortisol in less invasive biological matrixes such as in mucus or faeces, especially during rearing (Bertotto et al., 2010; Turner et al., 2003).

1.2.2.2 Glucose

The secondary responses occur as a direct result of the release of cortisol and catecholamines (including alterations in blood and tissue chemistry). An increase in blood glucose concentrations, or hyperglycaemia, is generally regarded as a reliable indicator of stress in fish (Barton, 1997;Wedemeyer et al., 1990). In vertebrates, this elevation of blood sugar is typically due to the action of catecholamines in function to provide caloric energy for the 'fight-or-flight' reaction (Pottinger et al., 2000). Catecholamines, such as adrenaline, rapidly direct the phosphorylation of the inactive form of glycogen phosphorylase resulting in an increase in glycogenolysis (Vijayan and Moon, 1992), with the primary source of glycogen being the liver and muscle (Wedemeyer et al., 1990). Gluconeogenesis may, however, gain greater importance when the glycogen stores of the liver have been depleted (Janssens and Waterman, 1988;Mommsen et al., 1988). The increase in circulating adrenaline levels is rapid and transient (Wells and Weber, 1990), and the hyperglycaemia almost immediate. Cortisol has also been demonstrated to cause hyperglycaemia in fish (Begg and Pankhurst, 2004; Leach and Taylor, 1980;Mommsen et al., 1999;Pickering and

Pottinger, 1995;Vijayan et al., 1997) following the activation by the HPI axis (Pickering, 1981;Sumpter, 1997), probably as the result of gluconeogenesis (Vijayan et al., 1991). Glucose is very easy to determine so it is frequently used as a stress indicator, although some authors have found a delay in its release (Barry et al., 1993).

1.2.2.3 Lactate

Higher energy mobilization and utilization, following the hypoxia conditions or due to the increased muscular activity, implies anaerobic glycolysis in white muscle, associated with a large build-up of lactic acid both in the muscle and blood, followed by hematic pH decrease. Such behaviour is the result of the response to the release of catecholamines (Milligan and Girard, 1993). Therefore the increase level of plasma lactate is used as stress index (Arends et al., 1999; Erikson et al., 1999; Lowe et al., 1993), even if fish stores most of the lactate in the muscle.

1.2.2.4 Haematocrit

In stress conditions, the increase in muscle activity and heartbeat requires higher oxygen intake that raises haematocrit (red blood cell number) and thus haemoglobin concentration and the oxygen-carrying capacity of the blood. Haematocrit is used as a stress index because of its easy detection, even if standard values have to be validated for each species before to be correctly used (Reddy and Leatherland, 1988).

1.2.2.5 Free Fatty Acids (FFA)

It seems that under stress conditions, CAs are involved in the mobilization of free fatty acids (FFAs), important energy substrates for fish (Pickering and Pottinger, 1995). Changes in plasma FFA could be a stress condition index, however, Sheridan (1988;1994) concluded that the effects of CAs on FFA levels in fish did not show a clear response, since these parameters are too variable to permit any general conclusion. For these reasons are not commonly used.

1.2.2.6 Reactive Oxygen Metabolites (ROM) and Antioxidant Power (AOP)

Stress condition can promote the oxidation of polyunsaturated fatty acid (PUFAs), abundant in fish, which may result in the production of reactive oxygen metabolites

(ROMs). The production of ROM is proved to induce severe alterations in nucleic acid, proteins, and lipids (Halliwell and Gutteridget, 1984) As a result, the nutritious value of post-slaughter fillet is reduced, due to the deterioration of both texture and flavour of the product assisted by the degradation and loss of PUFAs (Frigg et al., 1990; Waagbø et al., 1993). The ROMs production can be contrasted by an adaptive response such as the activation of the endogenous detoxification pattern in terms of anti-oxidant power mechanism (AOP). The determination of oxidative stress by ROMs and AOP intends to identify the early oxidation products (hydro-peroxides), thanks to the presence of reactive oxygen species and the correspondent unsuccessfully anti-oxidant power mechanism. Animals in good welfare conditions generally show a proportional and positive AOP response to ROMs release; on the contrary, animals compelled to cope with a prolonged oxidative stress show a non proportional and positive AOP response and animals with a major injury show a negative correlation (Poli, 2009).

A rapid death does not allow a completion of secondary effects of stress, and pre-slaughter stress always has to be considered in the stress evaluation at slaughter through blood parameters.

1.2.3 Tissue post mortem quality indicators

Operations concerning stunning and slaughtering processes, as well as the immediately prior stages, can cause particular stress and disturbances that may affect meat quality. It is fundamental to reduce muscle activity during transport and netting and to ensure stunning/slaughtering methods able to minimize *pre mortem* stress of fish since it may affect muscular energy reserves giving rise to an earlier onset and resolution of *rigor mortis* (Nakayama et al., 1996; Thomas et al., 1999), texture (Nakayama et al., 1996;Ando et al., 1992), fillet colour perception (Jittinandana et al., 2003; Robb et al., 2000a; Robb and Warris, 1997), shelf-life (Lowe et al., 1993), ATP degradation rate (Parkhouse et al., 1988) and K-value evolution(define as a later indicator of fish freshness) as reported by Izquierdo-Pulido et al. (1992). Severe stress caused by pre-slaughter practices can be so aversive to fish that it masks the benefits of good slaughter practices.

1.2.3.1 Lactic acid and pH

During strenuous activity anaerobic metabolism is activated and generates muscle lactate with consequent lowering of pH (Wood, 1991). Muscle tissue is still alive after the slaughtering process (Davey, 1983), as consequence the physiological condition of fish muscle prior to slaughter is also related to the tissue *post mortem*. As there is no possibility for the metabolism to recovery after death, the exhaustion of muscle energy reserves is inevitable and ends with *rigor mortis*. The first day *post mortem,* the amount of lactate increases at muscle level and, as consequence, the pH decreases. These changes are considered valuable early stress and muscular activity indexes (Lowe et al., 1993;Marx et al., 1997; Robb and Warris, 1997). From the third day of storage to the end of the shelf-life, differences are generally less marked (Lowe et al., 1993; Marx et al., 1997; Robb and Warris, 1997;Robb et al., 2000a;Sigholt et al., 1997).

1.2.3.2 ATP, ATP/IMP, AEC, K-value

The effect of a short-term stress on the health of an organism can be measured by the energy balance of the cells in that particular moment, represented by adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), ATP/IMP ratio, inosine (Ino) and hypoxanthine (Hx) amount. In stress condition, like transport, netting, stunning and slaughtering, muscle activity results in an increased energy demand, thus ATP reserves are depleted through the splitting of the phosphate bonds with the consequent transformation of ATP into ADP, and of ADP into AMP, and sometimes further to inosine monophosphate (IMP). The nucleotides in the muscular tissue's cells (ATP, ADP and AMP) begin the break down process immediately after the animal death, in more stable compounds such as IMP, Ino and Hx, which accumulate in the muscular tissue. The sequence ATP to IMP is generally completed within two days of storage in the ice after death. The loss of IMP can affect the flavour in fresh fish, since it is recognize as a flavour enhancer of meaty foods, especially the umami flavour (Kawai et al., 2002) and it is likely that IMP contributes to the sweet, creamy, meaty flavours of fresh fish (Bremner et al., 1988;Fletcher et al., 1990; Fraser et al., 1968; Fuke and Konosu, 1991; Hashimoto, 1965). Hx increase with the loss of freshness, to reach a maximum value and then decrease in the degradation phase. Hx amount gives information about freshness evolution in the first *post mortem* phases. Another important parameter to express the

cellular energy charge could be the Adenylate Energy Charge (AEC) = (ATP+0.6ADP)/(ATP+ADP+AMP). AEC can assume values between 0 and 1. When AEC is equal to 1, energetic reserves are all made of ATP, which means a "normal" consumption of ATP molecules, completely restored with the energetic synthesis. The condition where AEC = 0 corresponds instead to the only presence of AMP, due to an important consumption of energy reserves (ATP and ADP), not been restored in the energetic synthesis. Thus, considering the correspondence between AEC value and the physiological conditions of the organism, the study of this index can be regarded as indicator of a general external stressing event (aspecific).

All these indexes can be used as early stress indexes. The speed of nucleotide's catabolism is not constant but varies according to the temperature, the species and the physiological state of the fish. In some fish species more inosine is accumulated, in others instead more hypoxanthine, thus the only determination of Hx amount is not always sufficient to give information about fish freshness. For this reason, different Indexes (K, K_1, G, P, H, Fr) representing the ratio among the different nucleotides have been developed, to better highlight the evolution of the process and because they are better correlated to the sensory observations (Howgate, 2006). The K-value, defined as the ratio of the sum of the non-phosphorylated compounds, Ino and Hx, to the sum of all ATP-derived degradation products,and calculated by the following formula:

$$K\ (\%) = [(Ino + Hx) / (ATP + ADP + AMP + IMP + Ino + Hx)] * 100$$

has been much used as a later Index of freshness (Ehira and Uchiyama, 1986), but in almost all storage trials described in literature, concentrations of the adenine nucleotides are very low and a revised K-value, designated as K_1, is calculated as the ratio of the sum of Ino and Hx to the sum of IMP, Ino and Hx:

$$K_1\ (\%) = [(Ino + Hx) / (IMP + Ino + Hx)] * 100$$

In this case, K_1 Index monitors the loss of IMP (Howgate, 2005).

1.2.3.3 Rigor mortis phases

ATP in *post mortem* muscles is mainly derived from glycogen depletion to lactate as end product (Cappeln and Jessen, 2002). *Rigor mortis* development has been closely linked to ATP depletion as well as glycogen (Iwamoto et al., 1987;Mørkøre et al., 2006). The *post mortem* energy status depends on several factors such as the nutritional condition of the fish but also the stress exposure during the slaughter process. It is well documented that pre-slaughter handling stress has adverse effects on product quality such as accelerating the onset and the maximum *rigor* score, but also softening muscle texture (Ando et al., 1992; Kiessling et al., 2004; Robb, 2001; Sigholt et al., 1997;Skjervold, 2002). On the contrary with reduced pre-slaughter stress and activity, the onset of *rigor mortis* is delayed in the time, allowing the possibility to handling the fish before its entry into *rigor*, increasing fillets yield and decreasing the damage of the flesh. *Pre rigor* salmon fillets are thicker, firmer and often the colouration is more intense compared with their *post rigor* counterparts (Skjervold, 2002). The onset of *rigor mortis* is well established as indicator of fish freshness (Davey, 1983;Iwamoto et al., 1987). Many quality traits can change as affected by conditions at slaughtering time (pre slaughter and slaughter severity stresses) and during storage (handling and storage temperatures). The relative quality changes can be indicated by:

- Fish and fillet appearance (physical injuries, gaping and colour)
- Technological properties of the fish and fillet: *rigor* evolution, texture (hardness, cohesiveness, springiness), water holding capacity, fillet shrinkage, *rigor mortis* onset and resolution and texture, in particular, are important for flesh processing (Poli, 2009)
- Freshness indicators: dielectric properties, K-value, spoilage indicators as biogenic amines and lipid oxidation products, such as malonaldehyde
- Sensory qualities: of raw fish (appearance of the skin, eye and gills, *rigor* status, smell, mucus and flesh consistence), the shelf-life evolution, and sensory traits of cooked fillets as texture, taste, odour and flavour.

Like for other farmed animals, good fish rearing practices and welfare have to be guaranteed, according to the last European regulations. The main goal is to minimize and monitor the *pre mortem* and slaughtering stress, also by ensuring slaughtering practices able to render the fish unconscious until death without any excitement, pain

or suffering prior to killing. Slaughtering methods can be evaluated by considering many parameters, which however when isolated do not give enough information about fish welfare.

1.3 Stunning/slaughtering methods for farmed fish

When it comes to regulations and legislation governing welfare during farming and at slaughter, fish are treated as one species. It is fundamental to keep in mind that a wide number of fish species are farmed, with a large variety of ecological adaptations and evolutionary developments. This means that different species can react in a different way to similar situations: at a given environmental temperature, some species die quite quickly (trout) when removed from water into air, while others like eels or marine flatfish can take several hours. Aquatic animals, differently from the terrestrial ones, have in their environment a limited supply of oxygen, thus depending on the habitat fish have adapted to tolerate various degrees of hypoxic and hypercapnic environments. In general, some freshwater species have higher tolerance to hypoxia and hypercapnia due to more variation in their environment. Some fish species like eel, tilapia, cyprinids and goldfish can survive at least 1 hour in hypoxic and hypercapnic water, while rainbow trout survives only 10 min (Kestin et al., 1991). Similarly, eels require a much important amount of stunning current than trout or salmon to render them unconscious. Species differences need to be taken into account when considering particular procedures.

Many existing commercial slaughtering methods expose fish to substantial suffering over a prolonged period of time. It is required a certain knowledge by the operators, for the application of these stunning/slaughtering methods, otherwise the risk is to kill the animals in a non-human way even if the method is humane.

The European Food Safety Authority (EFSA, 2004)has classified the methods used to killed fish into 2 main groups:

- Stunning/slaughtering methods
- Slaughtering without stunning.

Stunning methods based on bleeding to achieve the death of the fish are seldom applied because the brain takes longer time before to lose its functions after bleeding (Robb et al., 2000b). Percussive and electrical stunning can be both primary stunning

methods and stunning/slaughtering methods depending on the parameters applied but generally are almost always stunning/slaughtering methods.

1.3.1. Stunning/slaughtering methods

1.3.1.1. Percussive method

Percussive stunning is frequently used in salmon industry and for flat fish. Fish is removed from the water, restrained and then hit with a blow or repeated blows on the top of the head above the brain by a club ("priest") or hammer until the fish is considered dead. Generally, fish are exposed to the air during the restraining and positioning process for 5-10 sec before the blow is delivered. This stunning/slaughtering method can be applied both manually (on single fish) and by semi-automatic percussive stunning devices (on large number of fish) equipped with a flat head pneumatic hammer (20 mm in diameter), which are becoming widespread in salmon industry. Percussive stunning using these devices is reported to be irrecoverable in more than 99% of case when it is applied correctly (EFSA, 2004). Fish are manually pushed head first into a guide and when the snout of the fish touches a trigger, the hammer delivers a hard percussive blow to the head of the fish, which is immediately rendered unconscious. The carcass is instantly removed and exsanguinated, both for quality reason and to prevent recovery in inadequately stunned fish. Current developments with semiautomatic percussive slaughtering, involved methods for percussively slaughtering fish straight into the water and push it to swim towards the apparatus intentionally, without need for an operator. The necessary impact energy to stun or kill the fish depends on the shape of the hammer, and generally a flat hammer is more efficient than a round one(EFSA, 2004) . When correctly applied with adequate force, percussive killing is an efficient and humane way to slaughter fish: loss of movement and VERs can be immediate and permanent in salmon and trout (Kestin et al., 1995; Marx et al., 1997;Robb et al., 2000a). When not properly applied or applied with insufficient force, unconsciousness is not immediate and consciousness can be recovered after short time (Kestin et al., 1995;Robb et al., 2000b). Not all fish species are suitable for percussive killing: in sea bream, African catfish or eels, for example, skull morphology seems to prevent sufficient energy reaching the brain to render the animal unconscious(Van De Vis et al., 2003). Many studies showed that fish killed by percussive blows show reduced physical activity at slaughter, slower onset of *rigor mortis* and *post mortem* muscle

pH decrease, compared to other commercial methods for fish slaughtering (Marx et al., 1997; Morzel and Van De Vis, 2003).

1.3.1.2 Spiking (coring or iki jime)

This method is similar to captive bolt stunning of mammals. Fish are hauled out of water, restrained and a spike is driven into the brain through the top of the head by a pneumatic pistol, in order to provoke its destruction. In some cases, fish are also pithed with a rod or wire to destroy the upper part of the spinal cord and reduce carcass convulsions (Robb and Kestin, 2002). Normally, between the capture and removal from water and spiking may elapse about from 10 sec to up a minute. High precision is required in its application, and it cannot be achieved, for example, in small fish like salmon; in this species the brain is harder to target, and considering that fish makes strong attempts to escape, the system can be inclined to erroneous application that do not disable the brain. Therefore this technique is applied in larger fish that can be individually restrained (Robb et al., 2000b). Manual spiking has been applied for several years on tuna, but the result, in contrast to the semi-automatic one, is a clear delaying in time of death, that is why this technique should not be manually performed.

1.3.1.3 Electrical stunning or stunning/slaughtering methods

The application of electrical current can be a stunning or stunning/slaughtering method, according to the considered current parameters and the fish species. Electrical stunning is generally applied on eels, but recently the research is developing commercial system for salmon and trout and currently some commercial electric slaughter systems are available. Fish are typically placed in a tank full of water where electrodes are attached to the opposite sides, and an electric field of 50-Hz (mains) is passed. The electrical field is uniform and strong enough to stun the fish; loss of movement (Marx et al., 1997; Robb and Kestin, 2002) and VERs or SERs are immediate (Kestin et al., 1995; Van De Vis et al., 2003). This system has the advantage that the fish are not removed from water before they are insensible or dead (Gregory, 1998). In some systems, mainly those used for salmon, fish are immediately exsanguinated until death, and it takes around 4-5 min to loose brain functions after gill cutting (Robb et al., 2000a); whereas, trout and eels are killed by

electrocution and exsanguination is not required. An alternative way to apply electrical stunning consists in the application of a weaker electric field for longer periods to de-watered fish placed in a tank. The result is that fish exposed to low voltages are not stunned but electro-immobilized and, after the current is switched off, strong aversive reactions are seen, and death can eventually occur as consequence of muscle energy reserves exhaustion (Kestin et al., 1995; Robb and Kestin, 2002). The mechanism of death as a result of electrical application is still not known. It seems not related to heart fibrillation (Kestin and Lines, pers. comm.), as normal cardiac rhythm could be recorded after stunning for a prolonged period. Death in these fish could be due to respiratory arrest or complete and irreversible depolarization of the nervous system. When fish are stunned and evoked responses are lost, the fish enters a stage of mild tonic and clonic spasms which last around 20-50 sec in salmon and trout (Kestin et al., 1995; Robb and Roth, 2003), eel (Lambooij et al., 2002c) and African catfish(EFSA, 2004). Robb et al. (2002) showed that if the process does not kill the fish, most trout are fully recovered after 3 minutes. Eels are particularly resistant to electrical stunning and require at least 5 min of exposition to high current to achieve unconsciousness. If at the same time nitrogen is flushed in the water, eels die without recovering consciousness (Lambooij et al., 2002c). Generally, according to the fish species, higher stunning current and longer time of exposition are associated with longer periods of unconsciousness and higher mortality. Electrical stunning has some potential advantages compared to other stunning methods: large batches of fish can be stunned or stunned/slaughtered with limited handling and restraint (Roth and Moeller, 1999); the process can be performed straight in the water avoiding further stress event. However, it has to be born in mind that intense electrical currents can damage the carcass (Kestin et al., 1997), causing hematoma, blood clots, spinal and vertebrae fractures (Kestin et al., 1995;Roth and Moeller, 1999; Wall, 2001).

1.3.2 Slaughtering methods without stunning

1.3.2.1 Carbon dioxide (CO_2) narcosis

CO_2 narcosis was commonly used in some salmonid farms. Fish are placed in a bath with CO_2 gas saturated water (> 400 mg / l with a pH of 5.0-5.5). The CO_2 dissolves in water to form H_2CO_3 acid, fish blood's pH is lowered and consequently the fall causes the destruction of the brain activity, narcosis and eventually death (Kestin et

al., 1995;Robb, 2001) in about 3-4 minutes (in salmonid),then fish are slaughtered by cutting the gills and bleeding. Researches have shown that several species of fish exhibit aversive behaviour towards CO_2 narcosis and loss of sensation may occur after few minutes, depending on the species. In -salmon the time needed is about 6 min (Robb et al., 2000a), resulting in the total exhaustion of the fish at the time of death (Erikson et al., 2006; Marx et al., 1997;Robb, 2001), which reach the condition of *rigor mortis* during the processing line, approximately two hours after death (Berg et al., 1997). Carp, trout and eels showed increased mucus production (Marx et al., 1997), which could be a further irritation index. Aversive reactions to CO_2 stunning heve been reported to cause injury and scale loss (Robb and Kestin, 2002; Roth et al., 2002). Modifications to the process outlined above include ice addiction to CO_2 saturated water in order to cool it at about 1 °C; in Atlantic salmon the consequence is a faster loss in physical activity, but the activity still continues for about one min (Robb pers. comm.). Immobility is reached before loss of consciousness, within 2-4 min, and it was demonstrated that fish remain conscious until stunning time, which is different according to the species: 2 min in salmon; 3 min in trout; 9 min in carp; 109 min in eel; 7-10 min in sea bass; thus the risk is to exsanguinate or gut the fish whilst it is still conscious (Kestin et al., 1995; Marx et al., 1997; Poli et al., 2002; Robb et al., 2000b). Industry recommends that fish should be left in the water for at least 4-5 min before exsanguination, but observations indicate that fish are often removed when all carcass movements cease after 2-3 min (Robb pers. comm.). When fish are removed from CO_2 saturated water, before respiratory movement have been lost, it can recover if placed in well-oxygenated water; whereas if the fish is left in CO_2 solution for a prolonged time, the process lead to death. There is no evidence that CO_2 has analgesic or anaesthetic effect, just narcosis that does not imply any reduction in pain or fear. Carbon dioxide narcosis is potentially a killing method but in commercial practices it is usually only a sedation method since fish are rarely left in the CO_2 bath for long enough to die (Robb pers. comm.).

1.3.2.2 Carbon monoxide (CO)[1]

Carbon monoxide has been used in animal euthanasia for a long time (Smith, 2001) but is not widely used in fish. However, recent data in Atlantic salmon (Bjørlykke et

[1]This part is extrapolated from the book chapter Concollato et al. (2015) (Annex 2).

al., 2011; 2013), tilapia (Mantilla et al., 2008), pollack (*Pollachius pollachius*), herring (*Clupea harengus*) and mackerel (*Scomber scombrus*) (Slinde et al., unpublished data)suggest that CO is an excellent fish sedative agent that does not appear to cause any visible stress response.

The mode of action of CO as sedative is not fully understood. However, it is well known that CO will bind to the hem group of haemoglobin and myoglobin displacing oxygen and producing carboxy-myoglobin (COMb) and carboxy-haemoglobin (COHb) that are incapable of oxygen transport. Both COMb and COHb are stable compounds, and it has been assumed that the animal will die due to oxygen shortage without sensing the deficiency. Recent data also suggest that CO binds to the oxygen-storage proteins in *Saccus vasculosus* and neuroglobin (Ngb) of the brain. It is believed that *Saccus vasculosus* is an oxygen depot with functions during hypoxia and stress (Burmester and Hankeln, 2009), while Ngb is an oxygen transporter mainly located in neurons of the central and peripheral nervous systems and in some endocrine tissues (Reuss et al., 2002). Blocking these with CO may induce immediate sedation and unconsciousness in fish.

1.3.2.3 Death in air or asphyxiation

Asphyxiation is the oldest slaughtering method characterised by a prolonged suffering period before death. It is considered aversive to the fish since it does not induce immediate unconsciousness and can cause deleterious change in shelf-life and flesh quality, that's why it cannot be considered humane whatever the circumstances. Smaller farmed fish with low individual economic values like trout (*Oncorhynchus* and *Salmo* spp.) or tilapia (*Oreochromis* spp.) are usually killed by this technique. Within the fish farming industry, this method is commonly used for emergency killing (Roth pers. comm.). Fish are removed from water and leave to die in the air; in most of the cases, fish exhibit a violent reaction and attempts to escape, followed by decreasing muscular activity and spasms, with death after protracted agony (Robb et al., 2002). The time need for fish to die depends on both species and temperature (Table 1). Generally, higher environmental temperatures results in faster death of the fish.

Table 1. The effect of killing by asphyxiation in air on time to loss of brain function and carcass movement.

Species	Temp (°C)	Time to loss of brain function (min)	Time to loss of carcass movement (min)
Rainbow trout	14	3	28.6
Rainbow trout	20	2.6	11.1
Gilthead sea bream	22	5.5	4

(Kestin et al., 1991; Robb and Kestin, 2002; Van DeVis et al., 2003).

1.3.2.4 Asphyxia in ice/ice slurry/thermal shock

Asphyxia in ice consists in the transferring fish from water at ambient temperature into chilled brine or ice/water slurry at a significantly lower temperature (temperature differential at least 10 °C), often followed by draining of the water, as to leave the fish surrounded by ice. The purpose is to simultaneously chill, sedate and kill the fish by asphyxia. This easy and rapid procedure is used in Mediterranean countries for small sized species such as gilthead sea bream (*Sparus auratus*), sea bass (*Dicentrarchus labrax*) (Smart, 2001), eel (Van De Vis et al., 2003), turbot and for rainbow trout in the UK. Fish body temperature, metabolic rate, movements, oxygen demand decrease rapidly, and time to death may be extended. Cold adapted species, such as rainbow trout, can survive in cold waters for many days by controlling their metabolism, as happened in nature during winter periods, thus are not affected by the ice slurry and will die by anoxia in the water. When fish are placed in ice slurry, reactions can be variable: some species move around before slowing and becoming immobilised as their muscles cool; other species, like eel and gilthead sea bream, show strenuous attempt to escape (Van De Vis pers. comm.).

Table 2. Effect of asphyxiation in ice on time to loss of brain function and carcass movement.

Species	Temp (°C)	Time to loss of brain function (min)	Time to loss of carcass movement (min)
Rainbow trout	2	9.6	198
Eel	1	>12	>1
Gilthead sea bream	0.1	5.0	>1

(Robb and Kestin, 2002;Lambooij et al., 2002a).

As reported in Table 2, asphyxiation in the ice does not induce immediate unconsciousness, that's why some authors have questioned the humane aspect of this method (Kestin et al., 1991). The difference between the ambient temperature of the fish and that of the ice slurry has to be great in order to cause the thermal shock and thus reduce the time to loss of brain function; that is what happened for rainbow trout (Table 1 *vs*. Table 2), where the thermal shock may have played a role in the shortening of time to loss of brain function. However in warm Mediterranean species, such as gilthead sea bream (Bagni et al., 2002) and European sea bass (Zampacavallo et al., 2003) it has been showed that this method does not seems to be so stressful, especially if compared with asphyxiation. Live chilled sea bass did not show any violent reaction; lower haematocrit, plasma lactate and glucose, muscle lactate, reduced metabolic rate, delayed in time the onset of *rigor mortis* and high AEC (Adenylate Energy Charge) was detected. Thanks to the rapid reduction of the body temperature, improved flesh quality and shelf-life were showed (Parisi et al., 2002; Poli et al., 2002; Zampacavallo et al., 2003). However in salmonids, elevated plasma cortisol levels, marked muscle pH drop (Skjervold et al., 2001), and over time plasma osmolarity disorder (Rørvik et al., 2001) have been reported, probably related to aversive reactions. Because of the induced and progressive muscle paralysis, behaviour cannot be considered an index of fish aversion toward this slaughtering method. This technique allows the brain to recover its function, when fish is removed from the cold water very soon; moreover, Robb and Kestin (2002) demonstrated that if fish, immediately after loss of VERs and SERs, is placed in water at normal temperature it can recover brain function and muscular activity very rapidly.

1.3.2.5 Dry salt or ammonia bath

This commercial method utilised for eel slaughtering consists in placing the fish in a bath with dry salt (NaCl - sodium chloride) or 1% ammonia solution. The intent is to cause desliming of the fish, as it interferes with processing. Eels react energetically to the chemical (Kuhlmann and Munkner, 1996; Van De Vis et al., 2003); they struggle around gradually losing the motor functions over a period of 10 min. Slime is produced abundantly and when fish are limp and still are removed and processed, after about 15 min. The main aim of NaCl or ammonia addition is not to kill the animal, but to help in the slime removal and render the fish motionless and suitable

for processing (Van De Vis et al., 2002), but if left longer fish are rendered unconscious and dead. In commercial practice, it's the gutting and filleting that actually kill the fish by bleeding. If the animals die as a result of the process, it is probably because of the osmotic shock, it is usual that the most of the eels treated with salt are processed before they are dead. For this reason slaughtering of eels in salt of ammonia is considered inhumane and it has been forbidden in Germany (EFSA, 2004) and Netherlands (Van De Vis pers. comm.) since April 1999 and 2006, respectively.

1.3.2.6 Bleeding out/exsanguination

This method is commonly used after stunning of large fish to improve flesh quality, but exsanguination without stunning is also performed to slaughter salmon (*Salmo salar* L.) (Robb et al., 2000a), large rainbow trout, cod, turbot and channel catfish(Boggess et al., 1973). Gills are cut or manually pulled out or, as in flatfish, the main blood vessels in the tail are cut to achieve exsanguination, and the fish returned to water to bleed for about 10-15 min (Wardle, 1997). In some cases the isthmus is cut or the heart pierced with a knife. When exsanguination is performed after stunning, it improves welfare without compromising quality. Bleeding without prior stunning of the fish is considered aversive; Atlantic salmon showed erratic behaviour during the first 30 sec and the loss of VERs after gills cutting took 4-5 min (Robb et al., 2000a). It was reported by Morzel et al. (2002) that turbot took more than 15 min before behavioural responses were lost after bleeding. Time to die post exsanguination is dependent on the temperature, for instance Robb et al. (2000a) showed that salmon at lower temperatures take longer.

Bleeding without stunning is not considered humane and should not be used, and when performed after stunning, major vessels should be cut as to ensure a rapid loss of consciousness and death.

An investigation regarding stunning and killing methods of farmed fish was sent out to organizations and competent authorities in 22 EU and EC countries (EFSA, 2009). EFSA received 6 answers from 4 countries (Norway, United Kingdom, Iceland and Greece) concerning the stunning and killing of farmed salmonids, Atlantic salmon and rainbow trout. The methods of stunning varies between countries: Iceland uses mainly ice slurry without CO_2 (75%) and some percussive stunning (25%). United

Kingdom uses only percussive stunning. Live chilling with CO_2 was the most common method in Norway (51%). Other methods used in Norway were exposure to CO_2 (20%), and percussive stunning (14%), electric stunning (7%), ice slurry without CO_2 (6%), and combinations of methods (3%).

All the considered countries reported to be exsanguination the most common slaughtering method for salmon. Salmon industry is subject to changes in legislation as well as in technical developments, so these figures are likely to change over time. For example the use of CO_2 is currently banned in Norway, so new perspectives and new horizons are now opening.

1.3.3 Pre slaughter immobilization methods used to facilitate killing or processing

Some commercial slaughter operations contemplate a pre-slaughter/handling step as to minimize fish activity, therefore to facilitate operations and improve carcass and meat quality.

1.3.3.1 Pre slaughter sedation with anaesthetics

This method is not considered a stunning or killing method but a pre-slaughter sedation step. In the EU is forbidden to produce or to import, from countries where the practice is allowed, fish slaughtered by the utilization of pre-slaughter anaesthetics (Council Directive 2001/82/EC; Council Regulation, EEC/2377/ 90). Humane killing by anaesthetics or sedatives based on eugenols is applied and marketed outside of the EU; a well-known anaesthetic is marketed under the name AQUI-S™. In New Zealand, Chile and Australia isoeugenol (the anaesthetic compound in AQUI-S™) is used for stunning of salmon in combination with exsanguination. When immersed in the agent, salmon lose motor function and responsiveness to stimulation after about 30 min (Robb et al., 2000b). Fish are then netted and killed by percussion or spiking without showing any physical activity or aversive reaction to handling (Goodrick et al., 1998). Isoeugenol has important anaesthetic properties (Robb pers. comm.) and fish sedated before slaughter appears to be less stressed than normal fish removed from water to stunning. Induction of sedation with AQUI-S™ does not appear to be stressful according to behaviour observations, if compared to anaesthesia induction with other anaesthetics such as

MS222 –metacaine- (Kestin, Robb and Van De Vis pers. comm.), even though at high concentrations eels showed escape attempts (Van De Vis pers. comm.). Fish can detect the agents due to their characteristic chemical properties; they may be sensed through taste and smell and can also irritate the skin. Moreover, when the anaesthetic starts to take its effect, loss of balance may also elicit a stress response, thus the length of time needed to induce anaesthesia is of great importance. Goodrick et al. (1998), Jerrett et al. (1996), Robb et al. (2000b) and Van De Vis et al. (2002) reported that salmon and rainbow trout, killed after sedation with AQUI-S™, presented improved flesh quality. However, since there is no evaluation on food safety aspects, these substances are not available for use on food fish produced or imported in the EU, according to the EU Regulation 2377/80.

1.3.3.2 Pre slaughter sedation by slow live chilling

Live chilling consists in the gradual lowering of the water temperature the fish is in (1.5 °C/hour; Michie pers. comm.), and by the supplying at the meantime sufficient oxygen to maintain consciousness and prevent hypoxia. The aim of this process is to chill and sedate the fish whilst maintaining it conscious and alive. If fish are rapidly chilled they become cold paralysed. Generally, salmon are sedated prior to slaughtering during transfer from production cages to slaughter station. As a result of cooling, fish are still aware not showing aversive behaviour or any response to handling (Roth, 2003).

A more rapid live chilling can be reached by transferring the fish straight from the farm into water between 1-5 °C, in a killing and processing station. In this case, oxygen can or cannot be supplied and, after a period of chilling, fish are transferred to a bath for CO_2 narcosis or exsanguination by gill cutting (Robb pers. comm.).

If the temperature of the water from which fish are moved is high (over 10 °C), the rapid drop in temperature results in vigorous activity of the fish at the moment of the entering in the chilled water; the fish then become exhausted in about 15 to 20 min, even if they can preserve some activity for maximum 30 min. If the drop in temperature is low (as happen during winter with salmonids and other temperate species) fish do not react and muscle activity does not result affected by cold.

This means that during exsanguination process, fish are fully conscious and active and respond with vigour to gills cutting (Robb, pers. comm.). Rapid live chilling increases plasma cortisol levels (Skjervold et al., 2001), and over time also disturbs

plasma osmolarity (Rørvik et al., 2001); it also causes a large drop in muscle pH, indicating strenuous muscle activity (Skjervold et al., 2001). Roth (2003) showed that, when salmon were exposed to 2°C in CO_2 saturated seawater, aversive behaviour and flight reactions were expressed. Erikson (2002) reported that Atlantic salmon maintained in chill seawater for a maximum of 4h prior of CO_2 narcosis resulted torpid when removed from the water. Live chilling thus raises many questions with regard to welfare: after live chilling the VOR reflex may be reduced or absent and respiratory movements are very slow, but according to the species the fish may still be aware (assessed by EEGs, Van De Vis, unpublished results). Therefore, should be pay great attention when monitoring reflexes.

1.4 Methods to evaluate fish quality

Seafood is most perishable than other high-protein food, and the overall quality is characterised of both wholesomeness and sensory acceptability of the consumer (Sikorski and Sun Pan, 1994). Chemical composition and microbiological aspects influence wholesomeness, whereas sensory factors are determined by flavour and texture (Sawyer et al., 1988; Sawyer et al., 1984).

The term "quality" is widely used in seafood literature and considerable efforts has gone on searching for various tests/methodologies to be used as indicators (Olafsdottir et al., 1997). The research believed that rapid, non-destructive tests/methods for quality/freshness assessment would have been generally accepted in industry (Olafsdottir et al., 1997).After fifty years of experimentation there are several indicators available in research or as diagnostic tool in investigation of complaints, but only a few are commercially performed, since many of them do not present the necessary features for commercial functionality (Bremner, 1997; Bremner et al., 1987).

The content in trimethylamine (TMA) (Tozawa et al., 1971), the total volatile basic nitrogen (TVB-N) (Antonacopoulos and Vyncke, 2000), the individual nucleotides(Haitula et al., 1993) and the nucleotides degradation products (K-value, K_1-value) have been used as quality indicators (Burns et al., 1985;Ehira and Uchiyama, 1986;Karube et al., 1984).Although the sensory method is still the most satisfactory and the official method for fish quality assessment, it presents some limits, therefore its application in fish processing and technology is quite limited (Alasalvar et al., 2001), and other instrumental methods are needed (Macagnano et

al., 2005). In the last few years several new instrumental techniques have been introduced to measure chemical, physical and biological parameters in fish such as texturometers, colorimeters, spectrophotometers, electronic noses and tongues (Macagnano et al., 2005).

1.4.1 Total Volatile BasicNitrogen (TVB-N)

Odour is one of the most important parameters used to evaluate fish freshness. TVB-N measurement is widely used for seafood quality assessment; it gives information about the specific volatile compounds used to monitor the freshness or spoilage state of fish. TVB-N analyses include measurements of trimethylamine (TMA), dimethylamine (DMA), ammonia and other volatile basic nitrogenous compounds associated with seafood degradation. TVB-N analyses generally reflect later stages of advanced spoilage and usually is considered not reliable during the first 10 days of chilled storage in several species (Huss, 1995). In fresh caught fish TVB-N level is usually between 5 and 200 mgN/100 g muscle, whereas levels around 30-35 mgN/ 100 g muscle are considered as the limit of acceptability for ice-stored cold water fish (Connell, 1995; Huss, 1988). TMA is produced during chilled storage of fish and is the most used index in evaluating freshness. This compound is very low in fresh fish, and its presence is associated with bacterial spoilage (Fernandez-Salguero and Mackie, 1987).Headspace methods for the analysis of volatile compounds require the collection and concentration of the volatiles for subsequent chromatographic separation to identify and quantify the separated compounds. Extremely volatile, low molecular weight compounds can be analysed by static headspace methods (Milo et al., 1995). More efficient, dynamic headspace methods are necessary for collecting and concentrating less-volatilecompounds (Refsgaard et al., 1998) such as those contributing to ‘fresh fish’ and ‘oxidized’ odours. Higher-boiling compounds require even more efficient isolation methods such as solvent extraction with organic solvent or supercritical carbon dioxide (Snyder and King, 1994). Other approaches are the simultaneous distillation and extraction in the gas phase (Chung and Cadwallader, 1994)or high-vacuum distillation(Milo and Grosch, 1996). Once the volatiles have been confined, they are transferred by thermal desorption or solvent extraction to a chromatograph for separation and identified by appropriate detectors. Although instruments with a high degree of automation are available for the trapping and

chromatography steps, the time required, complexity and costs of volatile analysis methods make them suitable only for specialized research and analytical laboratories.

1.4.2 K-value measurement

At fish industry level, the use of ATP metabolites as quality indicators is not very common because of the costs and time required in the measurements (see PART I, 1.2.3.2 ATP, ATP/IMP, AEC, K-value). Generally, after acid extraction and neutralization, metabolites are separated by ion exchange chromatography or HPLC and quantified by their absorbance. HPLC is considered the most reliable method, even if other methods have used enzymatic assays and biosensors (Gill, 1995).

1.4.3 Measurements of lipid oxidation in fish

The high amount of unsaturated lipids in fish is strongly subjected to oxidation, with consequent alterations in smell, taste, colour, texture and nutritional value. Oxidation starts immediately after catch but becomes considerable for shelf-life only at temperatures <0 °C (Harris and Tall, 1989), when oxidation rather than microbial activity become the major spoilage factor. Lipid oxidation rises from early *post mortem* changes in fish tissues, which compromises the natural balance between antioxidant, pro-oxidants, favours the accumulation of active oxygen species, the activation of haemoproteins, the increase in free iron and the consumption of antioxidant (Hultin, 1994). Lipid oxidation proportions can be followed using either the reactants or the products.Measurements of oxygen consumption can be checked with an oxygen electrode (Eriksson and Svensson, 1970), whereas the loss offatty acids and antioxidants can be measured using gaschromatography (GC) and high-performance liquid chromatography (HPLC) (Erickson, 1993). The peroxide value (PV) is the most common measure of lipid hydroperoxides, alsocalled primary lipid oxidation products. Other methods for analysis are HPLC in combination with chemiluminescence detection (Yamamoto et al., 1987) or, if conjugated double bondsare present, simple spectrophotometry (Gray, 1978). The primaryproducts easily break down into secondary products, such as aldehydes and ketones. The volatile nature ofthese compounds makes them suitable for both GC andsensory analysis. Aldehydes can also be measured using several colorimetric methods, such as the method that determines the anisidine value, or the widely used thiobarbituric-acid-reactive substances (TBARS) test (Gray, 1978).Tertiary products, arising from

interactions between oxidizinglipids and nitrogen-containing compounds, canbe followed using fluorescence spectroscopy or, in later stages, by visual assessment or colorimetry (Young and Whittle, 1985).

All of these techniques are applied in research, but only a few are regularly applied in the fish industry, because are time-consuming, require expensive laboratory equipment and trained personnel. To monitor the evolution of lipid oxidation, it is important to consider many methods, especially when comparing different types of fish products. Otherwise, the instability of the various oxidation products could make the results difficult to explain and extremely confusing.

1.4.4 Texture measurement

Texture measurements can be used to study structural changes, indeed texture parameters are modified by enzymatic but also chemical reactions that lead to toughness, softening or change in elasticity of muscle and fillet. Texture is an important sensory characteristic of fish flesh responsible of quality or acceptability in high-value products (Botta, 1991).There are many factors that can affect fish texture, ranging from the species, biological condition of the fish, capture, stunning and slaughtering methods, *post mortem* treatment, storage time and temperature (Careche and Barroso, 2009).Texture is represented by different properties derived from food structure, and can be described by physical properties (mechanical or rheological). It is perceived by the feeling of touch in the mouth and in some cases in the hands. Thus it makes more sense to consider textural properties rather than texture itself (Coppes et al., 2002). Texture of raw and cooked seafood is an important attribute, that is why several attempts has been performed to appropriately measure it(Botta, 1994).To assess objectively fish and seafood product texture many studies have used a wide variety of instruments (Barroso et al., 1997;Hall et al., 1998;Hyldig and Nielsen, 2001; Ofstad et al., 1990; Sigurgisladottir et al., 1999; 2000a; 2000b). These instruments, for example the Instron Universal Testing Machine, Texture Analizer TA.XT2, and the Reograph Gel, can measure different variables under controlled conditions. Szczesniak (1998) argued that texture makes sense only when considered as “how a food feels in the mouth”, and sensory tasting methods can be as precise and repeatable as instrumental ones. These evaluations can vary from informal quality checks, trained taste panellists, and more complex consumer tests (Giese, 1995). Most common tests are: Texture Profile Analyses, Quantitative Descriptive

Analyses and Anchored Descriptive Analyses; these methods are time consuming, expensive and complex, but provide a complete analysis as perceived by human senses. Even though chemical and instrumental tests of fish texture are of high scientific value, they cannot by themselves give any information about the final quality of the product. Thus complementary sensory analysis is often necessary to determine quality parameters and shelf-life of seafood products (York and Sereda, 1994).

1.4.5 Colour measurement

Colour is the most important sensory attribute because can influence consumer decisions on the purchase of fresh meat, so it is very important to be able to maintain the key colour attributes. Colour perception depends on several aspects such as species, genetics, diet, *post mortem* changes in muscles, *post mortem* storage temperatures and time, display and lighting variables during the marketing phase. However colour of food is not stable, because it changes with decreasing freshness. Colour in fish muscle is best measured with a colorimeter, especially when the flesh is fresh since the colour is quite uniform; on the contrary, if colour uniformity is gone and colorimeter readings will depend on the location chosen, it will be most appropriate to utilise a machine vision based colour which can measure the many possible colours in no uniform surfaces.

In 1976 the CIE (Commission Internationale de L'Eclairage) developed the CIE L*a*b* colour space, which describes all the colours visible to the human eye and was created to serve as a device-independent model to be used as a reference. With CIE L*a*b*,the colorimetric distances between the individual colours corresponds to the perceived colour differences, for example the distance between green and greenish-yellow is relatively large while that distinguishing blue and red is quite small. With the three dimensional Lab colour space (CIELAB colour space), colour differences one perceives correspond to distances when measured colorimetrically. The three coordinates of CIELAB system, L*, a* and b*, represent respectively the lightness of the colour (***L**** = 0 indicates black and ***L**** = 100 diffused white), its position between red and green (***-a**** values indicate green while ***+a**** values indicate red) and its position between yellow and blue (***-b**** values indicate blue and +***b**** values indicate yellow). Colours have properties like hue, lightness and saturation. Hue is colour description as we communicate it (red, green, yellow and blue), and it

develop when wavelength are reflected from a matrix surface (e.g. meat) to the detector; lightness is referred to the brightness or darkness of the colour; saturation to how dull or vivid the colour is. L* values can be traced for lightness and darkness determination, a* and b* values to establish the hue and saturation of a sample (meat for example) (AMSA, 2012).

Colour measurements may have a fundamental role in the fresh market of particular species like tuna (Ochiai et al., 1988) or salmon(Hatano et al., 1989)where retention of flesh colour and its perception is crucial for market acceptance.

1.4.6 Near Infrared Spectroscopy (NIRS)

Fish industry has been continuously seeking for a non-destructive, reliable, fast and cost-effective method for the analysis of fish quality. Fish quality assessment has traditionally been on either time-consuming and expensive laboratory analysis or sensory assessments (Cozzolino et al., 2002). NIRS analytical technology might overcome the abovementioned limitations.

In fishery, NIRS has been used to estimate the chemical composition of several species, such as halibut, cod, and salmon (Cozzolino et al., 2002; Solberg and Fredriksen, 2001) and to discriminate between rearing systems (Xiccato et al., 2004) and between wild and farmed (Fasolato et al., 2010; Ottavian et al., 2012) or fresh and frozen–thawed (Fasolato et al., 2012; Ottavian et al., 2013; Zhu et al., 2012) samples. On rainbow trout, Gjerde and Martens (1987), Lin et al. (2006), Rasco et al. (1991), estimated a limited number of parameters (fat, moisture, protein and spoilage);Dalle Zotte et al. (2014) accurately estimated chemical properties and also classifiedrainbow trout fillets according to rearing farm.

1.4.7 Sensory evaluation of fish freshness and Quality Index Method (QIM)

Sensory evaluation is the scientific discipline used to measure, analyse and interpret features of food as perceived by the senses of smell, sight, taste, touch and hearing. Sensory tests are usually distinguished in three groups: discriminative tests, descriptive tests and affective tests (Olafsdottir et al., 1997). Discriminative tests point out whether there is a difference between the samples and, together with the descriptive ones, are considered objective analytical tests in which a trained panel is used. Affective tests are subjective consumer tests based on a measure of preference or acceptance. The choice of one test than the other depends on the aim of the application and if it is used for product development, quality assessment, consumer

studies or research. The most common descriptive tests are structured scaling according to quality assessment and profiling for an accurate description of one or more attributes (Olafsdottir et al., 1997). Sensory changes take place on appearance, odour, taste and texture of fish when they degrade (Shewan et al., 1953). In Europe the most common applied method for raw fish quality assessment in inspection and fishing industries is the European Union Scheme (CouncilRegulation (EC), 1996).This scheme does not consider differences among the species because only general parameters are used. A European alternative scaling method into commercial practice is the Quality Index Method (QIM) (Luten and Martinsdottir, 1997). In this method a number of important attributes such as gill colour, odour, firmness, appearance, etc., are designated scores on a limited scale (usually 0 up to 3) and these scores are summed to a total QI (Quality Index) to provide an index which can also be used to predict residual shelf-life (Bremner et al., 1987). This is a fast and non-destructive method, based on the direct observation of the properties of the fish itself. In the fish industry, the categorization of raw fillets is also performed. However, sensory assessment of cooked fillets is most common, especially in research and in QA/QC (Quality Assessment/Quality Control), where experienced tasters are used to detect any unusual flavours rather than to determine the “freshness”; the Torry scheme (Shewan et al., 1953) is the most commonly used scale for freshness evaluation of cooked fish, both in the fish industry and in research laboratories of Europe.

1.4.8 Electronic tongue (e-tongue)

In food analysis field many methods with high accuracy, precision and reliability are applied for the detection of specific food compounds, but are expensive, time-consuming, may require the destruction of the sample, and could be not suitable for *in situ* or *at site* monitoring. *E*-tongues, *i.e.* arrays of gas sensors, go beyond these issues and have proved to be rapid, easy to use and very promising for food quality evaluation. Although, *e*-tongues showed good correlations with organoleptic scores given by trained panellists. Some good points of *e*-tongues are their objectivity, the fact that do not get tired or infected, can be used also for toxic samples, have higher sensitivity than human tongue since the taste system in human is less developed than the olfactory one (Escuder-Gilabert and Peris, 2010).*E*-tongue can be considered as analogous of both olfaction and taste, and can be applied for the determination of any

kind of dissolved compounds, including volatile ones which give odour after evaporation (Legin et al., 2002).*E*-tongue can be considered for application in: process monitoring, freshness evaluation and shelf-life investigation(Gil et al., 2008a; 2008b;Kress-Rogers, 2001; Rodríguez-Méndez et al., 2009), authenticity assessment, foodstuff recognition, quantitative analysis, and other quality control studies. Results obtained from applications of *e*-tongue for fish freshness evaluation, pointed out its usefulness for the *in situ* or *at site* evaluation, low-cost and time-consuming features.

1.4.9 Electronic nose (e-nose)

Electronic noses are instruments that mimic the sense of smell. They are typically array of sensors used to detect and accurately distinguish odours in complex matrices at low cost (Pearce et al., 2003; Stetter and Penrose, 2002).

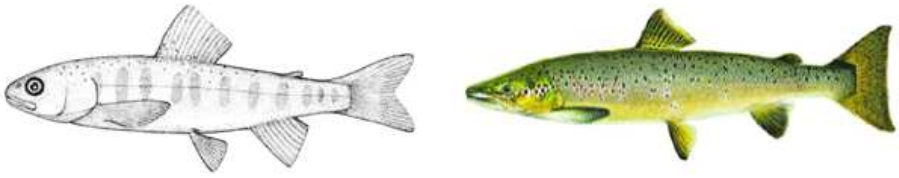
Most food aromas are difficult to characterize with conventional techniques such as gas chromatography or gas-chromatography-olfactometry. Sensory analysis performed by trained panellists results expensive since can work for only short periods of time; responses to odours are mainly subjective and it has to be considered the variability between judgements. Hence, the need of an instrument such as the electronic nose characterised by high sensitivity and correlation with data from sensory analysis. *E*-noses are easy to build, cost-effective, can perform analysis in very short time, and are considered non-destructive techniques for the characterization of food flavours (Peris and Escuder-Gilabert, 2009).The composition and concentration of volatile compounds emanating from fish depend on its freshness. Spoilage odours develop as a result of microbial growth and oxidation leading to the degradation of the tissue. Electronic noses can monitor the onset of spoilage of fish by detecting some of these volatile degradation compounds (Chantarachoti et al., 2006; Di Natale et al., 2001; Du et al., 2001; Olafsdottir et al., 2004). According to the results obtained from the studies above cited, the *e*-nose can be an alternative simple, fast and non-destructive tool for bacterial analysis in shelf-life determination (quality assessment) and spoilage classification (safety assessment).

1.5 Considered species in the research studies

1.5.1 Atlantic salmon

Atlantic salmon (*Salmo salar* Linnaeus 1758) (Figure 1) is a bony freshwater and marine fish typical of temperate and cold seas of the North Atlantic, belonging to the Salmonidaefamily. It is found naturally along both east and west coasts of the North Atlantic Ocean where it exist in both anadromous and non-anadromous freshwater resident forms. *Salmo salar* is the only species of salmon naturally found in the Atlantic Ocean. In the eastern areas, it is located between the Bay of Biscay to the south, and the Arctic Circle in the north, including the White Sea and the Barents Sea and Iceland; along the American coast occurs between Quebec and New England. Present in the Baltic Sea (Kottelat and Freyhof, 2007), is also found along the southern coasts of Greenland. In the past, go back up even the Spanish rivers where it is extinct. It was introduced in Chile, Argentina, New Zealand and Australia (Kottelat and Freyhof, 2007). It is completely absent from the Mediterranean Sea and the Italian and southern European freshwater.

Figure 1. Atlantic salmon (*Salmo salar* L.).



Found in all rivers where temperature rises above 10°C for about 3 months per year and does not exceed 20°C for more than a few weeks in summer (preferred temperatures 4-12 °C) (Kottelat and Freyhof, 2007). Although European Atlantic salmons are typically anadromous, there are examples of landlocked races or strains (Berg, 1985). Young salmon spends between 1 and 3 (usually 2) years in their natal river in Britain, but longer (up to 7 years) in colder regions such as Scandinavia and Canada (Gibson, 1993). When they are large enough (ca. 400-500 g), they undergo physiological changes where they change camouflage from stream-adapted with large grey spots, to sea adapted with shiny sides. They also undergo some endocrinological changes, to adapt for the change in osmosis process, from fresh water to salt water. Finally, the parr (young fish) will finish smoltification phase by swimming with the current instead of swimming against it. When this change of behaviour occurs, they are no longer called parr, but are referred to as smolt. In April–May when the smolts reach the sea, they follow sea surface currents and feed on plankton or fry from other fish species such as herring. After spending a few years in the sea (generally 5 for males and 7 for females) they can reach large sizes but they are typically 8-13 kg in weight when go back to their natal river to spawn.

Wild salmon disappeared from many rivers during the twentieth century due to overfishing and habitat change (Kottelat and Freyhof, 2007). By the year 2000, the number of wild Atlantic salmon had dropped to critically low levels (Dempson, 2001).

Atlantic salmon culture started in the 19th century in the UK in fresh-water as a means of stocking waters with parr specimens in order to favour wild returns for anglers. Sea cage culture was first used in the 1960s in Norway to raise Atlantic salmon to marketable size and then spread in the areas which lies within latitudes 40-70° in the Northern Hemisphere, and 40-50° in the Southern Hemisphere (www.fao.org) (Figure 2).

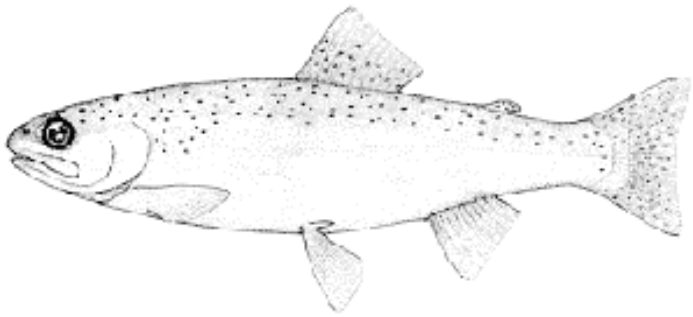
Figure 2. Main producer countries of *Salmo salar* (FAO Fishery Statistics, 2006).

Current worldwide production of farmed Atlantic salmon exceeds 1 000 000 tonnes and the major markets are currently Japan, EU and North America (www.fao.org). Salmon fillets are rich in n-3 PUFA that have beneficial health effects in humans (De Deckere et al., 1998), but are also rich in proteins, vitamins (B_6 and B_{12}), minerals, phosphorus, selenium and present a low amount in sodium.

1.5.1 Rainbow trout

Rainbow trout (*Oncorhynchus mykiss;* Walbaum 1792) (Figure 3) is a North American salmonid which typically lives in oxygenated and clear waters, but, thanks to its resistance to temperatures up to 20 °C, it proves to be able to adapt to many areas.

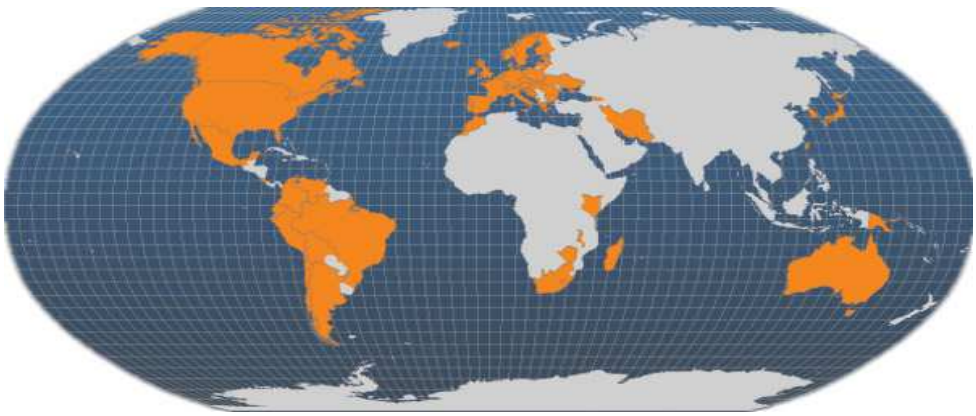
Figure 3. Rainbow trout (*Oncorhynchus mykiss*).



Production greatly expanding in 1950s and several local domesticated strains were developed. The production of rainbow trout has grown exponentially since the 1950s, especially in Europe and more recently in Chile. This is primarily due to increased inland production in countries such as France, Italy, Denmark, Germany and Spain to supply the domestic markets, and mariculture in cages in Norway and Chile for the export market. Chile is currently the largest producer. Other major producing countries include Norway, France, Italy, Spain, Denmark, USA, Germany, Iran and the UK (Figure 4) (www.fao.org).

The global aquaculture production of rainbow trout in 2012 has been 855 981 tonnes (FAOFishStat). The fresh fish market is large because the flesh is soft, delicate, and white to pink in colour with mild flavour. Preferences in meat vary globally with USA preferring white meat, but Europe and other parts in the world preferring pink meat generated from pigment supplements in aquafeed.

Figure 4. Main producer countries of *Oncorhynchus mykiss* (FAO Fishery Statistics, 2006).



2. AIM OF THE STUDY

The overall aim of this study was to assess the effects induced by different stunning/slaughtering methods on welfare and fillets quality by conventional and innovative methodologies, as well to test the discriminant ability of NIRS as innovative methodology to evaluate quality of fillets from two different farmed species of salmonids, *i.e.* Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*). The study points to inform both fish farmers and slaughtering/processing plant how important is to consider stunning/slaughtering methods able to reduce or minimize pre slaughter stress condition, not only for an ethical issue but also in order to preserve shelf-life of the fillets over in time.

To achieve these goals, 3 main research studies were set:

2.1 First research study

A first study on rainbow trout tested preliminarily the reliability of using near infrared spectroscopy (NIRS) as innovative methodology for the authentication of raw and cooked freeze-dried fillets of rainbow trout (*Oncorhynchus mykiss*) belonging to 5 different genetic strains and reared in 3 farms of Trentino Alto Adige region (Italy), characterized by different management. All this has been performed as a preliminary work to the second study (paper IV), by considering that the latter would have foreseenthe transferring ofsamplesindifferent sitesalso locatedatgreat geographical distances.

The aim of the study is synthesized in the paper produced with the results obtained, titled

- Authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) by means of near infrared spectroscopy and data fusion (PAPER I, Annex 1; published in: *Food Research International (2014): 60, 180-188*).

2.2 Second research study

The objective of this study was to investigate the effects of carbon monoxide (*CO) as stunning/slaughtering method in Atlantic s*almon (*Salmo salar* L.) on stress indicators (behaviour, adrenaline, noradrenaline, *rigor mortis* evolution), fillets

quality during shelf-life (evaluated by pH, colour, drip losses), sensory properties and to test the ability of instruments such as NIRS, *e*-nose and *e*-tongue on predicting quality traits. This experimental study was preceded by a short review summarizing current status of knowledge on the effect of CO in fish, with a focus on sedative and anesthetic treatment as well as on quality characteristics of fillet (PAPER II, Annex 2; published as chapter of the Book: *Processing and Impact on Active Components in Food (Victor R. Preedy Ed.). Elsevier, UK, pp. 427–431).*

The experimental study was organized into two parts:

- Effect of carbon monoxide for Atlantic salmon (*Salmo salar* L.) slaughtering on stress response and fillet shelf-life (PAPER III, Annex 3; published in: *Aquaculture (2014), 433:13-18*).
- CO Stunning salmon treatment revealed by electronic nose, electronic tongue and NIRS in differently prepared fillets influences *post mortem*catabolism and sensory traits (PAPER IV).

2.3 Third research study

On rainbow trout reared at two different temperature conditions (8 °C and 12 °C), the effects of stunning/slaughtering methods (carbon monoxide asphyxia; electroshock; asphyxia in the air) on *pre rigor mortis* (blood parameters, *rigormortis* development, fillet contraction changes, ATP depletion and Adenylate Energy Charge in muscle) and *post rigor mortis* (K-value, texture, lipid oxidation and sensory analysis) changes have been investigated.

The study was organized into two parts, where in details they have been considered:

- Effects of stunning/slaughtering methods on *pre rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions (PAPER V).
- Effects of stunning/slaughtering methods on *post rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions (PAPER VI).

3. MATERIAL AND METHODS

A range of different methods for assessments of fish welfare, handling stress or muscular activity immediately prior to slaughter, and quality of the derived flesh has been utilised in this thesis. An overview of these assessments is given in the Tables 3 and 4, and described in depth in the Part II, which collects the papers that have originated from the research work carried out during the PhD period.

3.1 First research study

Experimental set-up

A total of 150 farmed rainbow trout (*Oncorhynchus mykiss*) fillet samples was used in this study. Samples of five different genetic strains (indicated as IT1, IT2, IT3, USA and UK, according to origin) and three different rearing farms (in Trentino Alto Adige region in northeast Italy, indicated as farms A, B and C) were considered, for a total of ten samples per farm per genetic strain, i.e. N = 10 (samples) x 3 (farms) x 5 (genetic strains). Farm characteristics were as follows: farm A - indoor rearing tanks supplied with well water at a constant temperature (range: 11-14 °C) throughout the year; farm B - outdoor rearing (temperature range: 9-11 °C); and farm C - outdoor rearing (temperature range: 3-14 °C). Fish were collected after reaching average weight greater than 600 g (i.e., their commercial size). Twenty-four hours *post mortem*, fish were filleted and the fillets were transported in refrigerated condition to the laboratory and immediately processed. Left and right fillets of each specimen were both weighed and analysed: the former were used to evaluate raw fillet properties; the latter were used to evaluate cooked fillet properties. As regards the latter, prior to physico-chemical analyses, each sample was wrapped in aluminium foil and boiled in steamer for 10 minutes, then cooled at room temperature and re-weighed after broth removal. Cooking loss was then calculated and expressed as percentage of weight decrease.

The analytical methods performed in this trial were the followings:

Physical analyses

- Texture, measured by the Zwick-Roell® texture analyser (Zwick Roell, Ulm, Germany)
- Colour, instrumentally measured by using a Spectro-colour® meter (Dr. Lange, Düsseldorf, Germany).

Chemical analyses

- The measured chemical properties were: moisture (method 950.46; AOAC, 1995), crude protein (by Kijeldhal – method 976.05; AOAC, 1995), lipids (by Soxhlet – method 991.3; AOAC, 1995), ash (method 920.15; AOAC, 1995), total lipids (Folch et al., 1957)
- Fatty acids profile (Morrison and Smith, 1964).

NIRS scanning

- Scanning monochromator NIRSystem 5000 (FOSS NIRSystem, Silver Spring, MD, USA) was utilised to scan raw and cooked freeze-dried samples at the Animal Science Section of the Department of Animal Medicine Production and Health (MAPS) of the University of Padua, Italy

Statistical analysis

A multivariate data analysis technique was performed by using several chemometric tools:

- Principal component analysis (PCA; Jackson, 1991)
- Partial least-squares regression (PLS; Geladi and Kowalski, 1986)
- Partial least-squares discriminant analysis (PLS-DA; Barker and Rayens, 2003)
- Linear and quadratic discriminant analysis (LDA and QDA; Seber, 1984)
- *k* nearest-neighbour (*k*NN; Sharaf et al., 1986) for their classification
- Multi-block (MB) framework (Westerhuis et al., 1998).

3.2 Second research study

Experimental set-up

The study was performed at the facilities of the Institute of Marine Research (IMR), in Matre, Norway (Figure 5). Forty-five Atlantic salmon (*Salmo salar* L.) (1.07 ± 0.1 kg) were assigned to three experimental tanks containing 900 L seawater and fed with the same commercial extruded feed. Before the trial, they were starved for 24h. The temperature of seawater was constant at 7.3 ± 0.5 °C. Fish in tank 1 were used as control (C) and slaughtered by percussion; fish in tank 2 and 3 were flushed with 100% food grade CO (Yara Praxair, Oslo, Norway), using a ceramic diffuser (wedge lock base unit, Point Four Systems Inc., Richmond, Canada), for 8 (CO8) (tank 2) or 20 minutes (CO20) (tank 3) at 2-3 bar. The timing would have to coincide with the time of fish first responding to CO (8 min) and all fish being completely sedated (20 min). At the given time points, the fish were quickly hauled from the tanks and killed by percussion. During the experiment, the CO concentration in the air was monitored and measured by the use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA).

Figure 5. The facilities of Institute of Marine Research (IMR), in Matre, Norway.

The analytical methods considered for assessments of fish welfare, handling stress or muscular activity immediately prior to slaughter were the followings:

Behavioural analysis

During CO injection salmon's behaviour was recorded with a video camera then described according to Roth et al. (2003). Table 3 reports the stages of behaviour used as a reference (PAPER III).

Table 3. Different stages utilized for classification of the behaviour of Atlantic salmon recorded in the videos.

Stage	Description	Behavioural signs
0	Normal	Active swimming patterns Normal equilibrium Normal ventilation of operculum
1	Light sedation	Reduced swimming activity Problems with equilibrium Normal ventilation of operculum
2	Light narcosis	Weak swimming activity Slow and long ventilation rate Equilibrium loss with efforts to right
3	Deep narcosis	No swimming activity Problems of ventilation of operculum Total loss of equilibrium
4	Surgical anaesthesia	No swimming activity Ventilation ceases Total loss of equilibrium
5	Medullary collapse	Death ensues

Blood

Plasma adrenaline and noradrenaline

Analysed using BI-CAT® - ELISA kit (DLD - Diagnostika, GMBH, Hamburg, Germany), according to the manufacturer's instructions (PAPER III).

Rigor Index

Calculated according to Bito et al. (1983) (PAPER III).

pH

Measured by using a Mettler Toledo SevenGo pro™ pH-meter (Mettler-Toledo Ltd, Leicester, UK) equipped with an Inlab puncture electrode (Mettler-Toldedo, Ltd) (PAPER III).

The analytical methods performed in relation to flesh quality assessment were the followings:

Fillet Drip losses

Drip losses (%) were determined by weighing the fillets at different times during the shelf-life (T0, T7 and T14), and calculated by the formula:

$$\text{Drip losses} = ((D_0 - D_t/D_0) \times 100$$

where D_0 is the fillet weight immediately after filleting, while D_t corresponds to the fillet weight after "t" days of storage (PAPER III).

Fillet colour

Colour was measured by using a portable Hunterlab MiniScan™ XE Plus D/8S Color Analyzer Colorimeter (PAPER III).

Fillet Sensory analysis

Performed at time of *rigor* resolution by 12 trained panellists in two consecutive days. Data acquisition was performed by FIZZ software (Biosystemes- France) installed in the 12 terminals provided in laboratory's tasting booths (PAPERIV).

Electronic nose

An αFox (ALPHA MOS, Toulouse, France) type EN with 18 metal oxide sensors (MOS) was utilised (PAPER IV).

Electronic tongue

An αAstree II (Alpha-MOS, Toulouse, France) type ET with an LS 48 auto-sampler unit was applied to measure the characteristics of liquid samples (PAPER IV).

NIRS scanning

- NIRSystems 6500 spectrometer (FOSS NIRSystem, Silver Spring now Laurel, MD, USA) equipped with a sample transport module and small ring cup cuvette (IH-0307) was utilised to scan both raw fresh and raw freeze-dried samples at the Department of Pig and Small Animal Breeding of the University of Kaposvár, in Hungary (PAPER IV).

- Scanning monochromator NIRSystem 5000 (FOSS NIRSystem, Silver Spring, MD, USA) was utilised to scan raw freeze-dried samples at the Animal Science Section of the Department of Animal Medicine Production and Health (MAPS) of the University of Padua, Italy (PAPER IV).
- A Portable LabSpec 4 Standard-Res Lab UV-Vis-NIR Analyzer fiber optic diode array spectrophotometer (ASD, Analytical Spectral device Inc., Boudler CO) was used to scan raw freeze-dried samples over a 350-1025 nm range at the Agriculture Academy of Torino, Italy (PAPER IV).
- Raw thawed specimens were treated with ethanol (ETH) then scanned by using a FT-NIRS device (Quantum-One, PE), from 1000 to 2500 nm, that is, 2751 points in the interferograms at the Agriculture Academy of Torino, Italy (PAPER IV).

Statistical analysis

- Paper III: Data were analyzed using the general Linear Model procedures of the statistical analysis software SAS (2004) for Windows. A one-way ANOVA tested the stunning methods as fixed effects.
- Paper IV:
- Modified Partial Least Square Analysis (MPLS) (WinISI v. 1.04 software) of the two main factors (treatment and time), and of their combination was carried out for the *e*-nose (EN) and *e*-tongue (ET) traces, but also for the UV-Vis-NIR spectra from thawed, freeze-dried and ethanol prepared specimens with the dataset averaged by four replicates
- Paired Friedman compared the different instrumentations
- Fisher's Test compared the two main factors effects (Gas and Storage Time)
- Ward's Hierarchical Clustering Analysis (HCA) was performed via StatBox software v. 6.5 (Grimmer Logiciel, Paris) in order to agglomerate hierarchical clustering of objects based on distance measures of dissimilarity or similarity
- Partial Least Square Discriminant Analysis (PLS-DA) in order to get the reclassification % of the spectra from seven instrument-preparation
- Nonparametric Friedman's Test for independent samples was applied to ascertain the significance of the difference between the C and CO max *rigor* time
- PROC MIXED by SAS considered the 12 panelists as a random effect and Gas was considered as the fixed factor

- Modified PLS (WinIsI 1.5 software) was considered in order to compare the different experimental effects (Gas and Storage time) as appreciated by the different devices (ET, EN, NIRS) and to study connections with sensory scores and biological variables.

3.3 Third research study

Experimental set-up

The study was performed at the experimental farm of Edmund Mach Foundation, in S. Michele all'Adige, Trento, Italy (Figure 6). Five hundred rainbow trout (*Oncorhynchus mykiss*) were equally allocated in 5 tanks containing 3600 L of freshwater each. In tanks 1, 2, and 3 the water temperature was maintained at 12 °C whereas in tanks 4 and 5 the water temperature was maintained at 8 °C. Three stunning methods were applied on rainbow trout: asphyxia in the air (A) lasting about 15 min (fish in tank 2 and part of fish in tank 5), electroshock (E) performed by the electronic teaser GOZLIN TEQ002 (GOZLIN, Modena, Italy) for 30s at 180 V (fish in tank 1 and part of fish in tank 5), and asphyxia with carbon monoxide (CO) until death (fish tanks 3 and 4). Eighteen fish per experimental unit were sampled for the scheduled analyses. Fish from tank 1 (mean weight 740 ± 105 g) were captured, hauled out of water and immediately treated by electricity (E_12 °C); fish from tank 2 (mean weight 684 ± 95 g) were used as control group and treated by asphyxia in the air (A_12 °C); fish from tank 3 (CO_12 °C) and tank 4 (CO_8 °C) (mean weight 737 ± 120 g and 773 ± 101 g, respectively), were flushed with 100% food grade CO (SIAD, Bergamo, Italy). Due to the overall availability of only 5 tanks, from tank 5, 18 fish (mean weight 667 ± 97 g) were captured, hauled out of waterand then immediately treated by electricity (E_8 °C), afterwards other 18 fish (mean weight 760 ± 85 g) were sampled from the same tank and treated by asphyxia in the air (A_8 °C). All groups of fish were finally percussively slaughtered.

During the experiment, the CO concentration in the air was monitored and measured by the use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA) and by supplementary gas detectors in charge of the firemen of Trento province (Italy), who attended to the entire trial.

Figure 6. Experimental farm of Edmund Mach Foundation, in San Michele all'Adige, Trento (Italy).

The analytical methods considered forassessments of the stress associated to the slaughter method and fish welfare were the followings:

Plasma lactate and glucose

Analysed using MaxMat PL (MaxMat S.A., Montpellier, France) (PAPER V).

Cortisol

Determined using ELISA (RE52061, IBL International GmbH, Hamburg, Germany) (PAPER V).

Ions (K^+)

Analysed with selective ion electrodes (Cobas c111, Roche Diagnostics Ltd., Rotkreuz, Switzerland) (PAPER V).

Rigor Index

Calculated according to Bito et al. (1983) (PAPER V).

Fillet shape

Fillet shape changes during *rigor mortis* were measured by taking pictures at different times *post mortem* with a NIKON D3000 camera with lens Nikkor 18-55. The photographed fillets were analysed by the Software Adobe Photoshop CS4 for the following parameters: area, perimeter, maximum length and maximum height (PAPER V).

pH

Measured by using a Mettler Toledo SevenGo pro™ pH-meter (Mettler-Toledo Ltd, Leicester, UK) equipped with an Inlab puncture electrode (Mettler-Toledo, Ltd) (PAPER V).

ATP and Adenylate Energy Charge (AEC)

ATP was determined by a HPLC based on Burns and Ke (1985) method.

From ATP and related catabolites, Adenylate Energy Charge (AEC) = (0.5 ADP + ATP)/(AMP + ADP + ATP) (Atkinson, 1968) was also calculated (PAPER V).

The analytical methods performed in relation to quality assessment of fillets derived from differently treated fish were the followings:

Freshness indexes: K and K_1-value

K-value, defined as the ratio of the sum of the non-phosphorylated compounds, Inosine (Ino) and Hypoxanthine (Hx), to the sum of all ATP-derived degradation products was calculated according to Karube et al. (1984) with the formula:

$$\text{K-value} = [(Hx + ino) / (ATP + ADP + AMP + IMP + Ino + Hx)] * 100$$

whereas the K_1-value, was calculated as the ratio of the sum of Ino and Hx to the sum of IMP, Ino and Hx:

$$K_1\ (\%) = [(Ino + Hx) / (IMP + Ino + Hx)] * 100$$

(PAPER VI).

Fillet drip losses

Drip losses (%) were determined by weighing the fillets at three different times during the shelf-life (T0, T7 and T14), and calculated by the formula:

Drip losses = $((D_0 - D_t/D_0) \times 100$, where D_0 is the fillet weight immediately after filleting, while D_t correspond to the fillet weight after "t" days of storage (PAPER VI).

Fillet colour

Colorimetric attributes were measured by using a spectrocolorimeter (X-Rite, RM200QC; X-Rite, Incorporated, Neu-Isenburg, GermanyC) (PAPER VI).

pH

Measured by using a Mettler Toledo FiveEasy™/FiveGo™ pH meter (Mettler-Toledo Ltd, Leicester, UK) (PAPER VI).

Texture Profile Analysis (TPA)

Carried out using a Zwick Roell® 109 texturometer (software: Text Expert II, version 3), equipped with a 1kN load cell (PAPER VI).

Lipid oxidation products (TBARS Index)

The determination of the thiobarbituric acid reactive substances (TBARS) was carried out according to the method described by Siu and Draper (1978) and modified by Luciano et al. (2013) (PAPER VI).

Fillet sensory analysis

Twelve trained panellists performed a Discriminant Sensory Analysis on fillet from fish differently slaughtered with the aim to identify differences in sensory characteristics due to the slaughter method. This analysis was carried out at the time of *rigor* resolution, in two consecutive days. Data acquisition was performed by FIZZ software (Biosystemes - France) installed in the 12 terminals provided in laboratory's tasting booths (PAPER VI).

Statistical analysis

Both for paper V and VI data were analysed using the General Linear Model procedures of the statistical analysis software SAS 9.1 (2004) for Windows. A two-ways ANOVA tested the stunning/slaughter methods (three levels: A, CO and E) and the water temperatures (two levels: 8 and 12 °C) as fixed effects. The stunning/slaughter method (S) x water temperature (T) interaction was also tested. In paper VI was also performed a multivariate discriminant analysis on sensory data, by considering treatments as discriminant variable (SAS 9.1, 2004).

An overview of the fish welfare and stress assessments, as well as of the quality parameters considered in the different trials and in the different papers is given in Tables 4 and 5.

Table 4. Overview of the fish welfare and stress assessments carried out in the different trials and found in the different papers.

Parameters	***PAPER I***	***PAPER III***	***PAPER IV***	***PAPER V***	***PAPER VI***
Behavioural observations		***x***			
Blood:					
Catecholamines		***x***			
Cortisol				***x***	
Glucose				***x***	
Lactate				***x***	
Ions K^+				***x***	
Muscle:					
ATP & AEC				***x***	
pH		***x***		***x***	***x***
Rigor mortis		***x***		***x***	

ATP: Adenosine Triphosphate; AEC: Adenylate Energy Charge.

Table 5. Overview of the quality assessments carried out in the different trials and found in the different papers.

Parameters	***PAPER I***	***PAPER III***	***PAPER IV***	***PAPER V***	***PAPER VI***
K-value					***x***
Drip losses		***x***			***x***
Fillet shape changes				***x***	
Colour	***x***	***x***			***x***
Proximate composition	***x***				
Fatty acid profile	***x***				
Texture Profile Analysis	***x***				***x***
Lipid oxidation products					***x***
Sensory analysis		***x***	***x***		***x***
NIRS	***x***		***x***		
E-nose			***x***		
E-tongue			***x***		

E-nose: electronic nose; E-tongue: electronic tongue.

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5. LIST OF PAPERS

PAPER I:

Dalle Zotte, A., Ottavian, M., Concollato, A., Serva, L., Martelli, R., Parisi, G. (2014). Authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) by means of near infrared spectroscopy and data fusion. *Food Research International, 60, 180-188.*

PAPER II:

Concollato, A., Bjørlikke, G.A., Kvamme, B.O., Sørheim, O., Slinde, E., Olsen, R.E., 2015. The Effect of carbon Monoxide on Slaughter and Processing of fish. In: Victor R. Preedy (Ed.), Processing and Impact on Active Components in Food. Elsevier, UK, pp. 427–431.

PAPER III:

Concollato, A., Parisi, G., Olsen, R. E., Kvamme, B. O., Slinde, E., Dalle Zotte, A. (2014). Effect of carbon monoxide for Atlantic salmon (*Salmo salar* L.) slaughtering on stress response and fillet shelf-life. *Aquaculture, 433,* 13–18.

PAPER IV:

Salmo salar L. CO stunning treatment revealed by electronic nose, electronic tongue and NIRS in differently prepared fillets influences *post mortem*catabolism and sensory traits.

PAPER V:

Effects of stunning/slaughtering methods on *pre rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions.

PAPER VI:

Effects of stunning/slaughtering methods on *post rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions.

PART II

PAPER I

Authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) by means of near infrared spectroscopy and data fusion

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Annex 1

Authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) by means of near infrared spectroscopy and data fusion

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Abstract

This study investigated the possibility of using near infrared spectroscopy (NIRS) for the authentication of raw and cooked freeze-dried rainbow trout (*Oncorhynchus mykiss*) fillets. Latent variables models applied on the spectral data were developed and used to estimate proximate composition, fatty acid profile, fillet yield and cooking loss and to classify the available dataset according to the rearing farm and the genetic strain each sample belongs to.

Results showed that NIR spectra can be used both to accurately estimate several chemical properties and to classify the samples according to the rearing farm. In order to classify the samples according to the genetic strain, instead, a data fusion approach was used where color and mechanical information were combined with the spectral data. No major differences were observed between the results obtained from raw freeze-dried fillets and those obtained from cooked freeze-dried fillets, with the exception of the estimation of some chemical constituents of interest such as C22:6 n-3 and content of polyunsaturated fatty acids, both better estimated from cooked freeze-dried fillets.

Keywords: *Oncorhynchus mykiss*; near infrared spectroscopy; authentication; PLS; data fusion; *k*NN

1. INTRODUCTION

Rainbow trout (*Oncorhynchus mykiss*) is a North American salmonid which typically lives in oxygenated and clear waters but, thanks to its resistance to temperatures up to 20 °C, it proves to be able to adapt to many areas. The first ten countries producing farmed freshwater trout (*O. mykiss* and *S. trutta* above all) are Turkey, Iran, France, Italy, USA, Denmark, Spain, Germany, Poland and China. In 2006, these countries produced about 75% of all farmed freshwater trout, for an overall value of about 1.3 billion USD (www.worldwildlife.org/aquadialogues).

Fish industry has been constantly seeking for a non-destructive, reliable, fast and cost-effective method for the analysis of fish quality. Traditionally, in fact, the evaluation of fish quality has been based either on time consuming and expensive laboratory analysis or on sensory assessments (Cozzolino, Murray, & Scaife, 2002), which needs a trained panel of experts that might be costly and unavailable in some situations and/or environments (Nilsen, Esaiassen, Heia, & Sigernes, 2002). Near infrared spectroscopy (NIRS) is a well-known analytical technology that is intended to overcome the abovementioned limitations. Its use has been constantly increasing, and plenty of applications can be found in the literature in very diverse fields (Dalle Zotte, Berzaghi, Jansson, & Andrighetto, 2006; Giunchi, Bardinelli, Ragni, Fabbri, & Silanghi, 2008; Huang, Yu, Xu, & Ying, 2008; Nicolaï, Beullens, Bobelyn, Peirs, Saeys, Theron, & Lammertyn, 2007; Rodriguez-Otero, Hermida, & Centeno, 1997). In fishery, NIRS has been used to estimate the chemical composition of several species like halibut, cod, salmon (Cozzolino et al., 2002; Mathias, Williams, & Sobering, 1987; Nortvedt, Torrissen, & Tuene, 1998; Solberg & Fredriksen, 2001), to discriminate between rearing systems (Xiccato, Trocino, Tulli, & Tibaldi, 2004), or between wild and farmed (Ottavian, Facco, Fasolato, Novelli, Mirisola, Perini, & Barolo, 2012) or fresh and frozen-thawed (Fasolato, Novelli, Salmaso, Corain, Camin, Perini, Antonetti, & Balzan, 2012; Ottavian, Fasolato, Serva, Facco, & Barolo, 2013; Zhu, Zhang, He, Liu, & Sun, 2012) samples. However, few applications can be found on rainbow trout (Gjerde & Martens, 1987; Lin, Mousavi, Al-Holi, Cavinato, & Rasco, 2006; Rasco, Miller, & King, 1991), dealing with the estimation of few parameters (fat, moisture, protein and spoilage).

The purpose of the present work was to evaluate the performance of NIRS as a fast, cost-effective and non-destructive method for the assessment of both raw and cooked rainbow trout (*Oncorhynchus mykiss*) fillets quality. Samples of five different genetic

strains from three different rearing farms were considered and, following Gjerde and Martens (1987) that showed that water absorption bands might interfere with important spectral bands of other analytes, fillets were freeze-dried before the NIR analysis. Partial least-squares regression (PLS; Geladi & Kowalski, 1986) was used in the estimation of the chemical composition (proximate composition and fatty acid profile), whereas partial least-squares discriminant analysis (PLS-DA; Barker & Rayens, 2003), linear (LDA) and quadratic discriminant analysis (QDA; Seber, 1984), and *k* nearest neighbor (*k*NN; Sharaf, Illman, & Kowalski, 1986) models were developed to classify the samples according to the rearing farm and genetic strain they belong to. Furthermore, since the classification accuracy obtained from the NIR spectra was found to be poor with respect to the genetic strain, a data fusion approach was adopted to improve the results. The spectral information was fused with mechanical properties and colorimetric data within a multi-block framework (Ottavian et al., 2013; Westerhuis, Kourti, & MacGregor, 1998), resulting in an higher classification accuracy. To the author's knowledge, this is the first study attempting at classifying the samples according to their genetic strain using NIR spectra.

The paper is organized as follows. The Materials and Methods section describes the available data and the statistical techniques used for their manipulation. The Results and Discussion section presents firstly a preliminary analysis of the dataset, and then the estimation and classification results for both raw and cooked freeze-dried samples.

2. MATERIALS AND METHODS

2.1 Sampling and sample treatments

A total of N =150 farmed rainbow trout (*Oncorhynchus mykiss*) fillet samples was used in this study. Samples of five different genetic strains (indicated as IT1, IT2, IT3, USA and UK, according to their provenience) and three different rearing farms (in Trentino Alto Adige region, in the north-east of Italy, indicated as farm A, B and C) were considered, for a total of ten samples per farm per genetic strain, i.e. $N = 10$ (samples) $\times$ 3 (farms) $\times$ 5 (genetic strains). Farm characteristics were as follow: farm A - indoor rearing tanks supplied with well water at a constant temperature (range: 11-14 °C) throughout the year; farm B - outdoor rearing (temperature range: 9-11 °C); and farm C - outdoor rearing (temperature range: 3-14 °C).

Fish were collected after they achieved an average weight greater than 600 g. Twenty-four hours *post mortem* fish were filleted and fillets were transported to the laboratory and immediately processed. Leftand right fillets were both weighted: one was used to evaluate the raw fillets properties, whereas the other one was used to evaluate the cooked fillets properties. For the latter, prior to the physicochemical analyses each sample was wrapped in an aluminium foil and boiled in a steamer for 10 minutes, then cooled at room temperature and weighed after broth removal. Cooking loss was then calculated and expressed as percentage weight decrease.

2.2 Sample analyses

A list of all measured quality attributes for each sample is given in Table 1, while details on the analyses are given in the following subsections.

2.2.1 Physical analyses

Texture and color information were collected using a Zwick-Roell® texture analyzer (Zwick-Roell, Ulm, Germany) and a Spectro-color® meter (Dr. Lange, Düsseldorf, Germany), respectively.

The compression test was repeated three times in three different fillet positions (epaxial, ventral and caudal, indicated as E, V and C in Figure 1, respectively) using a cylindrical probe, a 200 N load cell and at 20 mm/min (constant) speed. The shear stress test was carried out in the middle of the fillet (position A in Figure 1), using a linear blade, a 200 N load cell and at 30 mm/min (constant) speed. Data were collected in terms of compression force or shear stress at different percentage of deformation (with respect to the original dimension) and at different absolute deformation (in mm).

CIELAB L^*, a^* and b^* (i.e. the three colour indexes obtained from the colorimeter; CIE, 1974) were measured in positions E, V and C (see Figure 1), by averaging from three replicates for each measurement point. Hue angle ($\tan^{-1}(b^*/a^*)$) and Chroma ($\sqrt{(a^{*2}+b^{*2})}$) values were derived from a^* and b^*(see Table 1).

2.2.2 Chemical analyses

For each sample, the chemical properties analysed were moisture (method 934.01; AOAC, 2002), protein (method 992.15, AOAC, 1993), total lipid content (method 920.39 – AOAC, 2002) and ash (942.05; AOAC, 2002). Fatty acid profiles of freeze-dried samples were analysed by gas chromatography (Morrison & Smith, 1964) after Folch extraction (Folch, Lees & Sloane-Stanley, 1957).

2.2.3 NIRS analysis and spectra pretreatments

After the freeze-drying process, fillets were ground twice with a Retsch Grindomix GM 200 (Retsch GmbH, Hann,Germany) at 4000 rpm and then at 8000 rpm per 10s. Two aliquots per sample were placed in a 50 mm diameter ring cup and scanned in reflectance mode at 2 nm intervals from 1100 nm to 2500 nm using a scanning monochromator NIRSystem 5000 (FOSS NIRSystem, Silver Spring, MD, USA). For each aliquot of a sample, a mean spectrum was obtained by averaging from 32 multiple scans; then, the spectrum of the sample was obtained by averaging those of the two aliquots.

Mathematical pretreatment reduced the light scattering caused by the sample particles and removed the additional variation in baseline shift typically present in diffused reflectance spectra. Standard normal variate and first- and second order derivates were used to this purpose (Barnes, Dhanoa, & Lister, 1989; Savitzky & Golay, 1964).

2.3 Multivariate data analysis techniques

Several chemometric tools were used to analyze the available data: principal component analysis (PCA; Jackson, 1991) for preliminary data analysis, partial least-squares regression (PLS; Geladi & Kowalski, 1986) for estimating chemical properties from NIR spectra, and partial least-squares discriminant analysis (PLS-DA; Barker & Rayens, 2003), linear and quadratic discriminant analysis (LDA and QDA; Seber, 1984) and *k* nearest-neighbor (*k*NN; Sharaf et al., 1986) for their classification. Furthermore, in order to improve the classification accuracy, data from different instruments (spectra, mechanical properties, color information, etc.) were fused (Cozzi, Ferlito, Pasini, Contiero, & Gottardo, 2009; Ottavian et al., 2013; Zhu et al., 2012) within a multi-block (MB) framework (Westerhuis et al., 1998).

To validate the proposed models, the data were split into two groups: 120 samples were used in the calibration step, while the remaining 30 (2 samples per farm per genetic strain) for model validation. Model parameters were selected in cross-validation (Wold, 1978) of the calibration data, using a venetian blind algorithm.

Please note that with the exception of the PCA models used in the preliminary data analysis, all models were built on raw and cooked freeze-dried fillets separately.

2.3.1. Exploratory analysis

Principal component analysis (PCA; Jackson, 1991) was used as an exploratory tool of the available data. PCA returned a compact representation of the data and

highlighted the existing correlation among samples and variables. Given a generic matrix **X** [*N*×*M*], its PCA decomposition is given by

$$\mathbf{X} = \mathbf{T}\mathbf{P}_{\mathrm{PCA}}^{\mathrm{T}} + \mathbf{E}_{\mathrm{X}} \quad , \tag{1}$$

with **T** [*N*×*A*], $\mathbf{P}_{\mathrm{PCA}}$ [*M*×*A*] and $\mathbf{E}_{\mathrm{X}}$ [*N*×*M*] being respectively the scores, loadings and residual of the model built on *A* principal components (PCs), and the superscript $^{\mathrm{T}}$ indicating the transpose of a matrix. Note that the data in **X** need to be properly scaled before transformation (1) is carried out. PCA summarizes the information stored in the **X** matrix by defining a low-dimensional space (called latent space), whose axes (of which the *A* loadings $\mathbf{P}_{\mathrm{PCA}}$ are the direction cosines) represent the directions of maximum variability of the original data. The scores **T** = [$\mathbf{t}_1$, $\mathbf{t}_2$, …, $\mathbf{t}_A$], i.e. the projections of **X** onto the latent space, represent the new variables.

2.3.2. Estimation of chemical properties

Given a matrix **Y** [*N*×*I*] of *I* quality attributes (i.e. the measured chemical properties listed in Table 1) of the *N* samples of **X**, the PLS model finds the main driving forces that are most related to the response by maximizing the correlation among the projections of **X** and **Y** onto a common latent space (the model space). Formally,

$$\mathbf{X} = \mathbf{T}\mathbf{P}_{\mathrm{PLS}}^{\mathrm{T}} + \mathbf{E}_{\mathrm{X}} \tag{2}$$

$$\mathbf{Y} = \mathbf{T}\mathbf{Q}^{\mathrm{T}} + \mathbf{E}_{\mathrm{Y}} \tag{3}$$

$$\mathbf{T} = \mathbf{X}\mathbf{W}^{*} \tag{4}$$

where $\mathbf{P}_{\mathrm{PLS}}$ [*M*×*A*] and **Q** [*I*×*A*] are the loadings relating the projections in the model space **T** to the data matrices **X** and **Y** (respectively). $\mathbf{W}^{*}$ [*M*×*A*] is the weight matrix, through which the data in **X** are projected onto the latent space to give the scores **T**. $\mathbf{E}_{\mathrm{X}}$ [*N*×*M*] and $\mathbf{E}_{\mathrm{Y}}$ [*N*×*I*] are the residual matrices, and account for the mismatch in the reconstruction of the original data in the *A*-th dimensional PLS model space. Both **X** and **Y** data need to be scaled prior to being transformed.

The variable importance in projection (VIP) index (Chong & Jun, 2005) can be used to identify the most influential variables. The VIP index for the *m*-th predictor is given by

$$\mathrm{VIP}_m = \frac{\sqrt{M \sum_{a=1}^{A} R^2_{\mathbf{Y},a} w^2_{m,a}}}{\sqrt{\sum_{a=1}^{A} R^2_{\mathbf{Y},a}}} \quad (5)$$

where $R^2_{\mathbf{Y},a}$ is the variance of the response matrix **Y** explained by the *a*-th latent variable (LV), and $w_{m,a}$ is the weight of the *m*-th variable on the *a*-th LV of the PLS model. Variables with VIP greater than 1 are typically considered of great importance.

2.3.3. Classification

The four classification strategies considered in this study are detailed below. Two of them (PLS-DA and LDA) are linear classifiers, while the other two (QDA and *k*NN) are non-linear ones. Please note that while PLS-DA models are calibrated directly on the available data, LDA, QDA and *k*NN models are calibrated on the scores obtained from their PCA decomposition. In the latter case, cross-validation is used to optimize the number of PCA factors (and, for *k*NN, the number *k* of neighbors to consider; Balabin, Safieva, & Lomakina, 2010).

PLS-DA

The **Y** [*N*×*L*] response matrix of the PLS-DA model (which is formally identical to the PLS of (2-4)) is built with *L* columns, being *L* the number of classes of the specific classification problem. The class (one out of *L*) of each sample was coded by *L* binary strings, i.e. class *l* was represented as

$$[\underbrace{0\ldots0}_{l\text{-}1}, 1, \underbrace{0\ldots0}_{\mathrm{L}\text{-}l}]. \quad (6)$$

Since the output of the PLS-DA model was not in the form of 0's and 1's, but instead a real number that spanned a range wider than [0,1], a threshold was chosen to define class membership. Following a Bayesian approach (with the assumption that the predictions within each class are approximately normally distributed), the threshold value was determined in such a way as to return the best possible split among classes with the least probability of false classification of future predictions (Fawcett, 2006). The number of LV to retain was selected by maximizing the classification accuracy (i.e. the percentage of correctly classified samples) in cross-validation.

LDA

LDA aims at determining the linear combinations of features which best separate the samples of the calibration set. LDA works assuming that the variability within each class follows a normal *m*-variate distribution with the same covariance matrix (which is estimated using the data of all classes), which in turns implies that the separation surface formed by joining the points characterized by the same probability of belonging to a given class is an hyperplane.

As an example, for a problem involving the classification of samples between two classes *J* and *K*, the *n*-th sample $\mathbf{x}_n$ is attributed to class *J* if

$$a + \mathbf{x}_n b > 0 \tag{7}$$

where *a* and *b* are respectively the constant term and the linear coefficients of the separating hyperplane.

QDA

QDA is closely related to LDA. Unlike in LDA, in QDA there is no assumption that the covariance of each class is identical, hence implying a quadratic separation surface. Eq. (7) defining the attribution of the *n*-th $\mathbf{x}_n$ sample to class *J* is modified as

$$a + \mathbf{x}_n b + \mathbf{x}_n \mathbf{C} \mathbf{x}_n^{\mathrm{T}} > 0 \tag{8}$$

to include the quadratic coefficient matrix **C**.

kNN

In this method, the *n*-th $\mathbf{x}_n$ sample is assigned to the most common class label among those of its *k* closest neighbors. The closest neighbors are determined by means of a distance function. The Euclidean distance was used in this study, i.e. the distance D_{ns} between $\mathbf{x}_n$ and another sample $\mathbf{x}_s$ was defined as

$$D_{ns} = (\mathbf{x}_n - \mathbf{x}_s)(\mathbf{x}_n - \mathbf{x}_s)^{\mathrm{T}}. \tag{9}$$

Data fusion

In order to enhance the classification accuracy obtained from PLS-DA, LDA, QDA and *k*NN applied to the spectral information, the different types of available data (see Section 2.2) were fused within a multi-block framework. Namely, the matrices containing each piece of information were concatenated horizontally and block-scaled, i.e., each variable was scaled according to

$$x_{n,m_b} = \frac{x_{n,m_b} - \bar{x}_{m_b}}{\sigma_{m_b}\sqrt{M_b}} \quad ,(10)$$

where $\bar{x}_{m_b}$ and σ_{m_b} represent the mean and standard deviation of the *m*-th variable of the *b*-th block. The division by the square root of the number of columns (variables) of the block (M_b) ensured the same representativeness of each block.

3. RESULTS AND DISCUSSION

3.1 Exploratory analysis

The average NIR spectra for both raw and cooked fillets are shown in Figure 2.

The main result of the cooking process is a downshift of the spectra. Figure 2 reveals the existence of two regions (around 1400 nm and around 1900 nm) where the difference is minimal, which is consistent with the freeze-drying treatment of the samples, since water absorbance is usually reported for these regions (Murray, 1986).

The score plots of a 3 PCs PCA model calibrated on the [300×700] matrix of the spectra (raw and cooked fillets, with no spectra pretreatments applied) are shown in Figure 3. PC1, explaining 95% of the total variance, mainly accounts for the difference between raw and cooked samples (see Figure 3a). The loading values on PC1, in fact, are almost the same for the entire spectral range considered, indicating that the difference between raw and cooked samples can be mainly related to the average absorbance, as it was clearly observed in Figure 2.

In Figure 3b-c the score of the raw samples are highlighted according to the farm (Figure 3b) and genetic strain (Figure 3c) they belong to (a similar behaviour was observed also for the cooked samples). The plots suggest that, at least in the PC1-PC2 plane, samples of different farms or genetics strains are not linearly separable, i.e. non-linear classifiers (such as QDA and *k*NN, see Section 2.3.3) might be necessary.

3.2 Estimation of chemical properties

The PLS estimation results are given in the following sections. For each chemical property, results are presented in terms of average (measured) value, standard deviation (SD), standard errors (SEC, SECV, SEP) and coefficients of determination (R^2_{cal}, R^2_{cv}, R^2_{p}) for model calibration, cross-validation and validation, respectively.

Since the estimation of the physical parameters returned poor results, results are not shown.

3.2.1 Raw freeze-dried fillets

Results for raw samples are given in Table 2.

For proximate composition, excellent prediction ability was observed for moisture and lipid content, while unsatisfactory results were obtained for ash and fillet yield. Similar results were reported also by other authors, though referring to different fish species (Majolini, Trocino, Xiccato, & Santulli, 2009). As for the ash content, it should be considered that its prediction is known to be challenging (Prieto, Roehe, Lavìn, Batten, & Andrés, 2009).

With regard to the fatty acid profile, Table 2 shows that satisfactory estimates were obtained for the polyunsaturated fatty acids of the n-3 (ΣPUFAn-3) and n-6 series (ΣPUFAn-6), and for the C18:2n-6c (present in plant oils used in the feed of cultured fish), C22:6n-3 (DHA), C18:3n-3 (α-linolenic acid) and C18:1n-9 (the prevailing among MUFAs; Alasalvar, Taylor, Zubcov, Shahidi, & Alexis, 2002; Fasolato et al., 2010; Testi, Bonaldo, Gatta, & Badiani, 2006) content. A lower accuracy was observed in the estimate of C16:0 (the most abundant among the saturated fatty acids) and, analogously, in the estimate of ΣSFA. With respect to C20:5n-3, the estimate obtained was quite unexpectedly poor, especially considering its high content (as it usually characterizes fish species typical of cold waters).

As a general comment on the results of Table 2, it should be said that the evaluation of the goodness of the fitting of the measured data should not be based solely on the analysis of the coefficients of determination (R^2_{cv} and R^2_{p}), since the coefficients of variation (i.e. the ratio between the standard deviation and the average value of a given quality attribute) are different for different chemical attributes. Indeed, the quality of two estimates can be very different even if their R^2 values are similar. Hence, when referring to the Table, also the standard error (SECV and SEP) should be taken into account and compared with the standard deviation (values of the ratio between SD and SEP greater than 2 are usually considered satisfactory).

3.2.2 Cooked freeze-dried fillets

Results for cooked samples are given in Table A1 in the Appendix. With respect to the proximate composition, estimates are generally worse than those obtained for the raw fillets. As for the fatty acid profile, instead, better estimates (on average) were observed for cooked fillets, particularly for the content of C17:0, C18:1n-9c, C18:2n-6c, C20:1n-7, C20:4n-6, ΣSFA, C22:6n-3 and ΣPUFA.

Despite the differences observed in the estimation accuracy of the quality attributes of raw and cooked fillets, a comparison with other literature results was not possible as (to the authors knowledge) there are no applications dealing with NIR applied on cooked fish samples. However, a similar analysis was reported by Bajwa, Kandaswamy & Apple (2009) for beef meat patties: the authors attributed the lower accuracy obtained for cooked samples to the cooking loss. As for the cooking loss, please observe that the estimate obtained in the present study could not be considered satisfactory, though the accuracy was higher than that reported by other studies (for example, by Prevolnik, Čandek-Potokar, & Škorjanc, 2010 on pork intact meat samples).

Figure 4 presents the VIP index for the PLS model of some selected properties of economic and nutritional interest. Fillet yield (Figure 4a) exhibits a peak at 1600 nm, cooking loss (Figure 4b) in the range of 1500-1600 nm, the total lipid content around 1200, 1700 and 2300 nm (Figure 4c), whereas C20:5n-3, C22:6n-3 and ΣPUFAn-6 mainly at 1700 and between 2200 and 2400 nm (Figure 4d). The carbon-hydrogen (CH) stretch second overtone is usually reported at 1202 nm, and thus the region around 1200 nm was related to the absorbance of CH, CH_2, and CH_3 groups. In the region around 1700 nm, the first overtone stretch bonds of groups CH, CH_2 (1722 and 1760 nm), and CH_3 are represented: hence, these peaks were especially related to the samples lipid content. The region around 2200 nm was characterized by CH and CH_2 combination bands, which could be related to fatty acids, protein, and peptide groups (Ottavian et al., 2012). Eventually, protein absorbance is reported at at 1550, 2055, 2180 nm (Khodabux, L'Omelette, & Jhaumeer-Laulloo, Ramasami, & Rondeau, 2007).

3.3 Classification

Results of the four classification strategies are given in the following subsections. In each case, model parameters (LVs of the PLS-DA models, PCs of the PCA models for LDA, QDA and *k*NN, and *k* for *k*NN), spectra pretreatment (no pretreatment, SNV and/or its combinations with the derivatives D1 and D2, i.e. the four combinations tested), and calibration, cross-validation and validation accuracies are presented.

3.3.1 Raw freeze-dried fillets

Results for the classification by farm using the NIR spectra are given in Table 3. With the exception of the PLS-DA model, the classification accuracies were found to

be fairly similar among models. It is interesting to notice that not only NIR allowed to discriminate between Farm A (indoor rearing, hence with more uniform and controlled fish farming conditions) and Farms B and C (outdoor rearing), but also between Farm B and C, that differed in terms of water temperature, altitude (400 and 700 m a.s.l. respectively) and dissolved oxygen (8.25 and 10.35 ppm, respectively).

As a confirmation of the conclusions drawn from the preliminary analysis (see Section 3.1), a high number of PCs were retained for the classification (with the difference between the PLS-DA model and the other models being mainly the pretreatment on the spectra).

Results for the classification by genetic strain for raw samples are given in Table 4 for the NIR spectra and in Table 5 for proximate composition, colour and mechanical properties.

Cross-validation and validation accuracies (which better resemble the practical use of the models on unknown samples) were found to be not satisfactory, with values generally below 60% (with few exceptions). Note that, as suggested from the PCA analysis, non-linear classifiers (*k*NN in particular) had better performances.

In order to improve the results, the available information (proximate composition, NIR spectra, colour and mechanical information) were fused together. Since *k*NN returned the highest classification accuracy (see Tables 4 and 5), it was used also to classify the combined information. Results are given in Table 6 for three different data combinations.

It should be noticed that higher classification accuracies were obtained when fusing the available information (with respect to those obtained using each piece of information alone).

The combination of NIR spectra, colour and mechanical information represented the best choice, as the proximate composition analyses (whose addition to the fused information did not improve the cross-validation accuracy, on which the selection of the best model was based) are much more time consuming. The fact that the addition of the proximate composition did not improve the classification accuracy was somewhat expected, as the information on the genetic strain carried by the compositional data was found to be very poor (see Table 6).

The confusion matrix for the validation data for the best model of Table 6 is given in Table 7. Recall that the confusion matrix represents, at each row-column intersection, the number of samples belonging to the class specified by the row that were assigned

to the class specified by the column (Fawcett, 2006). Five out of thirty samples were misclassified, and the majority of the errors involved samples of the genetic strain IT2. Please note that the use of the *k*NN classifier limits the interpretability of the results obtained, as no statistics such as the VIP index are available.

3.3.2 Cooked freeze-dried fillets

The results for the classification exercises on the cooked samples are given in the Tables A2-A6 in the Appendix. As a general comment, it can be noticed that the accuracy of the models was approximately the same obtained for raw samples, i.e. the cooking process did not alter the discriminating capabilities previously observed.

Samples could be easily discriminated according to their rearing farm, with poor classification accuracies obtained only for PLS-DA modelling.

The discrimination of the samples according to their genetic strain, as for the raw samples, was improved by adopting the data fusion approach (Table A5). The combination of NIR spectra and colour and mechanical properties within a multi-block *k*NN model return an almost 100% cross-validation accuracy, with only 4 (out of 30) misclassifications within the validation dataset. As it can be observed from the confusion matrix (see Table A6), errors were found to be concentrated for the genetic strain IT2.

4. CONCLUSIONS

This study was intended to investigate the capability of NIR spectroscopy in the authentication of raw and cooked rainbow trout (*Oncorhynchus mykiss*) fillets.

PLS models were built to estimate proximate composition, fillet yield, cooking loss and fatty acid profile. No relevant differences were observed between the estimates obtained from raw and cooked fillets, with the exception of some constituents of interest (such as C22:6n-3 and ΣPUFA), for which the models calibrated from cooked samples showed a higher accuracy.

PLS-DA, LDA, QDA and *k*NN models were built to classify the samples according to the rearing farm and the genetic strain they belong to. As a general result, non-linear classifiers (*k*NN in particular) overperformed the linear ones (PLS-DA and LDA). In order to improve the accuracy of the classification by genetic strain, a data fusion approach was developed, where NIR spectra, colour and mechanical information were combined within a multiblock framework. With respect to the

classification exercises, no relevant differences were observed between raw and cooked rainbow trout fillets.

Appendix A

This Appendix reports the results obtained on cooked freeze-dried samples, which were not included in the manuscript for the sake of conciseness.

Table A1 presents the PLS estimates of the chemical properties listed in Table 1, while Tables A2-A6 shows the results of the classification exercises.

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http://www.worldwildlife.org/aquadialogues

Table 1. List of the measured quality attributes.

Quality attributes	Type	Quality attributes	Type
Compression force	Physical	C16:1n-7	Chemical
Compression force @ 10% deformation	Physical	C17:1	Chemical
Compression force @ 20% deformation	Physical	C18:1n-7	Chemical
Compression force @ 30% deformation	Physical	C18:1n-9ct	Chemical
Compression force @ 40% deformation	Physical	C20:1n-7	Chemical
Compression force @ 50% deformation	Physical	C20:1n-9	Chemical
Compression force @ 3 mm deformation	Physical	C20:1n-11	Chemical
Compression force @ 5 mm deformation	Physical	C22:1n-7	Chemical
Compression force @ 7 mm deformation	Physical	C22:1n-9	Chemical
Compression force @ 9 mm deformation	Physical	C22:1n-11	Chemical
Deformation at maximum compression force	Physical	∑MUFA[(2)]	Chemical
Shear stress	Physical	C16:2n-4	Chemical
Shear stress @ 10% deformation	Physical	C16:3n-4	Chemical
Shear stress @ 20% deformation	Physical	C16:4n-1	Chemical
Shear stress @ 30% deformation	Physical	C18:2n-4	Chemical
Shear stress @ 40% deformation	Physical	C18:2n-6ct	Chemical
Shear stress @ 50% deformation	Physical	C18:3n-3	Chemical
Deformation at maximum shear stress	Physical	C18:3n-4	Chemical
L*	Physical	C18:3n-6	Chemical
a*	Physical	C18:4n-1	Chemical
b*	Physical	C18:4n-3	Chemical
Hue	Physical	C20:2n-6	Chemical
Croma	Physical	C20:3n-6	Chemical
Fillet yield	Physical	C20:4n-6	Chemical
Cooking loss	Physical	C20:3n-3	Chemical
Lipids	Chemical	C20:4n-3	Chemical
Protein	Chemical	C20:5n-3	Chemical
Ash	Chemical	C22:2n-6	Chemical
Moisture	Chemical	C21:5n-3	Chemical
C14:0	Chemical	C22:4n-6	Chemical
C15:0	Chemical	C22:5n-6	Chemical
C16:0	Chemical	C22:5n-3	Chemical
C17:0	Chemical	C22:6n-3	Chemical
C18:0	Chemical	∑PUFA[(3)]	Chemical
C20:0	Chemical	∑PUFAn-6	Chemical
C22:0	Chemical	∑PUFAn-3	Chemical
∑SFA[(1)]	Chemical		

[(1)]Saturated fatty acids;[(2)]Monounsaturated fatty acids;[(3)]Polyunsaturated fatty acids.

Table 2. PLS models performance: raw samples.

Quality attributes[1]	Average	SD	SEC	R^2_{cal}	SECV	R^2_{cv}	SEP	R^2_p
Fillet yield	51.39	2.91	1.98	0.54	2.35	0.36	1.51	0.44
Moisture	73.01	1.43	0.28	0.96	0.34	0.95	0.38	0.96
Protein	20.82	0.97	0.38	0.85	0.50	0.73	0.40	0.68
Lipids	5.64	1.13	0.24	0.96	0.27	0.94	0.33	0.95
Ash	1.34	0.07	0.05	0.53	0.05	0.47	0.04	0.17
C14:0	3.97	0.33	0.16	0.78	0.20	0.65	0.17	0.66
C15:0	0.28	0.02	0.01	0.62	0.02	0.45	0.01	0.55
C16:0	13.28	0.66	0.28	0.83	0.36	0.71	0.35	0.60
C17:0	0.24	0.02	0.02	0.56	0.02	0.35	0.01	0.52
C18:0	3.14	0.21	0.12	0.69	0.15	0.53	0.13	0.31
∑SFA	21.12	1.02	0.42	0.83	0.60	0.66	0.51	0.59
C16:1n-7	5.63	0.37	0.16	0.81	0.21	0.68	0.27	0.65
C17:1	0.16	0.01	0.01	0.72	0.01	0.53	0.01	0.48
C18:1n-7	2.54	0.13	0.06	0.79	0.08	0.64	0.05	0.88
C18:1n-9ct	11.05	1.02	0.40	0.85	0.50	0.76	0.55	0.70
C20:1n-7	0.20	0.02	0.01	0.65	0.01	0.51	0.01	0.57
C20:1n-9	0.51	0.06	0.04	0.48	0.04	0.42	0.04	0.32
C22:1n-11	0.19	0.04	0.02	0.84	0.02	0.76	0.01	0.84
∑MUFA	20.62	1.09	0.58	0.72	0.63	0.67	0.65	0.60
C16:2n-4	0.64	0.05	0.03	0.60	0.04	0.38	0.03	0.69
C16:3n-4	0.55	0.06	0.03	0.69	0.04	0.55	0.03	0.56
C16:4n-1	0.74	0.11	0.05	0.79	0.07	0.62	0.05	0.71
C18:2n-4	0.38	0.03	0.02	0.78	0.02	0.68	0.02	0.76
C18:2n-6ct	10.53	2.17	0.69	0.90	0.87	0.84	1.03	0.76
C18:3n-3	1.54	0.23	0.10	0.83	0.12	0.72	0.12	0.71
C18:3n-4	0.42	0.04	0.03	0.42	0.03	0.30	0.02	0.37
C18:4n-1	0.63	0.09	0.06	0.53	0.06	0.45	0.05	0.45
C18:4n-3	1.19	0.10	0.04	0.81	0.05	0.67	0.06	0.61
C20:2n-6	0.43	0.08	0.05	0.62	0.06	0.37	0.05	0.37
C20:3n-6	0.31	0.04	0.02	0.71	0.02	0.56	0.03	0.21
C20:4n-6	0.99	0.05	0.03	0.67	0.03	0.53	0.03	0.27
C20:5n-3	11.90	1.17	0.55	0.78	0.71	0.64	0.72	0.49
C21:5n-3	0.53	0.04	0.02	0.83	0.02	0.71	0.02	0.66
C22:5n-3	2.95	0.27	0.13	0.76	0.16	0.66	0.16	0.67
C22:6n-3	22.59	1.57	0.69	0.81	0.72	0.80	0.77	0.72
∑PUFA	57.92	1.57	0.61	0.85	0.80	0.75	1.04	0.62
∑PUFAn-6	12.89	2.25	0.74	0.89	0.94	0.82	1.06	0.77
∑PUFAn-3	41.62	2.26	0.57	0.94	0.71	0.90	0.93	0.82

[1]Fillet yield and proximate composition are expressed as percentage; fatty acids are expressed as percentage of total fatty acid methyl esters.

Table 3. Classification by farm from NIR spectra of raw samples: results.

Model	Model parameters	Spectra pretreatment	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
PLS-DA	5 LV	SNV & D2	85.8	69.2	86.7
LDA	16 PC	No preprocessing	100	100	100
QDA	12 PC	No preprocessing	100	100	100
*k*NN	*k* = 5, 16 PC	No preprocessing	98.3	98.3	96.7

Table 4. Classification by genetic strain from NIR spectra of raw samples: results.

Model	Model parameters	Spectra pretreatment	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
PLS-DA	17 LV	SNV & D2	62.5	44.2	36.7
LDA	21 PC	No preprocessing	92.5	66.7	60.0
QDA	9 PC	No preprocessing	85.8	62.5	53.3
*k*NN	*k* = 5, 14PC	No preprocessing	90.8	90.8	60.0

Table 5. Classification by genetic strain from proximate composition and color and mechanical properties of raw samples: results.

Data	Model	Model parameters	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
Proximate composition	PLS-DA	4 LV	40.8	33.3	26.7
	LDA	4 PC	38.3	33.3	23.3
	QDA	3 PC	35.0	30.0	30.0
	*k*NN	*k* = 3, 1 PC	89.2	89.2	26.7
Colour	PLS-DA	15 LV	65.0	53.3	60.0
	LDA	14 PC	70.0	54.2	53.3
	QDA	10 PC	76.7	49.2	56.7
	*k*NN	*k* = 1, 12 PC	81.7	81.7	63.3
Mechanical properties	PLS-DA	20 LV	64.2	41.7	43.3
	LDA	11 PC	55.8	45.8	53.3
	QDA	11 PC	82.5	47.5	46.7
	*k*NN	*k* = 3, 4 PC	82.5	82.5	43.3

Table 6. Multi-block *k*NN classification by genetic strain from all available information: results for raw samples.

Fused data	Model parameters	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
Colour, NIR	*k* = 3, 8 PC	84.2	84.2	66.7
Colour, NIR, Mechanical properties	*k* = 3, 7 PC	91.7	91.7	83.3
Color, NIR, Mechanical properties, Proximate compositio	*k* = 1, 13 PC	91.7	91.7	73.3

Table 7. Confusion matrix for the best multi-block *k*NN classifier (raw samples).

	IT1	IT2	IT3	UK	USA
IT1	5	0	0	1	0
IT2	0	3	1	1	1
IT3	0	0	5	0	1
UK	0	0	0	6	0
USA	0	0	0	0	6

Table A1. PLS models performance: cooked samples.

Quality attributes[1]	Average	SD	SEC	R^2_{cal}	SECV	R^2_{cv}	SEP	R^2_p
Fillet yield	51.93	3.02	2.10	0.52	2.24	0.46	1.67	0.40
Moisture	69.44	1.48	0.62	0.83	0.68	0.79	0.66	0.82
Protein	25.01	1.26	0.52	0.83	0.66	0.72	0.80	0.46
Lipids	5.46	0.96	0.24	0.94	0.25	0.93	0.65	0.56
Ash	1.35	0.08	0.06	0.52	0.07	0.28	0.05	0.28
Cooking loss	12.94	3.68	2.09	0.68	2.37	0.58	2.45	0.45
C14:0	4.22	0.35	0.18	0.75	0.22	0.60	0.20	0.53
C15:0	0.32	0.03	0.02	0.75	0.02	0.59	0.02	0.60
C16:0	16.14	0.86	0.50	0.66	0.62	0.49	0.45	0.46
C17:0	0.30	0.06	0.02	0.91	0.02	0.86	0.03	0.73
C18:0	3.82	0.23	0.12	0.73	0.15	0.58	0.13	0.47
∑SFA	25.09	1.49	0.73	0.76	0.95	0.60	0.66	0.73
C16:1n-7	6.48	0.39	0.20	0.72	0.26	0.54	0.19	0.52
C17:1	0.16	0.03	0.02	0.58	0.02	0.41	0.02	0.15
C18:1n-7	3.01	0.15	0.07	0.79	0.09	0.67	0.07	0.78
C18:1n-9ct	12.67	1.31	0.47	0.87	0.60	0.79	0.60	0.74
C20:1n-7	0.20	0.02	0.01	0.78	0.02	0.60	0.01	0.60
C20:1n-9	0.56	0.05	0.04	0.36	0.04	0.34	0.03	0.21
C22:1n-11	0.19	0.08	0.07	0.20	0.08	0.16	0.04	0.07
∑MUFA	23.52	1.35	0.56	0.83	0.70	0.73	1.00	0.40
C16:2n-4	0.66	0.07	0.04	0.68	0.05	0.53	0.04	0.27
C16:3n-4	0.59	0.07	0.04	0.67	0.05	0.45	0.04	0.26
C16:4n-1	0.80	0.12	0.06	0.74	0.08	0.61	0.07	0.46
C18:2n-4	0.38	0.04	0.01	0.88	0.02	0.79	0.02	0.60
C18:2n-6ct	11.12	2.34	0.73	0.90	0.98	0.82	0.91	0.77
C18:3n-3	1.44	0.22	0.10	0.80	0.12	0.71	0.09	0.68
C18:3n-4	0.39	0.04	0.02	0.64	0.03	0.43	0.02	0.37
C18:4n-1	0.58	0.10	0.06	0.64	0.07	0.49	0.06	0.38
C18:4n-3	1.09	0.09	0.05	0.67	0.07	0.45	0.07	0.21
C20:2n-6	0.45	0.08	0.07	0.17	0.08	0.13	0.04	0.03
C20:3n-6	0.26	0.04	0.02	0.66	0.03	0.44	0.02	0.13
C20:4n-6	1.01	0.06	0.03	0.69	0.04	0.55	0.03	0.47
C20:5n-3	9.29	0.94	0.38	0.83	0.49	0.73	0.65	0.31
C21:5n-3	0.50	0.05	0.03	0.74	0.03	0.56	0.03	0.45
C22:5n-3	3.34	0.40	0.22	0.69	0.25	0.61	0.22	0.50
C22:6n-3	18.08	1.95	0.69	0.88	0.78	0.84	0.79	0.83
∑PUFA	51.33	1.90	0.83	0.81	1.05	0.69	0.78	0.85
∑PUFAn-6	13.32	2.36	0.80	0.88	1.04	0.81	0.95	0.73
∑PUFAn-3	34.65	2.45	0.68	0.92	0.92	0.86	0.95	0.82

[1]Fillet yield and proximate composition are expressed as percentage; fatty acids are expressed as percentage of total fatty acid methyl esters.

Table A2. Classification by farm from NIR spectra of cooked samples: results.

Model	Model parameters	Spectra pre-treatment	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
PLS-DA	11 LV	No pre-processing	82.5	78.3	80.0
LDA	7 PC	No pre-processing	100	100	100
QDA	12 PC	No pre-processing	100	98.3	93.3
*k*NN	*k* = 1, 7 PC	No pre-processing	100	100	90.0

Table A3. Classification by genetic strain from NIR spectra of cooked samples: results.

Model	Model parameters	Spectra pre-treatment	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
PLS-DA	15 LV	SNV & D2	75.8	51.7	33.3
LDA	23 PC	No pre-processing	85.0	66.7	60.0
QDA	11 PC	No pre-processing	94.2	59.2	56.7
*k*NN	*k* = 2, 9 PC	SNV	90.8	90.8	63.3

Table A4. Classification by genetic strain from proximate composition and color and mechanical properties of cooked samples: results.

Data	Model	Model parameters	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
Proximate composition	PLS-DA	4 LV	45.0	36.7	36.7
	LDA	4 PC	46.7	40.0	33.3
	QDA	3 PC	48.0	43.3	46.7
	*k*NN	*k* = 5, 4 PC	74.2	74.2	40.0
Colour	PLS-DA	13 LV	63.3	48.3	40.0
	LDA	9 PC	59.2	55.8	43.3
	QDA	11 PC	76.7	50.8	43.3
	*k*NN	*k* = 3, 2 PC	86.7	86.7	53.3
Mechanical properties	PLS-DA	25 LV	71.7	52.5	46.7
	LDA	14 PC	60.0	50.8	60.0
	QDA	14 PC	90.8	40.0	43.3
	*k*NN	*k* = 3, 5 PC	89.2	89.2	40.0

Table A5. Multi-block *k*NN classification by genetic strain from all available information: results for cooked samples.

Fused data	Model parameters	Calibration accuracy (%)	Cross-validation accuracy (%)	Validation accuracy (%)
Colour, NIR	*k* = 3, 4 PC	89.2	89.2	63.3
Colour, NIR, Mechanical properties	*k* = 1, 19 PC	97.5	97.5	86.7
Colour, NIR, Mechanical properties, Proximate compositio	*k* = 5, 8 PC	90.0	90.0	70.0

Table A6. Confusion matrix for the best multi-block *k*NN classifier (cooked samples).

	IT1	IT2	IT3	UK	USA
IT1	6	0	0	0	0
IT2	0	3	1	2	0
IT3	0	1	5	0	0
UK	0	0	0	6	0
USA	0	0	0	0	6

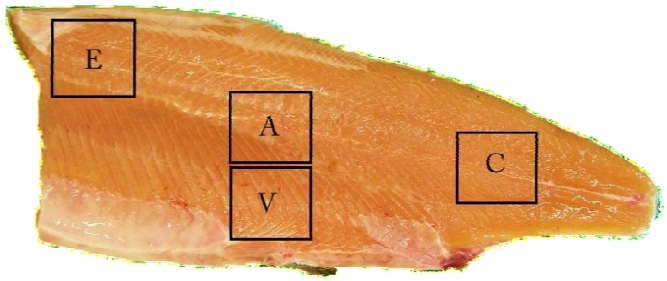

Figure 1. Rainbow trout fillet with indication of the measurement points: E (epaxial), V (ventral), C (caudal) and A (central).

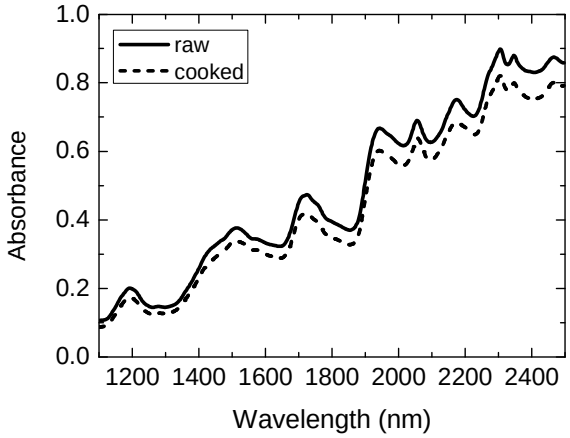

Figure 2. Average raw and cooked freeze-dried rainbow trout samples.

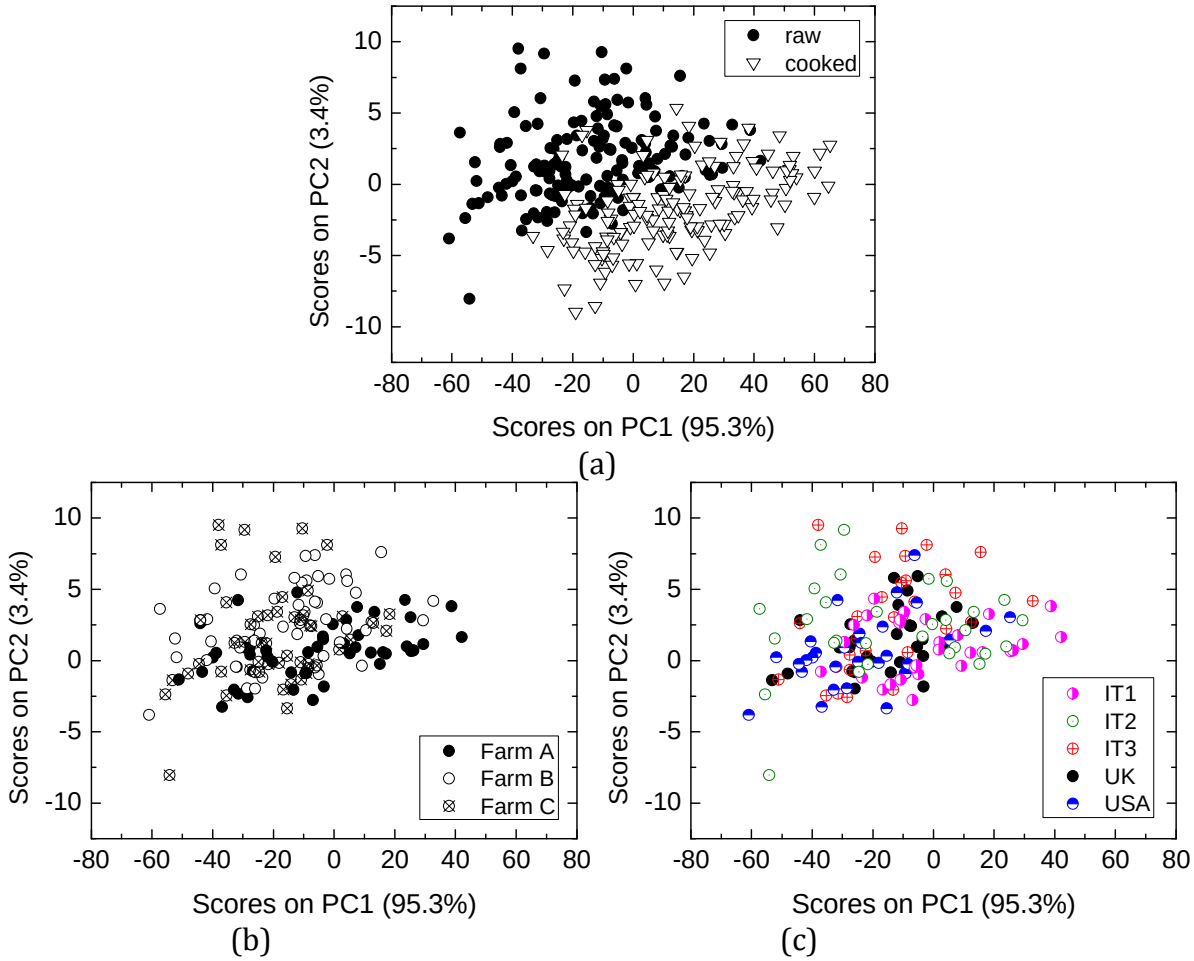

Figure 3. PC1-PC2 score plot of the preliminary PCA model built on the spectra matrix. In (a) raw and cooked samples are highlighted differently. In (b) and (c) raw samples belonging to different farms and genetic strains (respectively) are highlighted differently.

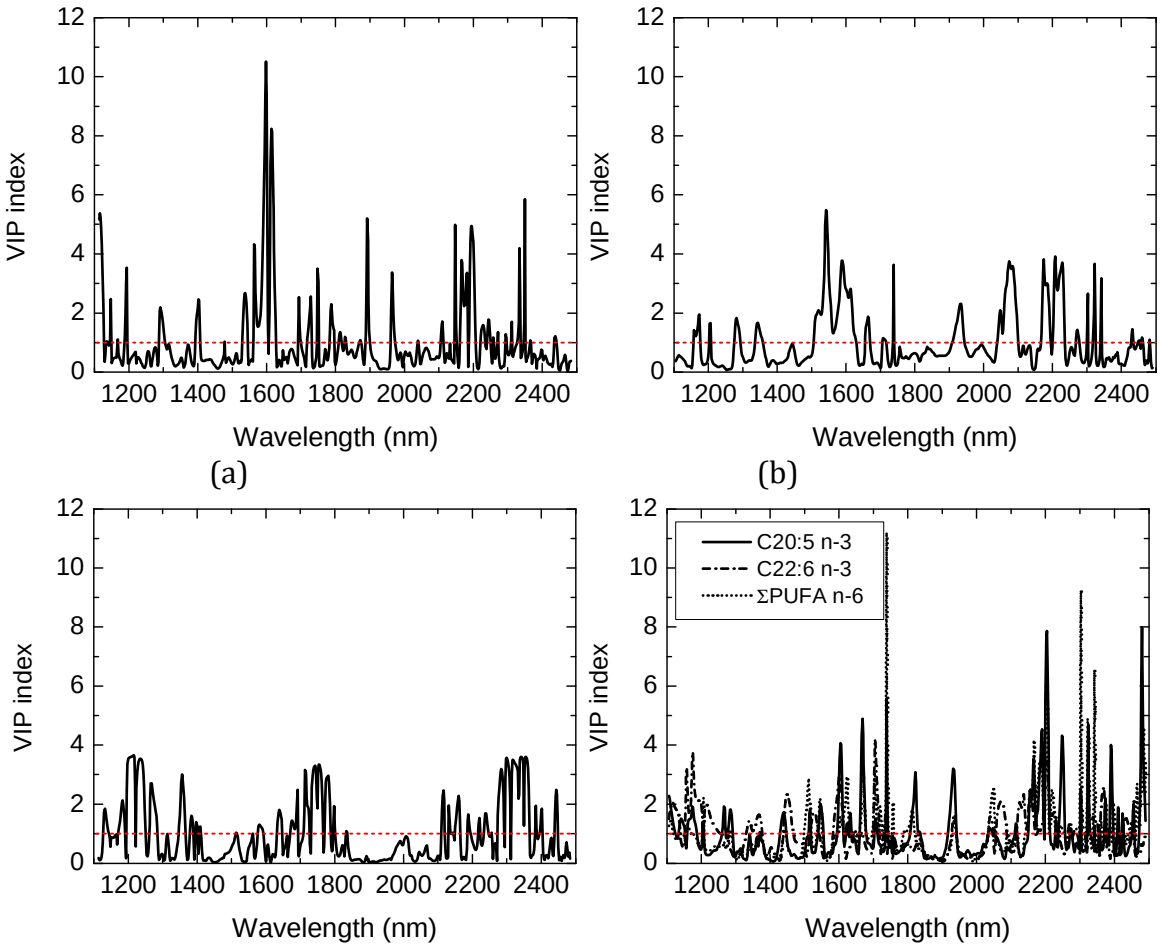

Figure 4. VIP index for (a) fillet yield, (b) cooking loss, (c) lipids content and (d) C20:5n-3, C22:6n-3 and PUFAn-6 (PLS models built using data from the cooked samples).

PAPER II

The effect of carbon monoxide on slaughter and processing of fish

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Annex 2

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Abbreviated title of paper: Effect of CO on fish

Key Words: carbon monoxide, fish quality, slaughter, color, chromatography, visible and near infrared reflection spectrophotometry

Abstract

The use of carbon monoxide (CO) to anaesthetize fish is not in common use. However, CO is an efficient sedative that does not produce aversive reactions in fish. Combined with other slaughter methods (electrical stunning), CO is a promising candidate for future and humane fish slaughter. New data suggest that CO causes brain death due to oxygen displacement in the heme-groups of neuroglobin and *Saccus vasculosus*, two recently discovered brain structures in fish. CO enhances flesh color by preventing discoloration caused by myoglobin and hemoglobin oxidation, and may improve quality in salmon and white fish. Using filtered smoke with CO, and CO packaging, favor quality. CO inhibits bacterial growth and reduces the contribution of heme-proteins to lipid oxidation. Chromatographic measurement of CO content in water enables continuous monitoring capabilities and excellent research possibilities. The effects of CO on fish quality can be assessed using Visible and Near Infrared Reflection spectrophotometry.

Introduction

Fish are regarded as a highly perishable food, since they are very susceptible to microbial and chemical decay. The type and rate of decay varies with fish species and is significantly influenced by the immediate handling before and after slaughter, and of processing and packaging systems. In general, it is important to keep the product cool at all times, and to limit oxygen availability. Optimizing these processes will lower the two main challenges, bacterial growth and lipid oxidation, the latter being particularly challenging in fatty fish species.

In the aquaculture industry, there is a growing awareness of maintaining animal welfare all the way through the slaughter process. The ethics involved in fish husbandry requires that the process proceeds with a minimum amount of strain. Normally, the fish should be anaesthetized before being slaughtered and bled. This has proven to be a challenge for the industry. The use of chemicals like clove oil had been suggested as a non-toxic anesthetic (Iversen *et al.*, 2003). However, the use of chemicals is troublesome as traces may remain in the flesh at consumption and will cause concern by some consumer groups. Further, the use of chemicals is likely to be banned in some countries while allowed in others. This creates a challenge when fish are sold on a global market. Consequently, in most countries the aquaculture industry

relies on non-chemical anesthetic methods. In Norway, only percussion and electrical stunning are allowed by the authorities, while other countries also use liquid ice and carbon dioxide (CO_2). Many of these methods have potential welfare issues that may limit their use in the future. For example, electrical stunning may cause muscular contraction promoting rapid onset of *rigor-mortis*. The force of contractions may also damage connective tissue, and cause detachment of myotomes (Robb, 2001; Jerrett *et al.*, 1996). However, when properly applied on sedated fish, the method is very useful (Roth *et al.*, 2003). Immersion in liquid ice is considered stressful by many authors and is a questionable approach (Robb, 2001; Kestin *et al.*, 1991). The method also requires a relatively high difference between temperature in holding-water and the ice-bath to efficiently immobilize the fish. The use of CO_2 is basically to asphyxiate the fish, and the exposure generally elicits a flight response, causing the fish to swim erratically, trying to escape. The method is therefore regarded as unacceptable (EFSA, 2009).

Recently, carbon monoxide (CO) has been suggested as an alternative sedative or anesthetic agent. CO treatment has proven advantageous in many aspects (Bjørlykke *et al.*, 2011), as it is not only an efficient sedative, but may also improve product quality and stability. In the following we are summarizing current status of knowledge on the effect of CO in fish, with a focus on sedative and anesthetic treatment.

Carbon monoxide, neuroglobulin and *Saccus vasculosus*.

In animals, the predominant heme containing groups are found in myoglobin and haemoglobin. The predominant states are oxy-myoglobin/hemoglogin (OMb/OHb)), deoxy-myoglobin/hemoglobin (DMb/DHb) and met-myoglobin/hemoglobin (MMb/MHb). The globins all have a central iron bound to them that is either ferrous (Fe^{2+}) or ferric (Fe^{3+}). In normal oxygenated tissue all globins are in their reduced state and bound to molecular O_2 (OMb/OHb). When oxygen is delivered to tissues, the deoxy form results. In tissues, often after slaughter, the ferrous iron will eventually oxidize to the ferric state, producing the met form (MMb/MHb).

When CO is added it will bind to the heme group of hemoglobin and myoglobin. The binding displaces oxygen and produces carboxy-myoglobin (COMb) and carboxy-haemoglobin (COHb) that are incapable of oxygen transport. Both COMb and COHb

are stable compounds, and the animal will die due to oxygen shortage, but without sensing the oxygen deficiency.

Over the past few years, there has been an increased interest in the use of CO for fish sedation and anaesthesia (Bjørlykke *et al*., 2011). The main reason is new data showing that CO may not only cause oxygen depletion, but also act directly on brain possibly causing brain death before slaughter. The data indicate that CO binds to oxygen-storage proteins in *Saccus vasculosus* below the brain, and neuroglobin (Ngb), a newly discovered globin, in the brain (Figure 1). *Saccus vasculosus* is found in fish and marine mammals and may function as an oxygen depot during hypoxia and stress. Ngb is mainly located in neurons of the central and peripheral nervous systems (Figure 1A) and in some endocrine tissues (Reuss *et al.*, 2002). It is a monomeric heme protein (Figure 1B) with a typical globin fold and a molecular weight of 17 000 (Bjørlykke *et al.*, 2012a). Ngb binds to ligands like oxygen, nitrogen-oxide, azide, cyanide and CO. The total concentration of Ngb in brain is fairly low (Bjørlykke *et al.*, 2012a), while the level of Ngb in the neuronal retina is relatively high (Schmidt *et al.*, 2003). Although the function of neuroglobin is still unknown, it appears to be involved in cellular stress regulation, and signalling, and possibly also in hypoxia signalling (Burmester and Hankeln, 2009).

Effect of CO on fish

CO has been used in animal euthanasia for a long time since it leads to a rapid and painless death with no awareness for the agent and little or no stress reaction (Smith, 2001). Compared to terrestrial animals, fish are hard to slaughter due to a general adaptation to a hypoxic water environment and their high capabilities for brain anaerobic energy metabolism (EFSA, 2009; Soengas and Aldegunde, 2002).

Despite the potential for use, CO has not been widely explored in fish. It has been demonstrated that CO can be used to sedate Atlantic salmon (*Salmo salar* L.) prior to killing without any visible adverse reactions or stress (Bjørlykke *et al.*, 2012b; Bjørlykke *et al.*, 2011). CO has also been used to anaesthetize tilapia (Mantilla *et al.*, 2008). Recently, we compared the effect of CO on pollack (*Pollachius pollachius*), Atlantic salmon, small herring (*Clupea harengus*) and mackerel (*Scomber scombrus*) using 100% food grade CO and a ceramic diffuser. With pollack, herring, and mackerel, the swimming pattern did not change with the treatment. After

approximately 5 minutes they showed signs of fatigue, and after 10 minutes they start to lose the equilibrium and swam near the surface. After approximately 12 minutes, all fish were laying at the bottom of the tank. The fish were hauled out of the tank and killed by percussion. In Atlantic salmon we observed the same behaviour, with one difference. After 12 minutes, most fish lost equilibrium and rolled over, while some briefly exhibited erratic swimming movements, before finally rolling over at the bottom of the tank. The fish were hauled from the tank and killed by percussion. As in previous experiments (Bjørlykke *et al.*, 2012b; Bjørlykke *et al.*, 2011), we could not observe any aversive or painful reactions, and the erratic unconscious movements are most likely due to irregular nerve pulses. In this respect, getting the correct dosage of gas mixtures is likely to be essential for best slaughter practices. Detecting the soluble gasses in the water (Figure 2) is therefore essential to control and reproduce the effect of CO on fish.

Processing of fish with CO

The dominant commercial method for applying CO to fish during processing is by pretreatment with filtered smoke (Kowalski, 2006). Fish rich in red muscle containing heme proteins, like tuna (various *Thunnus*) and mahi mahi (*Coryphaena hippurus*), are suitable for this technology. Filtered smoke is generated from natural sawdust by removal of some taste and odor components, carcinogen compounds and gases. Usually filtered smoke contains 15 – 40 % CO, and the fish is treated in chambers for 2 – 48 hours, depending on the size and thickness of fillets. Thereafter, the treated fillets are vacuum packaged, frozen and transported to the markets.

In addition to filtered smoke, fish may be pretreated, packaged or stored in high concentrations of CO, close to 100 %. Packaging of fresh meat with low levels of CO, up to 0.4 %, combined with high levels CO_2 and free of oxygen is well established, in particular in the USA (Cornforth and Hunt, 2008). Based on the beneficial experiences obtained with low CO packaging of meat over the last 2 – 3 decades, there is a potential for implementing this technology in the fish processing industry, yielding better color, longer shelf-life and inhibition of lipid oxidation. The application of CO already to the live fish is in this connection regarded as beneficial.

Effects of CO on fish quality

Color

In normal tissue, most color is caused by myoglobin. But some haemoglobin will also be present, particularly if fish have not been bled. Fresh tissue contains only OMb/OHb which has a bright red color. Shortly after death, oxygen is lost producing DMb/DHb that has a dark red color. With further decay, iron is oxidized to its ferric state, producing MMb/OHb which has a brown color. By consumers this color change is a little attractive feature, and producers therefore aim at maintaining the bright red color as long as possible.

When CO is added it binds directly to DMb, or to OMb displacing oxygen, producing COMb/COHb that has a cherry red color. They are stable compounds and the degradation to MMb/MHb takes a long time (Chow *et al.,* 1998) and will thus prevent discoloration. The attribute has been used by some producers to maintain color in products like tuna for a long period of time. The process (gas or filtered wood smoke) may also be used to stabilize globins of white flesh fish, improving the color appearance over time (Mantilla *et al.*, 2008; Kowalski, 2006).Table 1 shows redness, a* values, of salmon, herring and mackerel treated with CO compared to control groups. In herring and mackerel the COMb in the red muscle show persistent cherry red colour due to binding of CO. An important aspect of the trial was the typical rancid taste was not as pronounced in CO treated fish after 6 days as for the controls. Storage of Atlantic salmon in 100% CO and consequent binding to heme demonstrated that this pigment contributes slightly to the color in addition to the dominant astaxanthin pigment (Bjørlykke *et al.*, 2011; Ottestad *et al.*, 2011).

Visible (VIS) / Near Infrared Spectroscopy (NIRs) spectra of CO in fish

Ottestad *et al.* (2011) used spectroscopic measurements on mackerel muscle to study how spectral changes correspond to color variations under three different storage conditions air, vacuum and CO (Figure 3). The spectral color properties were dominated by myoglobin (and hemoglobin) at different oxidation states and bound to different ligands. The formation of COMb was positively correlated to the a* value on the L*a*b* scale (lightness, redness and yellowness). This implies the presence of different myoglobin species in fish, as reported by other authors (Mantilla *et al.*, 2008; Smulevich *et al.*, 2007). It will be interesting for future studies to use spectroscopy as

a non-destructive way for online measurements of water, lipid and protein (Folkestad *et al.*, 2008) together with visible color.

Lipid oxidation

After slaughter oxidative processes will start in meat. Oxymyoglobin is a relatively unstable compound and has the potential to contribute significantly to oxidation through several pathways. For example, OMb (Fe^{2+}) is easily oxidized to MMb (Fe^{3+}) producing superoxide anion that can dismutate to hydrogen peroxide and thus initiate lipid peroxidation. Next peroxides (lipid peroxides and hydrogen peroxide) are strong oxidizers that can oxidize OMb Fe^{2+}>Fe^{3+}(Tajima and Shikama, 1987) and thus propagate peroxidation. Finally, many peroxidation products like aldehydes, *eg* 4-hydroxy-2-noenal, may themselves attack sites on myoglobin facilitating its oxidation (Faustman *et al.*, 2010). COMb does not contain oxygen and will thus not as easily facilitate the production of superoxide radical. Introducing a ligand like CO to the 6^{th} coordination orbital increases the stability of the Fe^{2+} in the heme moiety. This will increase the shelf-life, and reduce lipid oxidation and browning of the product (Cornforth and Hunt, 2008; Hsieh *et al.*, 1998). This is especially important in fatty fish where the high level of unsaturated fatty acids makes them more susceptible to lipid oxidation.

Microbial growth

CO is known to have an inhibitory effect on microbial growth at levels above 5% (Gee & Brown, 1980). Fish treated with filtered smoke benefits from this by having extended microbiological shelf lives (Kowalski, 2006). In a study of aerobic bacteria in stored yellowfin tuna (*Thunnus albacares*),, filtered smoke efficiently reduced bacteria caused by high levels of CO, carbon dioxide and smoke components (Kristinsson *et al.*, 2007). The storage of tuna under 100 % CO reduced bacterial growth, but to a smaller extent than filtered smoke. The mechanism of CO induced inhibition on bacterial growth is still relatively unclear however. CO will affect cell respiration through inhibition of many enzymes (*e.g.*, cytochromes) with heme groups similar to Hb and Mb. With cytochromes, CO inhibits oxidative phosporylation and thus aerobic bacteria respiration and survival (Prescott *et al.*, 1996). During prolonged storage of marine fish with red muscles, bacteria may penetrate the flesh and convert free histidine to histamine. Although histamine is toxic at very low concentrations, it

does not cause appreciable visual or organoleptic changes. This increases the risk of intoxication by the consumer. Treating (directly or indirectly) fish with CO can reduce aerobic bacterial growth and histamine formation and increase shelf-life(Garner and Kristinsson, 2004).

Practical considerations

Human toxicity

It is important to be aware of the possible toxic effect of CO on humans. It is a colorless, odorless, tasteless and non-irritant gas. Inhalation of CO decreases the amount of O_2 delivered to the tissues. The affinity of hemoglobin for CO is over 200 times higher than its affinity for O_2. However, low concentration of CO is not considered a hazard. The uptake of CO to hemoglobin is reversible and the half-life of COHb is 4-6 hours. The rate of absorption and excretion of CO from the body is relatively slow. When working with CO a security alarm should be worn at all times. The administrative Norwegian working norm of CO is 25 ppm.

Legal issues

Presently, CO is not permitted for foods in the EU and Norway. The regulations in the use of CO in treatment and processing of muscle foods differ between countries and regions. The adoption of CO in treatment and processing differ in various countries due to regulatory limitations. United States Food and Drug Administration stated “tasteless smoke” or filtered smoke as GRAS (Generally Rrecognized as Safe) in 2000 (USFDA, 2000). Later, packaging of meat with up to 0.4 % CO har been permitted in the USA to master-bags and case-ready meat. Until 2004, low CO concentrations were widley used for packaging of meat in Norway, but at that time CO packaging was prohibited due to trade agreements with the EU. The positive effects of CO might cause a change in legislation in EU in the future. However, there are no regulations in EU on the use of CO as a sedative or anaesthetic component for fish.

Conclusion

- CO is an efficient sedative and anaesthetic agent in fish
- CO affects brain directly by binding to heme proteins in *Saccus vasculosus* and Ngb

- CO can be detected together with gases like nitrogen, oxygen and carbon dioxide using gas chromatography
- Visible and NIR spectroscopy can be used to study color and protein, fat and water content online
- CO stabilizes color of red fish muscle
- CO increases product stability by inhibition of microbial growth and lipid oxidation.

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Legend to figures.

Figure 1. A) Immunostaining using anti salmon neuroglobin in 1:70 dilution. Neuroglobin is found in perikardion (thick marker), and axon (thin marker) in thalamus of brain of Atlantic salmon. The arrows mark some of the positive staining. B) Western blot analysis using anti salmon neuroglobin detect recombinant salmon neuroglobin (Bjørlykke *et al.*, 2012a) at the expected Mw of 17000.

Figure 2. Detection of soluble gasses in water using an SRI dissolved gas analyzer-gas chromatography (DGA-GC) system equipped with a Thermal Conductivity Detector (TCD) and a Flame Ionisation Detector (FID). Two different standards (i.e. water samples) with different amounts of CO are shown, with the upper trace sample having twice the CO content as the lower trace sample. This detection method shows excellent reproducibility and allows detailed analysis of the water gas atmosphere. A detailed explanation of the system can be found at the SRI homepage (http://www.srigc.com).

Figure 3. Absorption spectra from mackerel fillet stored in vacuum, air and CO. The Soret maximum in Visible Spectra for mackerel packed in air was 421 nm; in CO 423 nm; and in vacuum 431 nm. This show the presence of the various myoglobin species depending on storage conditions. Fillet stored in vacuum (black solid line), air (dotted line) and CO (hyphened line). Courtesy of Jens Petter Wold, Nofima.

Figure 1

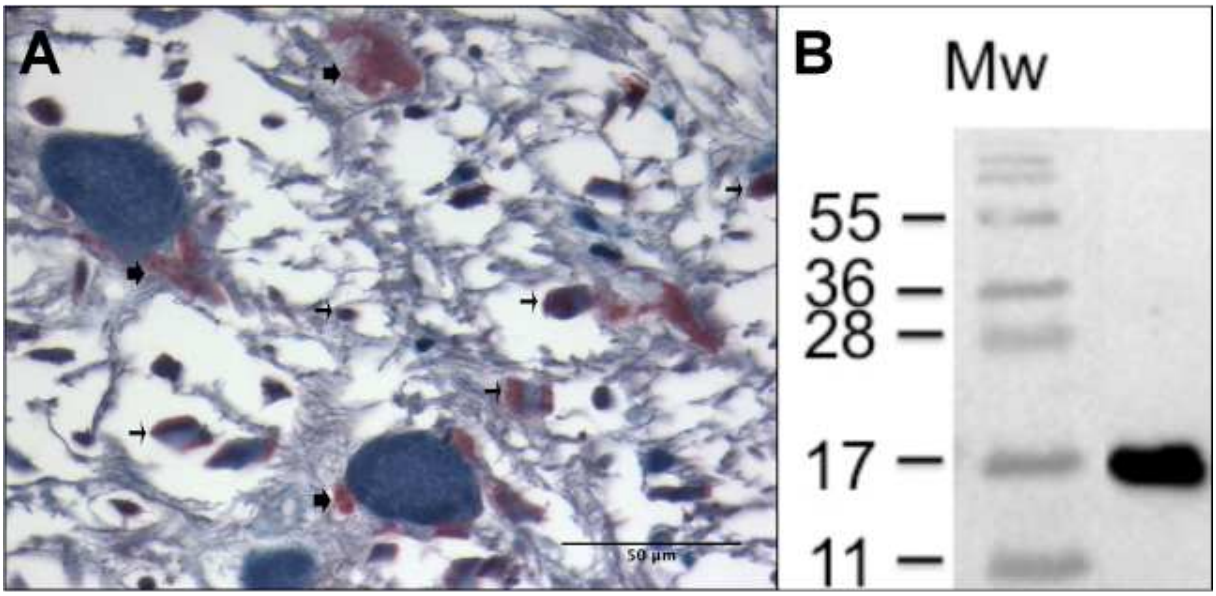


Figure 2

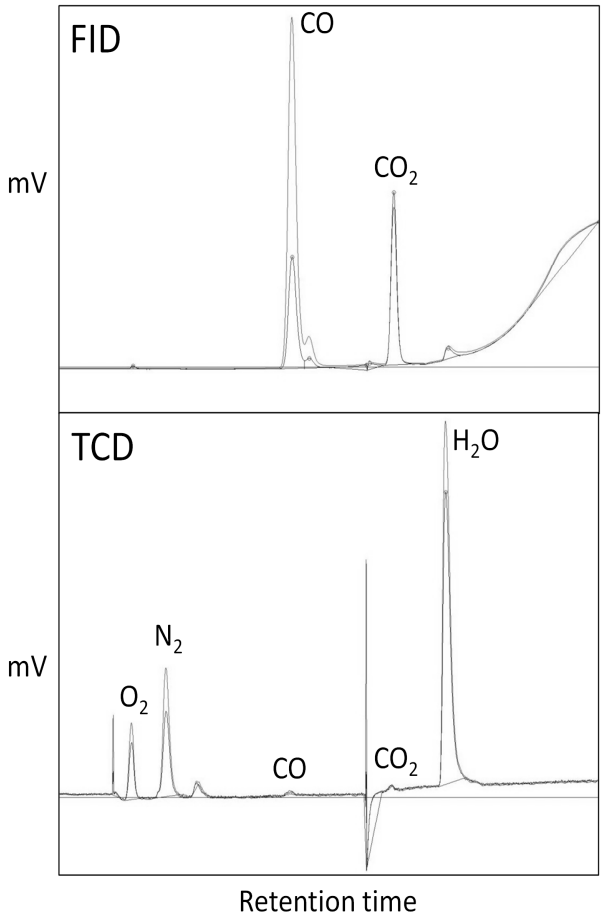
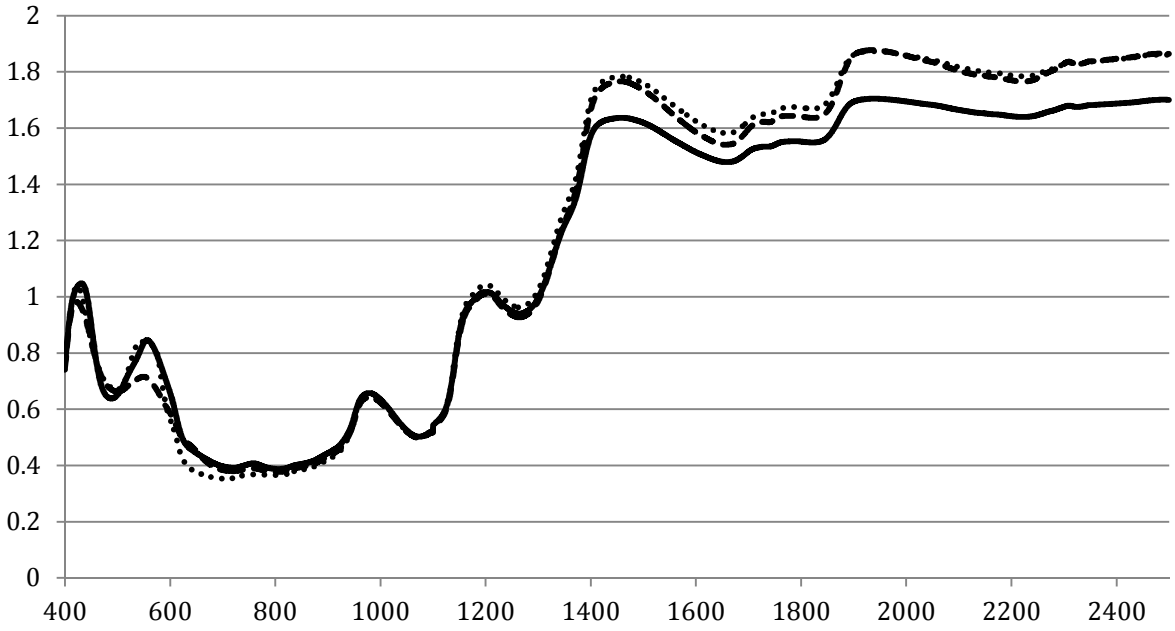


Figure 3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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
400
600
800
1000
1200
1400
1600
1800
2000
2200
2400

Table 1. Colour (a*, *i.e.* change in red colour) of cold room stored (4^{o}C) Atlantic salmon (1.30 kg), herring (0.16 kg) and mackerel (0.56 kg) after being anaesthetized with CO. The L* and b* values were very similar for treated and untreated fish.

Day	Salmon		Herring		Mackerel	
	Control	CO	Control	CO	Control	CO
1	22.3 ±0.7	23.0 ±2.0	2.7 ±1.1	10.0 ±2.0	9.5 ±1.0	8.9 ±2.0
6	19.4 ±1.0	21.9 ±2.0	3.7 ±0.3	10.3 ±2.0	5.1 ±0.9	10.0 ±0.7

PAPER III

Effect of carbon monoxide for Atlantic salmon (*Salmo salar* L.) slaughtering on stress response and fillet shelf-life

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Annex 3

Effect of carbon monoxide for Atlantic salmon (*Salmo salar* L.) slaughtering on stress response and fillet shelf-life

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Abstract

The effect of carbon monoxide (CO) as stunning method in Atlantic salmon (*Salmo salar* L.) on stress indicators (adrenaline, A; noradrenaline, NAD) and on fillets quality during the shelf-life has been investigated. The CO was dissolved into tanks with salmon for 8 and 20 minutes to obtain fish groups CO8 and CO20, respectively. These groups were compared to a non-stressed Control group (C). All the fish were hauled out from the tank and killed by percussion. Adrenaline content of CO20 group was 1.8 and 1.7-fold higher than CO8 and C groups respectively ($P<0.001$), which exhibited similar values. Noradrenaline content was higher in CO20 than in C group (8.1 *vs.* 5.4 ng/ml plasma; $P<0.0001$). The CO treatment resulted in a small significant increase in lightness and yellowness, not altering the overall "natural" colour of the fillet. CO treatment caused a rapid onset of *rigor mortis* and a small but significant increase in drip loss ($P<0.05$).

Keywords: Carbon monoxide, Stunning, Catecholamines, Fish quality, Shelf-life

1. INTRODUCTION

Fish quality can be influenced by *pre*, *ante* and *post mortem* conditions, including handling before slaughter, slaughtering methods and storage conditions.

Animal welfare has become a crucial issue for farmed fish. There are no optimal stunning conditions available today.

Carbon monoxide (CO) has proven not to provoke the aversive reactions (Smith, 2001) as seen with CO_2 (Poli et al., 2005). The effectiveness of CO is due to its displacement of oxygen on heme proteins (hemoglobin (Hb), myoglobin (Mb) and neuroglobin (Ngb)), causing tissue hypoxia (Brunori and Vallone, 2007; Devenport, 2002; Kalin, 1996). The effect is quick sedation and unconsciousness and the animal will die due to O_2 shortage without sensing the deficiency. It is also believed that CO binds to the oxygen-storage proteins in *Saccus vasculosus,*a well-vascularized organ situated in the ventral side of the brain with several putative functions during hypoxia and stress, but also as oxygen depot and transport(Burmester and Hankeln 2009; Yanez et al., 1997; Sanson, 1998).

CO has been used for decades as food preservative in food industry (Sørheim et al., 2001). However, CO has also been demonstrated to mask spoilage as the cherry red colour can last beyond the microbiological shelf-life of the meat (Kropf, 1980). Consequently, the use has been discontinued for meat in many countries (Wilkinson et al., 2006).

CO is also known to improve colour stability in red muscles (Chow et al., 2008; Kowalski, 2006), reduce microbial growth (Gee and Brown, 1980) and lipid oxidation (Cornforth and Hunt, 2008; Hsieh et al., 1998) even when live fish is exposed to CO (Mantilla et al., 2008). The latter is particularly interesting in fatty fish like salmon, which is vulnerable to lipid oxidation due to the high level of unsaturated fatty acids. When CO is added, it binds directly to oxymyoglobin/oxyhemoglobin (OMb/OHb), displacing oxygen, producing COMb/COHb that has a cherry red colour. They are stable compounds and the degradation to meth-forms MMb/MHb takes longer time (Chow et al., 1998) and will thus prevent discoloration. In Atlantic salmon, herring and mackerel anaesthetized by injecting CO in seawater, redness (a* value) was more persistent than the control groups; moreover CO treated fish did not develop the typical rancid smell even after 6 days of cold storage as was the case of the controls (Concollato et al., 2014). The autoxidation of heme protein to meth-forms is also a

critical step in lipid oxidation. MMb/MHb reacts with peroxides and stimulates formation of chemical compounds able of initiating and propagating lipid oxidation (Shahidi and Botta, 1994; Everse and Hsia, 1997; Mantilla et al., 2008), which is a major cause of quality deterioration in seafood, contributing to the formation of off-odours, off-flavours and texture declining. Since CO is expected to retard lipid oxidation of Hb and Mb to the meth-forms, it is possible that this treatment may extend the shelf-life of the product.

Bjørlykke et al. (2011) observed that Atlantic salmon did not take any notice of the gas once injected in the tank by diffusers. One limitation of killing fish with CO is its relative low solubility in water. It was reported by Daniels and Getman (1948) and Lide (2005) that CO has a 1.7×10^{-5} mole fraction solubility in water at 25 °C and 101 kPa. This solubility however is similar to that of O_2 in water (2.2×10^{-5} mole fraction solubility at 101 kPa) (Lide, 2005). This suggests that O_2 and CO dissolved volumes are almost equal. The volume of O_2 or CO dissolved in water is dependent by the partial pressure of the gas and temperature, the solubility of which increases as the temperature decreases (Mantilla et al., 2008).

Stress is a biological response elicited when an animal make abnormal or extreme adjustments in its physiology or behaviour in order to cope with adverse aspects of its management (Terlouw, 2005). During exposure to internal and environmental stressors, catecholamines like adrenaline (AD) and noradrenaline (NAD) are released by modulating cardiovascular and respiratory functions in order to maintain adequate levels of oxygen in the blood. Catecholamines also initiates breakdown of glycogen to increase available energy input during stress. This leads to physical responses including unsettled movements. In addition, stress may turn the metabolism in a more anaerobic one, which result in a lower glycogen content giving a faster pH decrease and onset of *rigor mortis* (Van Laak et al., 2000).

The aim of this study was to expose Atlantic salmon to CO before slaughtering in order to provide information on how this gas can affect adrenaline (AD) and noradrenaline (NAD) plasma levels and the fillet's quality changes during the shelf-life, in comparison with fish percussively slaughtered.

2. MATERIALS AND METHODS

2.1 Experimental set-up

The trial was carried out at the Institute of Marine Research, in Matre (61° N, western Norway). A total of forty-five Atlantic salmon (*Salmo salar* L.) (1.07 ± 0.1 kg) were assigned to three experimental tanks containing 900 L seawater, and were fed with the same commercial extruded feed. One week prior to the experiment, the ceramic diffusors (wedge lock base unit; Point Four Systems Inc., Richmond, Canada), were placed into the tanks, and used to deliver oxygen twice a day to get the fish accustomed to the bubbles. Before the trial, they were starved for 24h. The temperature of seawater was constant at 7.3 ± 0.5 °C. Fish in tank 1 were used as control (C) and slaughtered by percussion; fish in tank 2 and 3 were flushed with 100% food grade CO (Yara Praxair, Oslo, Norway), for 8 (CO8) (tank 2) or 20 minutes (CO20) (tank 3) at 2-3 bar. The timing would have to coincide with the time to fish first responding to CO (8 min) and all fish being sedated (20 min). At the given time points, the fish were quickly hauled from the tanks and killed by percussion. During the experiment, the CO concentration in the air was monitored and measured by the use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA).

The experiment was approved according to "The Regulations in Animal Experimentation" in Norway and carried out by certified personnel.

2.2 Behavioural analysis and measurement of CO

During CO injection salmon's behaviour was recorded with a video camera then described according to Roth et al. (2003). Table 1 reports the stages of behaviour used as a reference. Seawater CO analysis was performed as described in Concollato et al. (2014). Calibration was performed using standard gas containing 0.01, 0.1 and 1.0 % CO.

2.3 Plasma adrenaline (AD) and noradrenaline (NAD)

Immediately after slaughter, heparinised blood samples were collected from the caudal vein of 5 fish per tank (total No. = 15 fish). Samples were placed on ice and plasma prepared by centrifugation (13.500 rpm for 2 min) and frozen at -80 °C until the analyses. AD and NAD were analysed using BI-CAT® - ELISA kit (DLD - Diagnostika, GMBH, Hamburg, Germany), according to the manufacturer's instructions.

2.4 Rigor Index, pH, colour and drip loss

After slaughter, salmons from tank 1 (C group) and tank 2 (CO20 group) were individually tagged, weighed and stored in polystyrene boxes with ice. *Rigor mortis* and pH were determined on 6 fish/treatment at 0, 3, 9, 15, 24, 30, 40, 48 and 64 hours *post mortem*. *Rigor mortis* was measured by tail drop, and Rigor Index (RI) was calculated according to Bito et al.(1983), using the following formula:

$$RI\ (\%) = [(L_0 - L_t)/L_0] \times 100$$

where L_0(cm) is the vertical distance between the base of the caudal fin and the table surface measured immediately after the death, whereas L_t (cm) is the vertical distance between the base of the caudal fin and the table surface at the selected time intervals.

The pH was measured on the cranial part of the epaxial neck region, using a Mettler Toledo SevenGo pro™ pH-meter (Mettler-Toledo Ltd, Leicester, UK) equipped with an Inlab puncture electrode (Mettler-Toldedo, Ltd). After *rigor mortis* resolution (64 h *post mortem*, Time 0 – T0), all the 30 fish were gutted, filleted and weighed, then right fillets were vacuum packed and stored at -20 °C for further analyses, whereas the left fillets were stored for 14 days (T14) in PEHD (Poly-Ethylene High Density) trays with absorbent pads on the bottom, in a cold room at 2.5 °C. From T0 until T14, every second day, colour (L*a*b* values) and pH were measured. Flesh colour was measured using a portable Hunterlab MiniScan™ XE Plus D/8S Color Analyzer Colorimeter Spectrophotometer instrument, calibrated with a white and a black standard. The tristimulus L*a*b* measurement mode was used, where the L* value represents lightness, the a* value represents the redness and the b* value represents the yellowness indexes (Hunter and Harold, 1987).

Drip losses (%) were determined by weighing the fillets at T0, T7 and T14, and calculated by the formula:

$$\text{Drip losses} = ((D_0 - D_{7,\,14})/D_0) \times 100$$

where D_0 is the fillet weight immediately after filleting, while D_7 andD_{14} correspond to the fillet weight after 7 or 14 days of storage, respectively.

2.5 Statistical analysis

Data were analysed using the General Linear Model procedures of the statistical analysis software SAS 9.1 (2004) for Windows. A one-way ANOVA tested the stunning method as fixed effect.

3. RESULTS

3.1 Behavioural analysis and measurement of CO

The water samples indicated that the content of CO in the water was 0.1% after 8 min., and 0.6% after 20 min. All these values indicate super saturation, as the amount of CO at equilibrium is 0.028%.

Salmon showed a normal swimming activity before CO injection in the tank. Fish behaviour was very similar for both experimental groups, CO8 and CO20, in the first 8 min. As CO injection started, all fish behaved normally, with many swimming through the gas. At about 2 min, salmon showed a slight increase in motility, but still keeping normal swimming pattern and ventilation, which refers to stage 0 of consciousness (Roth et al., 2003). At 7 min a light sedation set in (stage 1; Table 1), as some fish had slight problems with equilibrium, whereas others laid on the bottom of the tank for few seconds. At 8 min. all fish expressed abnormal erratic swimming behaviour and uncontrolled convulsions. At this time fish from CO8 group were hauled and killed by percussion. In tank 3 (CO20 group) from 8 min. onward, as in CO8 group, salmons showed the same erratic swimming behaviour followed by circular movements near the surface, and then dive back in the water again. At 10 min., narcosis level 3 was reached (stage 3), and some fish started to lay on the bottom with abdomen up, little convulsions, and little operculum ventilation. Other fish looked like unconscious for some seconds and then suddenly swam showing convulsions. After 20 min. all the fish had reached stage 4-5 having no swimming activity or ventilation. They were then hauled from the tank and killed by percussion.

3.2 Plasma adrenaline and noradrenaline

Fish treated for 20 min. with CO showed significantly higher ($P<0.0001$) levels of catecholamines compared to C and CO8 fish (Table 2). Plasma AD level in CO20 group was significantly higher than C and CO8 groups (4.8 *vs*. 3.1 and 4.8 *vs*. 3.0 ng/ml plasma; $P<0.001$), the latters not differing between them. Plasma NAD level

was higher in CO20 than in C (8.1 *vs.* 5.4 ng/ml plasma; P<0.0001) while CO8 group presented an intermediate value.

3.3 Rigor Index, pH, drip loss and colour

Rigor Index evolution showed that fish of the CO20 group had earlier onset of *rigor mortis* than those of C group (Figure 1A). Full *rigor* wasreached by CO20 fishapproximately 10 hours *post mortem*, whereas by C fish 24 hours *post mortem*. *Rigor mortis* evolution was quicker for asphyxiated salmon (CO20); indeed itsresolution was reached 48 hours *post mortem*, time at which C group was still in *rigor*.

C and CO20 groups had similar rate of muscle pH drop (Figure 1B) during the first 24 hours: 7.06 *vs.* 6.74, 6.67 *vs.* 6.65, 6.48 *vs.* 6.45, 6.38 *vs.* 6.31, and 6.38 *vs* 6.28 at 0, 3, 9, 15 and 24-h *post mortem*, respectively. Thereafter, at 30 and 64-h the CO20 group had significantly (P<0.05) lower pH (6.29 *vs* 6.51 and 6.33 *vs.* 6.51).

The drip loss after 14 days of chilled storage is given in Table 3. Treatment increased drip loss in the CO20 group compared to the Control, since a slight but significantly higher loss was observed in CO20 compared to C group after 14 days of chilled storage (4.3 *vs.* 3.7 %; P<0.05; Table 3).

In Table 4 have been reported CO effects on flesh colour only at day 0 and day 14 of storage in cold room (+2.5 °C), since no significant differences were detected for the other days. On fresh fillets, CO20 group of fish had significantly higher lightness (L*) and yellowness (b*), compared to the Control group. These differences disappeared over time, and no differences were found at T14. Treatment had no effect on redness (a*).

4. DISCUSSION

4.1 Behavioural analysis and measurement of CO

Even on first measurement of CO in the water, it appeared that the content was much higher than the maximum water solubility indicating super saturation. At present, it is not possible to calculate the actual amount of CO dissolved that is available for the fish through the gills, or if super saturation has an additional effect compared to fully saturated water.

There were no effects of CO on fish swimming activity for the first 5 min. This clearly shows that salmon do not sense or smell CO. At about 8 min fish started to lose

buoyancy, and responded by abnormal erratic swimming behaviour, swam in circles near the surface before diving again and had uncontrolled convulsions. Bjørlykke et al. (2011) detected similar behaviour in Atlantic salmon only after 12 min from CO injection into the tank. This could be related to the lower water temperature (5.8 ± 0.5 °C *vs.* 7.3 ± 0.5 °C) and the not negligible greater mean body weight (3.4 ± 1.4 kg *vs.*1.07 ± 0.1 kg) with respect to the present study. At lower temperature the CO solubility should increase, but it has to be considered that also animal's metabolism become slower, likely requiring longer time to obtain the same reaction. It was observed that Atlantic salmons, reared at water temperature (7.4 ± 0.2 °C) and body weight (0.8 ± 0.1 kg) similar to our conditions, once subjected to a sudden increase in CO levels by the influx of saturated water with high and medium CO concentrations, show the same intense reaction only approximately after 2-4 min. (Bjørlykke et al., 2013). This may indicate that a rapid CO saturation of the water generates a faster stunning of the animal by skipping the initial step of slow diffusion, during which fish probably has the time to sense critical environmental conditions. Atlantic salmon is an active swimmer normally responding to perceived reduction in O_2 availability by a strong escape reaction (Zahl et al., 2010), which has been also confirmed in our study. The observed escape behaviour and surface seeking are probably originated by secondary hypoxia sensing mechanism, since CO effectively replace O_2 and inhibit its use throughout the fish body due to its higher affinity for oxygen binding proteins than oxygen itself (Blumenthal, 2001; Goldstein, 2008). Secondary effects that may signal hypoxia acidosis are due to anaerobic metabolism that increase lactate concentration, decreased ATP or increased ROS production. All of these are putative oxygen sensing mechanisms, and may elicit strong aversive reactions, at least in mammals (Lahiri et al., 2006). At 10 min, presumably a higher narcosis level was reached (stage 3), when some fish were lying on the bottom with abdomen up, showing little convulsions repeated in time and problem of operculum ventilation; others fish looked like unconscious for some seconds and then suddenly swam showing convulsions. Bjørlykke et al. (2013) described the same behaviour 8 min. after CO diffusion. The causes of erratic swimming behaviour in salmon have yet to be solved. Further work in this area is warranted. Performing this trial has been very useful because information here obtained helped to understand an important limit: the slow diffusion of the gas into tanks containing fish seems to be stressful since death is delayed in the time. It could be helpful reliable measurements of actual dissolved CO in water and

possible improvements of CO delivery systems.This preliminary work has made it clear that further studies should consider stunning in water previously saturated with CO or else a common stunning method followed by slaughtering in CO saturated water.

4.2 Plasma adrenaline and noradrenaline

Adrenaline values similar to those obtained for CO20 group were found in resting rainbow trout by Nakano and Tomlinson (1967) after blood sampling by caudal peduncle decapitation, which is an undoubtedly traumatic method. Later on Iwama et al. (1989) observed that blood adrenaline concentrations increased significantly during the latter stages of deep anaesthesiain rainbow trout. Carbon monoxide exposure for 20 min significantly increased AD and NAD levels compared to C group while in CO8 group catecholamine concentration did not differ from those of C group. It is important to consider that C and CO8 groups presented AD and NAD levels beyond the threshold of physiological range (usually less than 10 nM). This can make us to hypothesize that, when fish are exposed for short time period (8 min.) to CO, the gas is not really perceived as such, but has almost the same stressful effect of net capture followed by percussion stunning/killing method, commonly used. NAD concentrations similar to those of C (5.4 ng/ml) and CO8 (6.4 ng/ml) groups were detected in rested rainbow trout (5.02 ng/ml) (Van Dijk and Wood, 1988) and stressed ones after 6 min. of violent chase (6.66 ng/ml) (Milligan and Wood, 1987), respectively.

The high values of AD and NAD found in CO20 exposed fish might depend on CO influence on oxygen metabolism. By considering the general behaviour of the fish observed during the CO injection in the water, no aversive reactions such as those evocate when treated with CO_2 (Robb and Kestin, 2002; Roth et al., 2002) were evidenced in our trial. In fact, during the first 7 min of CO exposure fish were looking like do not take any notice about the gas presence by swimming freely trough it; however, after this time, fish started to show erratic swimming behaviour suggesting the presence of death cramps. In a recent study, Concollato et al. (2014) argue the hypothesis that the CO affinity to Ngb may induce immediate sedation and unconsciousness in fish, covering an important role in stress management in fish.

However, from the results emerged in this trial it seems that CO treatment was stressful to fish as it increased catecholamine's secretion. The few studies on AD and NAD release in salmonids found in literature are those cited above (showing similar

data), but none considered the catecholamine's release in relation to the application of different pre-slaughter stunning methods on fish. That is why further insights are needed.

When conducting field studies concerning stress, an important challenge is represented by the practical difficulty in sampling blood samples from undisturbed fish; up to now this problem is still not overtaken.

4.3 Rigor Index, pH, drip loss and colour

The intense *rigor mortis* process and the significant final pH decline observed in CO20 group at time of rigor resolution resulted in a significantly higher drip loss. Heme protein's affinity for CO is at least 240 times higher than that for O_2 (Roughton, 1970), this implies a dramatic reduction in O_2 transport and, as a result, the metabolism quickly change from aerobic to anaerobic, the ATP is gradually depleted and lactic acid is accumulated leading to a decrease in pH (Fennema, 1996). This explains the fast pH decrease early *post mortem*, that turned out in an early onset of *rigor mortis* (Bjørlykke et al., 2011), denaturation of muscle proteins with subsequent lower water holding capacity and higher drip losses. This demonstrates that it is extremely important to avoid fast post mortem pH decline as it weakens tissues between the muscle blocks (the myosepta) which then break, blocks become separated, and "gaping" takes place (Robb et al., 2000). The extension of the pre-rigor period is considered an important factor to maximize fillet's yield, since it is reduced when the fish is processed during the rigor stage (Azam et al., 1990). Fish processing plants then evaluates the delay in the start of rigor positively, because the full rigor filleting leads to a reduction in the yield and because the loss of freshness begins at the stage of post-rigor.

At T0, exposure to CO led to a small but significant increase in L* and b* values in comparison to the C fillets, not altering the overall "natural" colour of the fillet. During the 14 days of chilled storage the C fillets, compared to the CO20 fillets, showed an increased of b* value in comparison to T0 likely attributed to both lipid and heme proteins oxidation. Heme proteins, once oxidized to MHb/MMb, can give a brown-yellowish appearance to the red muscle, thus explaining the increase in b* value (Kristinsson and Demir, 2003). The slightly higher, but not significant, a* value in CO20 at T0 could be attributed to CO binding to Mb or Hb, displacing oxygen, producing COMb or COHb that has a stable cherry red colour, and the degradation to

MMb or MHb could take longer time (Chow et al., 1998), preventing discoloration. Indeed after 14 days of storage the redness for CO20 group was almost unchanged, highlighting the positive effect of CO. It must be mentioned that salmon fillets contain astaxanthin that gives the characteristic red to orange colour, and it may have minimized the colour differences among the experimental groups (Bjørlykke et al., 2011; Ottestad et al., 2011).

5. CONCLUSIONS

Behavioural analysis showed that salmon do not sense the CO gas. At 8 to 10 minutes, the fish respond with aversive behaviour before becoming fully sedated. It is possible that the swimming behaviour is elicited as a response to loosing buoyancy, or a biological response to hypoxia. This is confirmed also from blood analysis, showing a general increasing level of catecholamines in the order C<CO8<CO20.

CO treated fish resulted in an earlier onset of *rigor mortis*, lower final *post mortem* muscle pH and higher drip loss after filleting. The assimilation of CO by Atlantic salmon's muscles, through injection in the water, slightly increased L* and b* values, limited however to the fresh samples (T0). None significant difference in redness (a*) at any considered time was found between CO and Control group, probably because of the content in astaxanthin that may have minimized the colour differences among the experimental groups.

Further studies are needed to improve CO application as stunning/killing method. This includes reliable measurements of actual dissolved CO in water and possible improvements of CO delivery systems, so that to minimize stress perception immediately before slaughtering. The solution of these issues could allow the direct application of CO for stunning/slaughtering fish. Otherwise it could be necessary the utilization of other stunning methods followed by slaughtering in CO saturated water.

Contributors

All authors contributed equally to this manuscript.

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Captions of Tables

Table 1. Modified protocol from Burka et al. (1997) to determine, based on behavioural observations of Atlantic salmon, different stages (0-5) of reaction to electrical exposure. Behavioural studies were based on signs of swimming activity, reactivity to visual and tactile stimuli, equilibrium efforts, and ability to ventilate.

Table 2. Mean Adrenalin (AD) and Noradrenaline (NAD) values (ng/ml plasma) in blood samples collected from Atlantic salmon (No.= 5/treatment): control (C), CO8 and CO20.

Table 3. Drip loss (DL, %) during cold storage of Atlantic salmon fillets from control (C) and exposed to CO for 20 minutes (CO20) groups.

Table 4. Colour parameters (lightness [L*], redness [a*], yellowness [b*]) at day 0 and day 14 of storage in cold room (+2.5 °C), measured in fillets of Atlantic salmon from control (C) and exposed to carbon monoxide for 20 minutes (CO20) groups.

Table 1.

Stage	Description	Behavioural signs
0	Normal	Active swimming patterns Normal equilibrium Normal ventilation of operculum
1	Light sedation	Reduced swimming activity Problems with equilibrium Normal ventilation of operculum
2	Light narcosis	Weak swimming activity Slow and long ventilation rate Equilibrium loss with efforts to right
3	Deep narcosis	No swimming activity Problems of ventilation of operculum Total loss of equilibrium
4	Surgical anaesthesia	No swimming activity Ventilation ceases Total loss of equilibrium
5	Medullary collapse	Death ensues

Table 2.

	Treatment			Significance	RSD(1)
	C	CO8	CO20		
AD	3.1^{a}	3.0^{a}	4.8^{b}	<0.0001	0.5
NAD	5.4^{a}	6.4^{ab}	8.1^{b}	<0.0001	0.9

(1) Residual Standard Deviation

Different superscripts in the same line indicate significant differences.

Table 3.

	Treatment		Significance	RSD[(1)]
	C	CO20		
DL 0-7 days	2.3	2.9	NS	0.7
DL 7-14 days	1.4	1.4	NS	0.5
DL 0-14 days	3.7[a]	4.3[b]	<0.05	0.8

[(1)] Residual Standard Deviation
Different superscripts in the same line indicate significant differences
NS: not significant

Table 4.

Time (days)		Treatment		Significance	RSD[(1)]
		C	CO20		
0	L*	50.7[a]	52.8[b]	<0.01	1.6
	a*	19.9	21.1	NS	1.7
	b*	18.2[a]	19.3[b]	<0.05	1.3
14	L*	49.8	50.7	NS	1.4
	a*	21.2	21.2	NS	1.7
	b*	19.8	19.2	NS	1.2

[(1)] Residual Standard Deviation
Different superscripts in the same line indicate significant differences
NS: not significant

Caption of Figure

Figure 1. Rigor Index (A) in Atlantic salmon of control (C) and exposed to CO for 20 minutes (CO20) groups. The values are presented as means (No.= 6/group) ± SD. Symbol (*) denotes significant differences (*=P<0.05; **=P<0.01).

Figure 2. pH values (B) in Atlantic salmon of control (C) and exposed to CO for 20 minutes (CO20) groups. The values are presented as means (No.= 6/group) ± SD. Symbol (*) denotes significant differences (*=P<0.05; **=P<0.01).

Figure 1

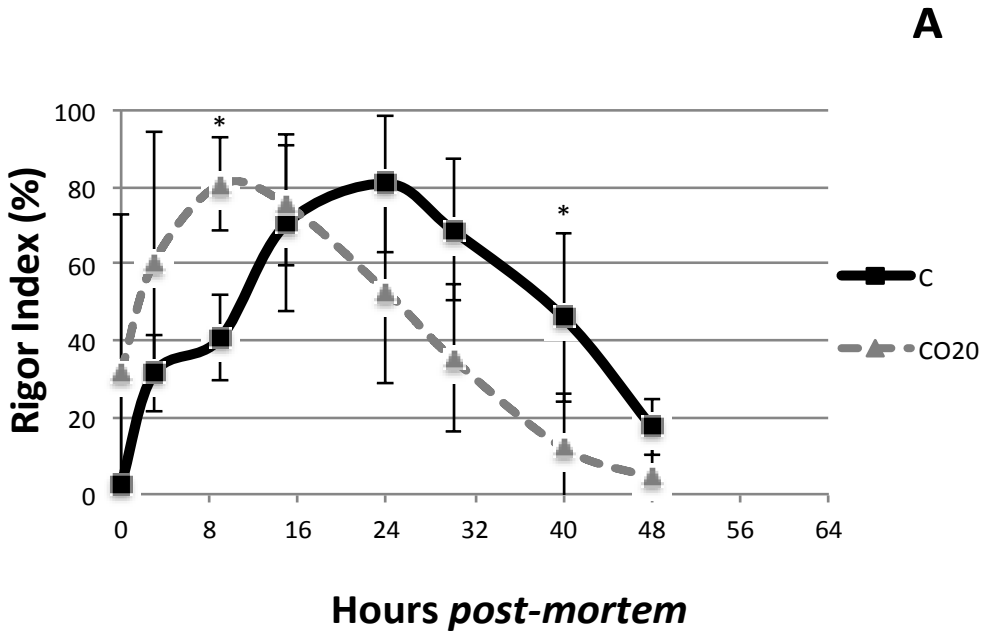
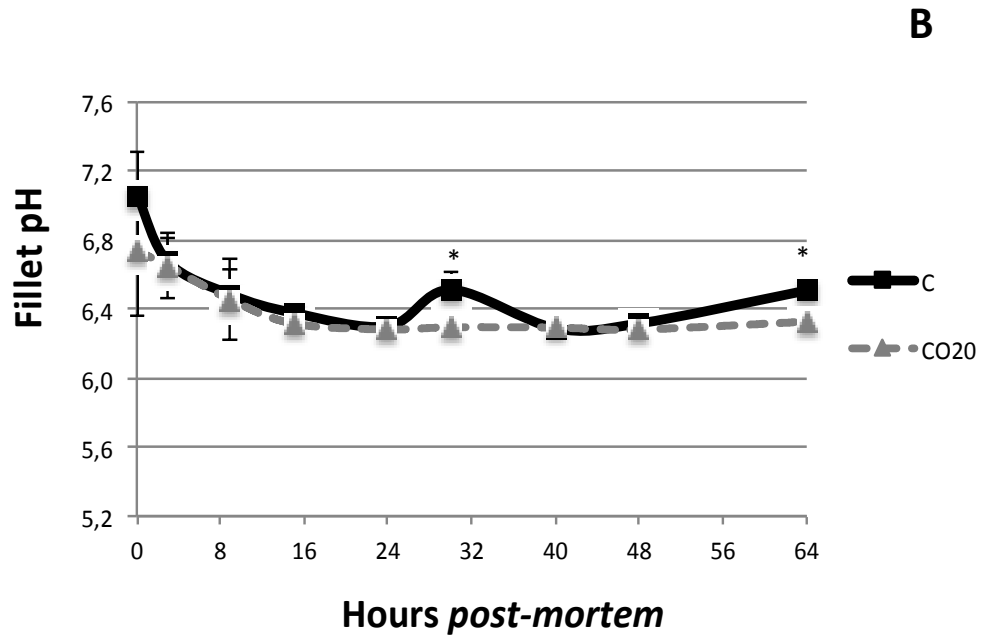


Figure 2



PAPER IV

Salmo salar L.CO stunning treatment revealed by electronic nose, electronic tongue and NIRS in differently prepared fillets influences post mortem catabolism and sensory traits

Abstract

The objective of this study was to evaluate and compare sensory analysis, NIRS, Electronic nose (EN) and Electronic tongue's (ET) ability in discriminating Atlantic salmon (*Salmo salar* L.) fillets according to stunning methods (percussion: Control; Carbon Monoxide: CO) and Storage Time (64 h, T1 or 14 days *post-mortem*, T2) and different preparation of the specimens (Thawed, in Ethanol and Freeze-dried). Samples were NIRS analysed by three different Research Units (RU): Hungary (H), Padova (PD), Torino (TO). As a general pattern, the Storage factor was the main source of effects for the instrumental discernment when compared to the Stunning effect. According to the two considered factors and their combinations for the seven instruments and preparations, the maximum efficiency was performed by the freeze-dried samples scanned by NIRS devices from the RU of TO, PD and H, also NIRS on thawed samples performed by the H RU was efficient; EN, ET and NIRS of ethanol specimens resulted to be the worse preparations and analysis methodologies. The PLS-DA and distance matrix confirmed these findings also. The correlation of maximum *rigor* time with the spectra resulted to be greater for freeze-dried and thawed samples according to all the different considered NIRS devices and for ethanol specimens. A general accordance between the spectral signature and the appreciation expressed by the panel for some sensory traits was observed, indicating that rheological but also taste and flavor properties are involved in this vibrational characterization. As general result freeze-dried preparation and NIRS devices resulted to be the best combination in samples discernment according to Storage time and Stunning factors, but also to maximum *rigor mortis* time and sensory scores.

Key words: *Salmo salar* L., carbon monoxide stunning, e-nose, e-tongue, NIRS, samples preparation

1. INTRODUCTION

All over the world, food safety and quality, are considered important issues directly related to people's health and social progress. Consumers are always more careful for quality labels and trust marks on food products, and il expects manufacturers and retailers to provide high quality products. These factors have driven the development of fast, efficient and reliable methods for food quality assessment. Among the vibrational techniques, that capitalize the fundamental properties of the organic bonds (C-H-O-N) in the electromagnetic spectrum, the FTMIR (Fourier Transformed Medium Infra Red, 2500-25.000 nm wavelengths) assumes absolute dominance in the milk world (Soyeurth et al., 2009). No similar development was extended by the IR in the solid agro-food media, mainly because lack of predictability of the method in complexes matrices, as grain, leaves, meat and cheese. The fundamental vibrations of the CHON bounds in the IR region are rebounded as overtones in the Near Infrared region (800-2500 nm wavelengths) and these vibrations may be de-convoluted and correlated - as causative - to the fundamental unique vibration originated in the IR band when the incident radiation strikes the organic molecules. The Near Infrared Reflectance Spectroscopy (NIRS) technology capitalizes the overtones and combinations of the constituents represented in the easily prepared or even in the intact samples, by using appropriate chemometric methods. NIRS represents a very common tool in the agro-world. The vibrational spectroscopy in the near infrared region is a very versatile device, with a paramount use in industry, farming and breeding, but also in experimental works. It provides a large amount of information from the spectra and this characteristic makes it a powerful tool for food analysis. It is widely used in many fields, as to identify adulteration of beverages (Paradkar, Sivakesava, & Irudayaraj, 2002; Pontes et al., 2006); evaluation of milk and dairy product quality (Cattaneo, Giardina, Sinelli, Riva, & Giangiacomo, 2005; Karoui et al., 2005; 2006); identification and constituent analysis of fruit, wine, meat, oil and corn; differentiation of wines on the basis of vintage year (Cozzolino, Smyth, & Gishen, 2003); determination of free fatty acids and moisture in fish oils (Cozzolino, Murraya, Chreeb, & Scaifec, 2005). Meat researchers have long sought non-destructive, objective techniques to predict meat quality. Several studies have focussed NIRS can be used to predict beef tenderness. A number of Authors nevertheless investigated off-line experimental procedures which were destructive in that they required excision of a muscle sample for spectroscopy (Hildrum et al., 1995; Liu et al., 2003; Rødbotten,

Nilsen, & Hildrum, 2000) or they were limited to sampling a very small area (4 cm^2) and thus, would be highly subject to error induced by non-representative sampling of the target muscle or interference from intramuscular fat. Their focus was altogether meat tenderness, but a number of other meat quality traits have been investigated with success by NIR Spectroscopy (Downey & Beauchene, 1997; Liu et al., 2003) and membership to ethnic groups of cattle (Andrighetto et al., 2004) or poultry (Fumière et al., 2000) or categories (Alomar, Gallo, Castaneda, & Fuchslocher, 2003). A special specimens of animal muscle were the ethanol preparate, that was preliminarily utilized in rabbits (Masoero, Brugiapaglia, Bergoglio, & Chicco, 2004), replicated in buffalo (Masoero, Bergoglio, Vincenti, De Stefanis, & Brugliapaglia, 2005) and in cattle (Masoero, Iacurto, & Sala, 2006) allowing significant results in easy and rapid NIR discrimination of two genetic origin for *Semitendinosus* and *Sternum mandibularis* muscle specimens from Friesian and Piemontese cattle**.** In forensic pathology purposes, an ethanol-based fixative method has been developed by Iesurum, Balbi, Vasapollo, Cicognani, and Ghimenton (2006), with a better DNA recovery in higher amounts compared with DNA extracted from formalin-fixed tissue.

Dalle Zotte et al. (2014) attained traceability results by NIRS and fusion data in the authentication of raw and cooked freeze-dried rainbow trout fillets.

The instrument of the ElectronicNose (EN) category, capitalize the electrochemical properties of low-weight molecules to excite complementary metal-oxide semiconductor (CMOS) sensors. EN consists of an array of chemical sensors, each with partial specificity to a wide range of odorant molecules. The signs of the sensory arrays produce the “fingerprints” of the given flavour, which are evaluated with chemometric methods. EN is widely used for foodstuff analysis, becoming promising towards industrial applications. In this particular field, electronic noses can help in freshness definition of product characterized by limited shelf-life, such as fish; distinction between fresh and thawed samples and the maintenance of a constant temperature during storage are of extreme importance. EN has been proven to be a valuable technique for food and drinks industry for product discrimination, classification, quality evaluation and control (Antoce, & Namolosanu, 2011; Peris, & Escuder-Gilabert, 2009; Torri, Migliorini, & Masoero, 2013). The Electronic Tongue (ET) has become established as rapid and easy-to-use tools, promising for evaluation of food quality from liquid media. Electronic tongues are still considerably far from natural taste sense, but they have shown good correlations with organoleptic scores

given by human panelists: artificial senses are not subjective, do not become tired or infected and can be used also for toxic samples. Moreover, ET can have better sensitivity than the human tongue and can detect substances undetectable by their natural counterparts. This because the taste system in humans is not as highly developed as the olfactory system (Escuder-Gilabert, & Peris, 2010). Legin, Rudnitskaya, and Vlassov(2002)pointed out that 'the electronic tongue can be thought of as analogous to both olfaction and taste and it can be used for the detection of all types of dissolved compounds, including volatile ones, which give odors after evaporation. This device can be used for process monitoring (Parra et al., 2006), freshness evaluation and shelf-life investigation (Ahlers, 2007), authenticity assessment (Dias et al., 2008), foodstuff recognition/characterization (Ciosek, Brzózka, & Wróblewski, 2004), quantitative analysis (Rodríguez-Méndez et al., 2008), and other quality control studies (Chen, Zhao, & Vittayapadung, 2008).

The objective of this work was to evaluate and compare sensory analysis, NIR, Electronic -Nose and Electronic Tongue's ability in discriminating Atlantic salmon fillets (*Salmo salar* L.) according to stunning methods, storage time, and different preparation of the specimens.

2. MATERIAL & METHODS

2.1 Experimental set-up

Atlantic salmons (*Salmo salar* L.) were farmed at the facilities of the Institute of Marine Research (IMR), in Matre, Norway. For the study, 30 salmons with a mean weight of 1.08 ± 0.09 kg were equally and randomly divided in 2 experimental tanks containing 900 L seawater each and maintained at constant temperature of 7.3 ± 0.5 °C. Fish in tank 1 were used as control (C) and killed by percussion; fish in tank 2 were flushed with 100% food grade CO (Yara Praxair, Oslo, Norway) using a ceramic diffuser (wedge lock base unit, Point Four Systems Inc., Richmond, Canada) for 20 minutes (CO) at 2-3 bar. Then, CO fish were hauled out of the tanks and percussively slaughtered. For personnel safety the air CO concentration was monitored and measured during the experiment by use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA). The experiment was approved according to "The Regulations in Animal Experimentation" in Norway and conducted by certified personnel.

Salmons from C and CO groups were individually tagged, weighed and stored in polystyrene boxes with ice. Immediately after slaughter, *rigor mortis* was determined on 6 fish/treatment at 0, 3, 9, 15, 24, 30, 40, 48 and 64 hours *post mortem* (interval T0-T1), during this time fish were stored in a cold room at +2.5 °C. *Rigor mortis* was measured by tail drop, and Rigor Index (RI) was calculated according to Bito, Yamada, Mikumo, and Amano(1983), using the following formula:

$$RI\ (\%) = [(L_0 - L_t)/L_0] \times 100$$

where L_0 (cm) is the vertical distance between the base of the caudal fin and the table surface measured immediately after the death, whereas L_t (cm) is the vertical distance between the base of the caudal fin and the table surface at the selected time intervals.

After *rigor mortis* resolution (64 h *post mortem*, Time 1 – T1) all fish were gutted, filleted and weighed. Right fillets were immediately *vacuum* packed and stored at -20 °C, whereas the left ones were stored for 14 days (Time 2 - T2, interval T1-T2 in days) in PEHD trays with absorbent pads on the bottom, in a cold room at 2.5 °C, then stored at -20 °C.

All right and left fillets of the 30 salmons (15 C and 15 CO) were delivered in dry ice at the Padova (Italy) Research Unit (RU; Department of Animal Medicine, Production and Health). Then all right (15 C-T1; 15 CO-T1) and left (15 C-T2; 15 CO-T2) fillets were divided, while still frozen, in three (cranial, central and caudal) and two (cranial and caudal) parts respectively, which were send in dried ice to the RU laboratories of Kaposvár University, Hungary (H; caudal part), Padova (PD; central part), for sensory analysis by trained panelists, and Torino (TO; cranial part).

Hungarian RU required from each raw sample about 8 and 60 g to perform EN and ET analyses (EN-H; ET-H), respectively, whereas to carry out NIRS scans first on raw (THAW-H) and then on the same raw but freeze-dried samples (FD-H), at least 40 g where necessary. Hungarian RU freeze-dried the raw samples and, after NIRS scan, the same samples were NIRS scanned again by devices in Padova (FD-PD) and Torino (FD-TO) RU, in order to compare the predicting ability of the three different instruments.

For sensory analysis only fresh samples (CT1 and COT1) were tested because it could have been risky for the panelists to taste samples after 14 days of chilled storage. For this analysis it was required at least 50 g from each thaw fillet.

When the 60 fillets samples, organized in 4 groups (C-T1, C-T2, CO-T1, CO-T2), arrived at HRU facilities, they were stored over night in chilled room at 4 °C, to ensure

a slow and proper thawing process. The following morning fillets were skinned, weighted (average weight 111.14 ± 21.51) and homogenized in a WARING 800 EG blender. After homogenization about 8 g were collected for electronic nose (EN) analysis, approximately 60 g were sealed in bags and cooked in water bath at 75 °C for 20 minutes in order to perform electronic tongue (ET) analysis, whereas the remaining part was used first for NIRS scanning on the raw matrix, then freeze-dried as aforementioned, for NIRS analyses.

When cranial samples arrived at the laboratory of Torino, after thawing a small 4 g sample was plugged in a 25 ml tube Ethanol 95% commercial solution and the tubes were stored in dark at + 4 °C. A rapid coagulation of the muscle protein and the substitution of the water in the tissue by the ethanol molecules give the appearance of cooked fish. The lecture of the specimens (ETOH-TO) was preceded by 1-hour ethanol evaporation at room temperature in order to intensify the vibrational response of the salmon matrices.

2.2 Instrumental analyses

2.2.1 Electronic nose

An αFox (ALPHA MOS, Toulouse, France) type EN with 18 metal oxide sensors (MOS) was used. The adsorption of volatile compounds onto the MOS surface generates a change in the electrical resistance that varies with the type of compound and its concentration in the headspace (HS). The multisensory arrays of EN are interfaced with computers, which collect the sensor signals via RS-232 ports. The raw EN sensor values were saved in the form of relative resistance changes ($\Delta R/R_0$). According to the applied static headspace (HS) technique, samples were placed in hermetically sealed vials of 10 ml. After the equilibrium has been established between the matrix and gaseous phase, an ALPHA MOS HS 100 auto sampler was used for sampling the HS. Synthetic air was used as a permanent airflow. The acquisition time and time between subsequent analyses were 120 and 1080 s, respectively. Four parallel measurements were performed (n = 4 x 4). During the EN method development the use of the following parameters resulted acceptable signal intensity values: sample quantity 2 g, sample temperature 60 °C, equilibration time 180 s, injection volume 3000 µl and the flow rate 150 ml/min.

2.2.2 Electronic tongue

An αAstree II (Alpha-MOS, Toulouse, France) type ET with an LS 48 auto sampler unit was applied to measure the characteristics of liquid samples. The equipment consists of an array of seven cross-selective chemical modified field effect transistor (CHEMFET) based potentiometric chemical sensors. In the presence of dissolved compounds, a potentiometric difference is measured between each of the seven sensors and the Ag/AgCl reference electrode. The multisensory arrays of ET are interfaced with computers, which collect the sensor signals via RS-232 ports. The basic ET parameters were formed by averaging the intensity values when sensors were in equilibrium. Since the broth collected after cooking resulted insufficient to test each sample, the cooked fillets were stored at -20 °C and one week later 6 samples from each group were randomly chosen to extract the liquid part needed for the ET measurements. From each of the 28 samples 3 g were sampled, diluted in Millipore water in the rate 1:20 and homogenized, then centrifuged (12.000 rpm for 5 min) and filtered with filtering paper (Sartorius Stedim Biotech, grade: 1289, diameter: 125 mm). Finally from each treatment group were obtained 6 sub-samples for a total of 28 ET measurements.

Once the samples were ready, those have been placed in 6 glass holders of 25 ml into which the measuring unit, namely the chemical sensors, the reference electrode and a stirrer were positioned. Six parallel measurements were performed (n = 4 x 6). The first element (K) of the sample series served as sensor conditioning. The measurement and the sensor cleaning times were 120 and 15 s, respectively. Millipore grade water was used for sensor cleaning.

2.2.3 NIRS analyses

Sixty (60) Atlantic salmon (*Salmo salar* L.) fillets were analysed in this study. Homogenized sample were scanned, in 4 repetitions, as thawed and as freeze-dried state at the HRU. NIRS spectra were collected in reflectance mode using NIRSystems 6500 spectrometer (FOSS NIRSystem, Silver Spring now Laurel, MD, USA) equipped with a sample transport module and small ring cup cuvette (IH-0307). Reflectance spectra were recorded from the 1100 to 2500 nm region and recorded as –log(*R*) at 2 nm intervals, with the WinISI II version 1.5 spectral analytical software (InfraSoft International LLC, Port Matilda now State College, PA, USA). In PD RU a similar FOSS NIRSystems 5000 (FOSS NIRSystem, Silver Spring, MD, USA) was used. In

TO RU Vibrational examination was conducted using a portable Model LSP 350-2500P LabSpec Pro portable spectrophotometer (ASD; Analytical Spectral Devices, Inc.; Boulder, CO), which was equipped to collect spectra from 350 to 2500 nm. The probe was an ASD Model A122100 high-intensity reflectance probe that served as an external light source (2900 K colour temperature quartz halogen light) to illuminate the object of interest. This probe can be used to collect reflectance spectra on an area as large as 25 mm in diameter. Reflected light was collected through a ASD Model 04-14766 1-m long fiber optic jumper cable that consisted of a bundle of forty-four 200-lm fibers.

2.3 Sensory method applied

For the sensory analysis it has been chosen the descriptive method to detect information needed to establish the presence or absence of perceived differences between the two stunning methods (C and CO).

A total of 30 right and central fillets (15 from C-T1, 15 from CO-T1) were used. Each fillet (with skin) was placed in aluminium trays (12.0 cm x 14.5 cm) previously drilled on the bottom so as to prevent the cooking of the fillets straight in their fluid; then they were covered with an aluminium foil on the top. Fillets were cooked in ventilated electric oven preheated at 200 °C, by placing on the bottom a pan with water in order to collect the cooking losses; cooking time was set up at the achievement of an internal temperature of the sample of 75-85 °C determined by a temperature probe. When cooked, the trays were placed in an incubator at 50 °C, and then served to the panelists at the request, according to the random distribution sequence.

The trial involved 12 trained panelists with experience in determination of sensory profile of different food matrices. They were subjected to training sessions for the purpose of familiarize with the matrix of interest, select the appropriate descriptors and define on a scale of measure the relative perceived intensity (Table 1). Olfactory, tactile, gustative and textural sensory aspects were evaluated and for each of them different descriptors were chosen after an accurate bibliographic research. Global odour and aroma intensity (olfactory descriptors); friability and tenderness (tactile descriptors); saltiness and sourness (taste descriptors); adhesiveness, fibrousiness and tenderness (textural descriptors) were evaluated by scored, linear and continuous scale of measure (0-10). Brackish/marine, “fishy”, animal feed, cardboard, stale, boiled potatoes, salmon and others (olfactory descriptors); bitter, astringent and metallic

(taste descriptors); stringy, unctuous and low solubility (textural descriptors) were evaluated by categories (presence/absence, Table 1).

The panel was trained with fresh purchased salmon, portioned into pieces and placed in the freezer at -18 ° C in individual plastic bags, like the samples to analyse. The evaluation sheet, the distribution of samples to the judges and the acquisition of the data was performed using FIZZ software (Biosystemes - France) installed in 12 terminals in the tasting booths of the lab. Thanks to this software, assessments made by the panelists (mouse click on the scale of measurement) are automatically transferred to the sheet of data collection; in this way no paper sheets are filled in by the panelists. The purchased sample in the evaluation of the treatments represented the reference standard, assigning an arbitrary score for each descriptor: this sample was cooked together with the samples to analyse. To each panelist were offered in 2 consecutive days 1 to 2 fillets of 50g each, corresponding to the reference and treatment, respectively, to evaluate successively. During the evaluation the panelists used unsalted crackers and natural water in order to neutralize any residual sensation between a sample and thenext.

3. DATA ANALYSIS

3.1 Instruments

All the digital signals produced by the instruments, which were recorded in native formats by specific software, were then imported into the WinISI II 1.03 software for chemometric elaboration. The replicate spectra were averaged before any chemometric elaboration.

3.2 Qualitative discrimination analysis based on spectra, comparison of the instrumental efficiency and differences between two 1-VR coefficients

The bi-factorial design with the Gas factors at 2 levels and the Storage time also with 2 levels (T1 and T2) produced four groups, which were considered as dummy values (1-4) and also contrasted each other in order to build a distance matrix. The calibration process was performed by the Modified Partial Least Squares (MPLS) method using NIRS II software, version 1.04, from Infrasoft International (ISI, State College, PA, USA) using a cross-validation system to assess the optimal number of latent variables to be included into the equations, permitting one passage for elimination of outliers ($t>2$; $H>10$). The prediction capacity of the calibrated models was then evaluated with the 1-VR parameter, which is routinely used by WinISI users and researchers (Mentink, Hoffman, & Bauman, 2006) and statistics as a cross-validation and a Relative Prediction Deviation (RPD) were considered for performance evaluation (Williams, 1987).

On the distance matrix a Ward's Hierarchical Clustering Analysis (HCA) was performed via StatBox software *vs*. 6.5 (Grimmer Logiciel, Paris) in order to compare the relative average dissimilarity patterns (Jobson, 1992). HCA performs agglomerate hierarchical clustering of objects based on distance measures of dissimilarity or similarity. In order to rank two independent factors the z-score obtained from by Fisher transformation according Preacher (2002) was used to testify the differences between two 1-VR values, with two-sided alpha< 0.05 limit. Because an objective judgments about the instruments and preparations is needing we have used the nonparametric paired Friedman tests (StatBox 1.5, Grimmer Logiciels, Paris), considering the variables as the key for pairing the observations; the observed value of Kruskal-Wallis H, is distributed as a chi^2 (df = 1); this test being one-sided, the *P*-value is compared at the signification limit: alpha= 0.05.

3.3 Rigor mortis test

The *rigor mortis* condition was examined in 6 salmons at the time 0, 3, 9, 15, 24, 40, 48 hours, from T0 to T1 (Concollato et al., 2014).

For each salmon an individual parabolic curve was fitted to establish the time at the maximum rigor and that variable in hours was retained (Figures 1 and 2).

A nonparametric Friedman's test for independent samples was then applied to ascertain the significance of the difference between the C and CO maximum *rigor mortis* time.

A regression of the variable on the dummy values 1 (C) and 2 (CO) established the R_{square} limit value for the discrimination in the real conditions.

3.4 Panel test analysis

The 12 panelists were considered as random effects in a mixed model (PROC MIXED by SAS, 2007). The fixed factor was the Stunning method (Stun). The scores on raw fillets were considered for the Stun effect elaboration in a linear model. Moreover in order to fit the instrumental spectra (ET, EN, NIRS) the panelist score, were standardized as follows:

$$S\_c = (C\text{-}CO) / 2 / std.dev + 1$$

$$\text{and } S\_co = (CO\text{-}C) / 2 / std.dev + 1$$

Where C and CO = estimated score for the C and CO salmons for the i^{th} panelist; S_c and S_co = standardized score for the C and CO salmons from the i^{th} panelist.

3.5 Correlation of the spectra from the different devices (ET, EN, NIRS) and preparations with the rigor mortis and sensory variables

The set of the spectra of the samples was multiplied as much times as were the number of the recorded *rigor mortis* maximum time (i.e. 6 times) or the number of panelists (i.e. 12 times).

As reference to the panelists case, the first subset was coupled to the set-scores of the first panelist, the second subset of spectra set was coupled to the second panellist set-scores and merged below the previous, and so on till to twelfth. In an analogous manner each of the 6 *rigor mortis* score was applied to all the spectra registered in the companion salmons, so the data set was multiplied by 6.

Calibration and cross-validation was performed by the MPLS method in the WinISI 1.5 software, with mathematical pre-treatment of standardization and 1^{st}-derivation,

allowing one passage to eliminate the outliers, with t set at a limit > 2.0. The 1-VR coefficient was considered in order to compare the different experimental effects (Stun and / or storage Time) as appreciated by the different devices (ET, EN, NIRS) as well as to study the connections with the sensory scores and the biological variables.

3.6 Comparison of the 1-VR efficiency of the spectroscopies in the whole set, or separately for the two Time of storage

If the spectra registered at the Time1 are different from the spectra registered at the Time2 an unique relationship between the spectra and the measured values may be doubtful. Then we must compare the 1-VR values of the common regression with the 1-VR values of the two Time and the two Time together. Furthermore we must compare the different devices within the spectra or within the Time2 and the Time2 categories already. In order to perform these comparisons we have considered as suitable the nonparametric Friedman tests (StatBox 1.5, Grimmer Logiciels, Paris), considering the variables as the key for coupling the observations; the observed value of Kruskal-Wallis H, is distributed as a chi^2 (df = 1); this test being one-sided, the *P*-value is compared at the signification limit: alpha= 0.05.

3.7 Comparison of the 1-VR efficiency of the spectroscopies for the different sensory variables

In a similar process, as that above mentioned, we can rank the ten sensory variables according their 1-VR values performed in the different devices. In this case we have to compare each couple of the ten variables in order to rank the 1-VR coefficients. The z-score obtained from by Fisher transformation according Preacher (2002) was used to testify the differences in the 1-VR values, with two-sided alpha< 0.05 limit.

4. RESULTS

4.1 Instrumental appreciation of the factors Stun and / or Storage

As a general pattern, the Storage factor was the main source of effects for the instrumental discernment when compared to the effects of the Stun. In fact the avg. 1-VR coefficients were showed in the Table 2: 0.835r *vs.* 0.488s values that correspond to avg. RPD coefficients 3.3 *vs.* 1.8. The two effects did not interact because the 1-VR

contrast of the two Stun condition is similar when examined at T1 or at T2 time (0.562t *vs.* 0.521t).

4.2 Instruments and preparations

As regard to the preparations (Table 2) the maximum of efficiency was attained by the freeze-dried preparation that was examined by the PD (1-VR avg.= 0.895a) similar to TO (0.848ab) and H (0.842b) URs. The thaw examination by the Hungarian team was also at that level (0.867ab). A step of lower efficiency distinguishes the ethanol specimens (0.467c) and the EN instrument (0.573c). The ET appears as the most inefficient instrument (0.180d).

As shown in the Table 3, the average distance matrices of the Thaw and of the FD preparations had very high 1-VR values (> 0.84); the ethanol preparation and the e-nose ranged around 1-VR 0.5, while the e-tongue had very low avg. distance matrix (0.19). As highlighted in Figure 3, the FD specimens strongly agree for the discernment of the four groups. In fact a symmetric pattern subordinates the minor factor (Stun) at the major factor (Storage Time); very similar symmetric patterns were enhanced in the examinations by e-nose, and by the e-tongue, but to a lesser extent, because its non significant reclassification ability (Table 4). In the NIRS spectra of the thaw specimens the two groups at the T1 appeared less symmetrically dispersed, while in the clusters derived from spectra of the ethanol specimens no homologous patterns were recognized, and in this preparation we can suppose that an interaction between the Stun and Time-storage factors could have appeared. Table 4 reports the full reclassification square, with the global significance test. All the instruments, except the e-tongue, were significant as regard to a non random distribution of the observations. According the seven groups records it was possible to calculate a relationships between the 1-VR coefficients and the reclassification percentage; as reported in Figure 4, the percentage of reclassification may be estimated as 0.98 detracted of the 0.78 * Log(1-VR), with R^2 0.81.

Figure 4 shows the high (R^2= 0.80) relationship between the two criteria considered for the evaluation of the results in the trial.

- On X-axis the 1st criteria: PLS of averaged spectra from seven instrument-preparations
- On Y-axis the2nd criteria: PLS-DA of averaged spectra from seven instrument-preparations.

4.3 Spectra of the NIRS instruments and preparations

Figure 5 presents the average NIR spectrum (as Log(1/R)) of Thaw, Ethanol and Freeze-dried specimens.

A flat curve means low absorbance because of a high reflection of the signal. Obviously the raw meat absorbs in the water a high part of the signal then resulting a salient curve. The freeze-dried specimens did not absorb the signal, chiefly because of their very low water content, then the curves appear more flat as respect to the raw; notice that the curves from the two FOSS instruments appear very superimposed. In the minor Vis-NIR band of the ASD instrument from Torino the ethanol specimens curves also appear superior to the freeze-dried, because the inherent major ethanol-water absorbance of the signal.

4.4Rigor mortis

As highlighted in Table 5 a very strong effect was apparent on the time of the maximum *rigor mortis* occurrence, as a consequence of the Stun treatment, which strongly anticipates the phenomenon. The unpaired Friedman test has P = 0.0037, disregarding the different amount in the standard deviations, limited to 2.53 h in the usual conditions or expanded to 7.52 in the CO use. The correlation of these values with the spectra of the devices and preparations are highlighted in Table 6, last 3 columns. Notice that the average 1-VR level is quite high (0.61) and in four cases surpasses the 0.74 limit of discrimination based on the real maximum *rigor mortis* time. As regard to the ability of the different instruments to collimate the real results the freeze-dried preparation, together with the thawed preparation and also the ethanol specimens prepared and examined in Torino, gained the highest fit (a, a, a respectively for All spectra, and storage time T1 and T2) with all the different NIRS devices utilized; however only two exceptions occurred: the NIRS of Padova did not appreciate the freeze-dried preparation of the All spectra (0.59b, 0.76a, 0.75a) and the minor capacity NIRS ASD of TO RU was less efficient in evaluating the storage time 1 (0.63ab, 0.56b, 0.74a).

The EN gave minor efficient results as compared to NIRS of the freeze-dried and thawed and ethanol preparations (0.47c, 0.63b, 0.47b), and even less efficient were the discrimination results for the ET (0d, 0.43c, 0.25c).

In conclusion we can observe that the effect of the anticipated *rigor mortis* on the muscle specimens, despite of different preparations and of different instruments, was

dragged on the vibrational spectroscopy of the equipollent tissues, examined after different storage time. Notice, that the storage time itself was also greatly apparent in the spectra composition (see previous results), but with proper and different vibrational signature.

4.5Sensory Test

As reported in Table 7, the positive aspects of the CO treatment were the reduction of aroma (-8%) and odour (-10%). Some negative aspects concerned a reduction of the tactile-crumbliness (-13%) and tactile-tenderness (-15%), while the treatment increased the salty taste (+14%).

Notice that the PLS fitting of the averages of the ten variable scores to a dummy value 1 (C) and 2 (CO) reaches a R^2 level of 0.48 (Figure 6).

As regard to the correlation of the spectra realized by different devices and preparations, some prominent considerations arise from the examination of the Table 8. In general many 1-VR coefficients appeared very highly significant, indicating a statistical indirect relationships of the traits into the spectra, but with no expectation of individual prevision ability.

The tenderness score (Avg. 1-VR = 0.45a) and the salty taste (0.41a) reached the maximum of correlation considering the whole set of calibrated instruments. Odour intensity (0.38b) and tactile tenderness (0.36c) were at almost comparable levels. Slightly lower precisions were attained for tactile crumbliness (0.27d), for the aroma intensity (0.20e), and for adhesiveness (0.17e). No correlation was established for the scores of sour, fibrousiness and overall acceptance.

All these correlations arise from a general indirect relationship of the spectra with the variation induced by the factor C *vs.* CO in the results of the panel test: as shown in Figure 7, the 1-VR values (Y) from all the devices are positively related with the probability of the gas factor (X = log 1/Prob) for the Stun factor in the ten sensory variables, pertinent to the whole instrumental set, that is pooling all the spectra produced at the two storage times, T1 and T2. Notice that the tactile tenderness and the salty taste are appreciated more than the common average function.

As regard to the ability of the different instruments to collimate with the panel, the results are provided in the last 3 columns of the Table 8. Notice that the average 1-VR level is poor because it derives from some insignificant sensory variables, apparently not affected by the Stun effects.

The freeze-dried preparation, together with the thawed preparation, gained the highest fit with all the different NIRS devices utilized. The ethanol preparation realized and examined in TO RU was alike the previous, but because of differences in the spectra pertinent to the Time1 and Time2 storage duration, the pooling for time was not as efficient (avg. 1-VR = 0.02c *vs.* 0.23a and 0.23a respectively for T1 and T2). The EN gave similar efficient results as the freeze-dried and thawed and ethanol preparations, for the storage time, T1 and T2 separated (0.16b, 0.26a, 0.25a). On the contrary the ET was not efficient, and the respective coefficients were 0.04c, 0.15b and 0.11b.

We have observed a general concordance of the spectral signature of the specimens with the appreciation expressed by the panel in some sensory traits. Not only rheological, but also flavour and taste properties are involved in this vibrational characterization.

5. DISCUSSION

5.1 Instrumental methods appreciation of Stun and/or Storage factors, instruments and preparations

Despite the differences observed between Storage and Stun factors, as the main source of effect for instrumental discernment in favour of Storage, and considering the wide set of instruments and preparations set up in this trial, a comparison with other literature results was not possible (at least at the authors knowledge). As showed in the clusters Figures, the Storage factor had an higher impact on samples, but the Stun effect was very similarly perceived at both the checked time, because the effects revealed at T1 was preserved and checked again at T2. In few cases, namely with the ethanol specimens, the groups were erratically confounded. Mantilla et al. (2008) showed that with increasing Storage time, CO concentrations in the biological matrix decreases, by confirming findings and favouring Storage time effect. Combinations of the two factors showed that on average CO both at T1 and T2 is not differently detected by the instruments, but it cannot be possible because at T1 there is no Storage time effect, which is present instead at T2 where it is summed to that of the Stun. This misrepresentation of the data may depend from the different efficiency of the considered instruments; indeed it is interesting to note that NIR scan of ETOH-TO specimens presented lower correlation with Gas at T1 when compared to T2, probably because the alcohol had a lower reaction with the fresh proteins (T1), that is the contrary of what happened for ET and EN which positively react to fresh proteins. In

general NIRS on freeze-dried samples resulted to be the most useful instrument and sample preparation combination. First probably because of the higher sensitivity of the method when compared to ET and EN, which are based on a global selectivity concept, able to recognize just a limited number of molecules (Smyth, & Cozzolino, 2012). Second, because the removal of water through the freeze-drying treatment further increases accuracy (Smyth, & Cozzolino, 2012).

5.2 Distance matrix and PLS-DA

The distance matrix and PLS-DA confirmed findings reported above: NIRS scanning of thawed and freeze-dried samples resulted as the most useful method and sample preparation in discerning specimens according to Storage time and Stun factors, with Storage time being the most discerning factor.

The high relationship between 1-VR coefficients and the PLS-DA indicate that the 2 criteria gives similar results, indeed in both cases NIR on raw and freeze-dried specimens performed in H, PD and TO resulted to be the best instruments in discerning samples, and in evaluating the presence and incidence of both experimental factors (Stun and Storage time).

5.3 Rigor mortis

When fish are killed, creatine phosphate is degraded before to the breakdown of ATP. When the creatine phosphate and ATP reach a similar concentration, ATP content decrease and *rigor mortis* starts till a full *rigor mortis* status when ATP diminishes to about 1 μmol/g. *Rigor mortis* occurs when in myofibrils ceases cross bridge cycling of myosin and actin, and permanent linkages are formed (Wang, Tang, Correia, & Gil, 1998).

Rigor mortis is resolved after some time. Possible causes of *post mortem* tenderization include a weakening of Z-discs of myofibrils (Seki, & Tsuchiya, 1991), a degradation of connective tissue, or a weakening of myosin-actin junctions (Yamanoue, & Takahashi, 1988). The effect of the anticipated *rigor mortis* on muscle specimens, caused from the anaesthetic effects of CO, despite of different preparations and of different instruments, was revealed by the vibrational spectroscopy of the equipollent animals and examined after different storage time from different NIRS instruments. NIRS resulted very useful in determining tissues changes correlated to earlier onset of *rigor mortis*, and reflected in the spectra over time. The rapid evolution of *rigor*

*mortis*is strictly linked to other changes that probably affected the spectra versus time, such as different levels of ATP, glycogen, lactate, pH, K-value and muscle texture (Ehira, & Uchiyama, 1986; Berg, Erikson, & Nordtvedt, 1997; Sigholt et al., 1997; Thomas, Pankhurst, & Bremner, 1999; Robb, 2001; Roth, Moeller, Veland, Imsland, & Slinde, 2002; Howgate, 2005). In the case of fish, no studies have been identified in literature where different NIRS devices recorded spectra at time of *rigor* resolution and after 14 days of cold storage in order to compare them and predict their correlation with time at maximum *rigor mortis*. In beef cattle (Lomiwes, Reis, Wiklund, Young, & North, 2010), in a range of normal commercial variation, the NIRS as an on-line method to quantify glycogen and predict ultimate pH (pH_u) of pre rigor beef M. *Longissimus dorsi* (LD) was unsuitable; in fact the spectra were poorly correlated against glycogen and pH_u (R^2)=0.23 and 0.20, respectively.

Notice that storage time itself was also greatly apparent in the spectra composition but with proper vibrational signature that not interact with the Stun effect.

Roth et al. (2002) observed that in Atlantic salmon stunned with carbon dioxide (CO_2), electricity, or percussion prior to slaughter the *pre mortem* stress during CO_2 stunning resulted in an earlier onset and resolution of *rigor mortis* followed by accelerated *post mortem* softening. In the present study the very early onset of the *rigor* was not followed by a softening, but by a hardening of the tissues, probably related to the long-term (20 min.) stress during CO exposition. Skjervold, Fjæraa, Østby, and Einen(2001) showed that pre-slaughtering stress affected salmon firmness depending on the severity and duration of stress: short term stress leads to muscle softening, whereas long term exhaustion leads to increase muscle firmness. This is in accordance with patterns of stress influence in mammal meat (Hedrik, Aberle, Forrest, & Merkel, 1994).

5.4 Sensory Test

In veal, Brugiapaglia, Destefanis, Lussiana, Giomo, & Masoero(2011) investigated the meat sample preparation methods and NIRS methodology to predict sensory scores of veal belonging to two ethnic groups and fed on different diets. Three preparations of *Longissimus thoracis* samples, i.e., thaw, ethanol-prepared, and freeze-dried, were studied. The distance matrices reached different 1-VR levels: 0.65 (thawed and ethanol samples); 0.42 (freeze-dried), whereas the panel was very distinctive (0.62).

Prediction of Panel scores were effective as 1-VR of ethanol specimens for Flavour (0.68) and Texture (0.68). It was concluded that NIRS scan of thawed samples anticipates results achieved by a wide set of laboratory analyses. NIRS analysis of ethanol samples exhibited strong predictive value of Panel scores. In the present study, as regard the ability of the instruments to collimate with the panel, freeze-dried preparations together with the thawed one, gained the highest fit with all the different NIRS devices.

6. CONCLUSIONS

This study meant to evaluate and compare sensory analysis, NIRS, Electronic Nose and Electronic Tongue's ability in discriminating Atlantic salmon (*Salmo salar* L.) fillets according to stunning methods and storage time and different preparation of the specimens. Generally, Storage factor resulted to be the main source of effects for the instrumental discernment when compared to the effects of the Stun. As regard the samples preparations and instruments used, the maximum efficiency was performed by the freeze-dried samples scanned by NIRS devices from the RU of PD, TO and H, also NIRS on thawed samples performed by the H RU was efficient; EN, ET and NIRS of ethanol specimens resulted to be the worst preparations and analysis methodologies. The PLS-DA and distance matrix confirmed these findings also. The correlation of maximum *rigor* time with the spectra resulted to be greater for freeze-dried and thawed samples according to all the different considered NIRS devices and for the ethanol specimens. A general accordance between the print of samples' spectra and the appreciation expressed by the panel for some sensory traits was observed, indicating that rheological but also taste and flavour properties are involved in this vibrational characterization.

It can be concluded that, as general result, freeze-dried preparation and NIRS devices resulted to be the best combination in samples discernment according to Storage time and Stun factors, but also according to maximum *rigor mortis* time and sensory scores.

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Table 1. Panel test: considered descriptors and measurement unit

Sensory aspect	Descriptors	Type of measurement	Unit
Olfactory	Global odour intensity	Scores, linear and continuous	0-10
	Global aroma intensity		
	Brackish/marine	Categories	Presence/Absence
	"Fishy"		
	Animal Feed		
	Cardboard		
	Stale		
	Boiled potatoes		
	Salmon		
	Others		
Tactile	Friability	Scores, linear and continuous	0-10
	Tenderness		
Taste	Saltiness	Categories	Presence/Absence
	Sourness		
	Bitterness		
	Astringent		
	Metallic		
Texture	Adhesiveness	Scores, linear and continuous	0-10
	Fibrousiness		
	Tenderness		
	Stringiness	Categories	Presence/Absence
	Unctuous		
	Low solubility		

Table 2. Chemometric analyses (by WinISI v. 1.04 software) of the two main factors Stun and Storage time, and of their combinations, for the seven instruments and preparations, according the average (Avg.) values of 1-VR in cross-validation. Paired Friedman's test compares the instruments (a>b>c>d) and Fisher's test compares the two main factors (Stun and Storage time) effects (r>s) and their combinations (t>u)

			ET-H	**EN-H**	**THAW-H**	**FD-H**	**FD-PD**	**FD-TO**	**ETOH-TO**	**1-VR**	**RPD**
		Factor	28	60	60	60	60	60	60	Avg.	Avg.
		Stun	0.000 s	0.439 s	0.853 s	0.832 s	0.862 s	0.626 s	0.170 s	0.488s	1.8
		Time	0.493 r	0.799 r	0.942 r	0.901 r	0.930 r	0.973 r	0.805 r	0.835r	3.3
		Group	0.000	0.596	0.845	0.787	0.825	0.749	0.236		
1_Control_T1	2_Control_T2	_12	0.310	0.662	0.944	0.844	0.865	0.968	0.663		
	3_Stunned_T1	_13	0.000 t	0.354 t	0.959 t	0.717 u	0.937 t	0.565 u	0.402 t	0.562t	2.3
	4_Stunned_T2	_14	0.000	0.596	0.945	0.946	0.946	0.983	0.804		
2_Control_T2	3_Stunned_T1	_23	0.082	0.813	0.739	0.830	0.905	0.951	0.008		
	4_Stunned_T2	_24	0.000 t	0.049 u	0.662 u	0.813 t	0.894 t	0.854 t	0.372 t	0.521t	1.8
3_Stunned_T1	4_Stunned_T2	_34	0.735	0.852	0.909	0.906	0.895	0.964	0.742		
		Avg.	0.180	0.573	0.867	0.842	0.895	0.848	0.467	0.662	2.5
			d	c	ab	b	a	ab	c		

a>b>c>d: Test of Friedman paired by rows, P<0.05; r>s: Test of Fisher for the main effects within instruments and preparations, P<0.05; t>u: Test of Fisher for the effects of the Stun factor in the two conditions of Storage time, within instruments and preparations, P<0.05.
FD-H: 400-2498 nm, 1049 digits; instrument: FOSS 6500, Hungary
FD-PD: 1100-2492 nm , 700 digits; instrument: FOSS 5000, Padova
FD-TO: 350-1025 nm, 1049 digits; instrument: ASD CCS-Aosta s.r.l.

Table 3. Distance matrix of the 1-VR coefficients by the PLS of the averaged (Avg.) spectra from seven instrument-preparation, and reclassification percentage of the individualsof thefour groups ([A] reclassification results from Table 4)

FD-H Group	1	2	3	4	Avg.	Reclassification % [A]
1_Control_T1	0	0.844	**0.717**	0.946	0.843	95%
2_Control_T2	0.844	0	0.83	**0.813**		
3_Stunned_T1	0.717	0.83	0	0.906		
4_Stunned_T2	0.946	0.813	0.906	0		
FD-PD Group	1	2	3	4	Avg.	
1_Control_T1	0	0.865	**0.937**	0.946	0.907	93%
2_Control_T2	0.865	0	0.905	**0.894**		
3_Stunned_T1	0.937	0.905	0	0.895		
4_Stunned_T2	0.946	0.894	0.895	0		
FD-TO Group	1	2	3	4	Avg.	
1_Control_T1	0	0.968	**0.565**	0.983	0.881	85%
2_Control_T2	0.968	0	0.951	**0.854**		
3_Stunned_T1	0.565	0.951	0	0.964		
4_Stunned_T2	0.983	0.854	0.964	0		
ETOH-TO Group	1	2	3	4	Avg.	
1_Control_T1	0	0.663	**0.402**	0.804	0.499	38%
2_Control_T2	0.663	0	0.008	**0.372**		
3_Stunned_T1	0.402	0.008	0	0.742		
4_Stunned_T2	0.804	0.372	0.742	0		
THAW-H Group	1	2	3	4	Avg.	
1_Control_T1	0	0.944	**0.959**	0.945	0.86	85%
2_Control_T2	0.944	0	0.739	**0.662**		
3_Stunned_T1	0.959	0.739	0	0.909		
4_Stunned_T2	0.945	0.662	0.909	0		
EN-H	1	2	3	4	Avg.	
1_Control_T1	0	0.662	**0.354**	0.596	0.554	60%
2_Control_T2	0.662	0	0.813	**0.049**		
3_Stunned_T1	0.354	0.813	0	0.852		
4_Stunned_T2	0.596	0.049	0.852	0		
ET-H	1	2	3	4	Avg.	
1_Control_T1	0.00	0.31	0.00	0.00	0.19	46%
2_Control_T2	0.31	0.00	0.08	0.00		
3_Stunned_T1	0.00	0.08	0.00	0.74		
4_Stunned_T2	0.00	0.00	0.74	0.00		

Table 4. Reclassification % in the four groups by the PLS-DA of the spectra from seven instrument-preparations, at the Time 1 and at the Time 2, and statistical significance

			FD-H (P <*0.00001*)					**FD-PD (P <*0.00001*)**					**FD-TO (P <*0.00001*)**				
Stun	Time	**Group**	**1**	**2**	**3**	**4**	Misses	**1**	**2**	**3**	**4**	Misses	**1**	**2**	**3**	**4**	Misses
C	1	**1**	15	0	2	0	2	15	0	0	0	0	13	0	5	1	6
C	2	**2**	0	15	1	0	1	0	14	0	0	0	0	15	0	1	1
CO	1	**3**	0	0	12	0	0	0	0	13	1	1	2	0	10	0	2
CO	2	**4**	0	0	0	15	0	0	1	2	14	3	0	0	0	13	0
			15	15	15	15	60	15	15	15	15	60	15	15	15	15	60
		Misses	0	0	3	0	3	0	1	2	1	4	2	0	5	2	9
		%	0%	0%	20%	0%	**5%**	0%	7%	13%	7%	**7%**	13%	0%	33%	13%	**15%**
			THAW-H (P <*0.00001*)					**EN-H (P <*0.00001*)**					**ETOH-TO (P = *0.01066*)**				
Stun	Time	**Group**	**1**	**2**	**3**	**4**	Misses	**1**	**2**	**3**	**4**	Misses	**1**	**2**	**3**	**4**	Misses
C	1	**1**	14	0	3	0	3	10	2	1	1	4	8	4	5	2	11
C	2	**2**	0	12	0	1	1	0	1	0	2	2	1	1	5	2	8
CO	1	**3**	1	0	11	0	1	5	2	13	0	7	4	4	4	1	9
CO	2	**4**	0	3	1	14	4	0	10	1	12	11	2	6	1	10	9
			15	15	15	15	60	15	15	15	15	60	15	15	15	15	60
		Misses	1	3	4	1	9	5	14	2	3	24	7	14	11	5	37
			7%	20%	27%	7%	**15%**	33%	93%	13%	20%	**40%**	47%	93%	73%	33%	**62%**

			ET-H (P = *0.10504*)				
Stun	Time	**Group**	**1**	**2**	**3**	**4**	Misses
C	1	**1**	1	0	2	0	2
C	2	**2**	1	4	2	2	5
CO	1	**3**	3	1	4	0	4
CO	2	**4**	2	2	0	4	4
			7	7	8	6	28
		Misses	6	3	4	2	15
			86%	43%	50%	33%	**54%**

Table 5. Values of the maximum *rigor mortis* time (in hours) in the selected examined Atlantic salmons.

	Stun	
Salmon #	**C**	**CO**
1	24	1
2	24	3
3	24	6
4	26	10
5	28	11
6	30	22
Avg.	26.00	8.83
St.dev	2.53	7.52
Unpaired Friedman's test P:	0.0037	
PLS-D of the C and CO groups R^2 based on the max *rigor morti* time	0.74	

Table 6. *Rigor mortis* maximum time correlated with the spectra of the different devices and Fisher's test of the 1-VR values.

						Fisher's test		
Device	**Sample**	**Spectra**	**N**	**(1-VR) Max-Rigor**		**All spectra**	**Time1**	**Time2**
Electronic Nose	Thawed	All spectra	360	0.47	b	c		
H	.	Time0	180	0.63	a		b	
.	.	Time2	180	0.47	b			b
Electronic Tongue	Thawed	All spectra	168	0	b	d		
H	.	Time0	84	0.43	a		c	
.	.	Time2	84	0.25	a			c
NIRS	Thawed	All spectra	360	0.74	b	a		
FOSS	.	Time0	180	0.81	a		a	
H	.	Time2	180	0.66	b			a
NIRS	Freeze-dried	All spectra	360	0.68		a		
FOSS	.	Time0	180	0.74			a	
H	.	Time2	180	0.72				a
NIRS	Freeze-dried	All spectra	360	0.59	b	b		
FOSS	.	Time0	180	0.76	a		a	
PD	.	Time2	180	0.75	a			a
NIRS	Freeze-dried	All spectra	360	0.63	b	ab		
ASD	.	Time0	180	0.56	b		b	
TO	.	Time2	180	0.74	a			a
NIRS	Thawed	All spectra	720	0.70		a		
ASD	Ethanol	Time0	360	0.79			a	
TO	.	Time2	360	0.73				a
.	Average	.	.	0.61	.	.	.	.

Within column: a>b>c>d, Fisher's bi-lateral test: P<0.05.

Table 7. Results of the mixed model analysis of the sensory scores of the 10 variables by the 12 panelists of the Atlantic salmon samples

Sensory variables	C	CO	*P*	CO/C
Sourness	1.97	1.86	*0.3742*	-6%
Adhesiveness	4.59	4.37	*0.2123*	-5%
Fibrousiness	4.89	4.85	*0.7488*	-1%
Tactile_Crumbliness	4.68a	4.05b	*0.0384*	-13%
Aroma_intensity	5.96a	5.48b	*0.0301*	-8%
Odour_Intensity	6.47a	5.82b	*0.0096*	-10%
Saltiness	3.15b	3.58a	*0.0546*	14%
Tenderness	5.78a	4.92b	*0.002*	-15%
Tactile_tenderness	5.12	4.82	*0.322*	-6%
Acceptability	4.65	5.37	*0.2765*	16%

Table 8. Sensory scorescorrelated with the spectra of the different devices and Friedman and Fisher test of the 1-VR values

															[A]Friedman's test			
Device	**Sample**	**Spectra**	**N**	**Average**	**Sour**	**Adhesiveness**	**Fibrousiness**	**Tactile_ Crumbliness**	**Aroma_ Intensity**	**Odour_ Intensity**	**Salty**	**Tenderness**	**Tactile_ Tenderness**	**Acceptance**	Within Instrument	**All spectra**	**Time1**	**Time2**
Electronic Nose	Thawed	All spectra	1440	0.16	0.00	0.05	0.00	0.20	0.23	0.26	0.28	0.36	0.21	0.00	s	u		
H	.	Time1	720	0.26	0.00	0.20	0.00	0.41	0.32	0.39	0.41	0.51	0.40	0.00	r		t	
.	.	Time2	720	0.25	0.00	0.12	0.00	0.36	0.33	0.42	0.43	0.53	0.36	0.00	r			t
Electronic Tongue	Thawed	All spectra	336	0.04	0.00	0.03	0.00	0.06	0.01	0.03	0.16	0.06	0.05	0.00	s	v		
H	.	Time1	168	0.15	0.00	0.10	0.00	0.19	0.18	0.20	0.23	0.36	0.21	0.00	r		u	
.	.	Time2	168	0.11	0.00	0.09	0.00	0.05	0.13	0.15	0.19	0.34	0.16	0.00	r			u
NIRS	Thawed	All spectra	720	0.26	0.00	0.16	0.00	0.32	0.31	0.41	0.47	0.57	0.37	0.00		t		
FOSS	.	Time1	360	0.27	0.00	0.24	0.00	0.30	0.20	0.47	0.53	0.52	0.44	0.00			t	
H	.	Time2	360	0.25	0.00	0.23	0.00	0.27	0.14	0.46	0.52	0.48	0.43	0.00				t
NIRS	Freeze-dried	All spectra	720	0.26	0.00	0.21	0.00	0.32	0.17	0.46	0.50	0.53	0.43	0.00		t		
FOSS	.	Time1	360	0.28	0.00	0.27	0.00	0.34	0.20	0.49	0.53	0.49	0.47	0.00			t	
H	.	Time2	360	0.29	0.00	0.29	0.00	0.41	0.25	0.47	0.52	0.54	0.44	0.00				t
NIRS	Freeze-dried	All spectra	720	0.26	0.00	0.19	0.00	0.28	0.20	0.47	0.50	0.51	0.44	0.00		t		
FOSS	.	Time1	360	0.29	0.00	0.25	0.00	0.44	0.27	0.47	0.52	0.53	0.44	0.00			t	
PD	.	Time2	360	0.28	0.00	0.29	0.00	0.38	0.22	0.47	0.52	0.51	0.44	0.00				t
NIRS	Freeze-dried	All spectra	720	0.25	0.00	0.25	0.00	0.23	0.19	0.47	0.52	0.44	0.45	0.00		t		
ASD	.	Time1	360	0.25	0.00	0.26	0.00	0.23	0.16	0.46	0.52	0.44	0.45	0.00			t	
TO	.	Time2	360	0.28	0.00	0.30	0.00	0.30	0.23	0.48	0.52	0.53	0.47	0.00				t
NIRS	Thawed	All spectra	1320	0.02	0.00	0.02	0.00	0.02	0.00	0.06	0.02	0.07	0.04	0.00	s	v		
ASD	Ethanol	Time1	660	0.23	-0.01	0.00	0.00	0.29	0.25	0.43	0.36	0.54	0.41	0.00	r		t	
TO	.	Time2	660	0.23	0.00	0.05	0.00	0.33	0.25	0.41	0.35	0.52	0.42	0.00	r			t
Averages variables				0.22	0.00	0.17	0.00	0.27	0.20	0.38	0.41	0.45	0.36	0.00				
[B]Rank, (Fisher's test)					f	e	f	d	e	b	a	a	c	f				

[A]In ranking average 1-VR by device: Fisher's test; r>s; t>u>v, P<0.05; [B]in ranking of sensory variable instrumental correlation: paired Friedman's test: a>b>c>d>e>f, P<0.05.

Figure 1. Fitting of the maximum *rigor mortis* time in the 6 C Atlantic salmons.

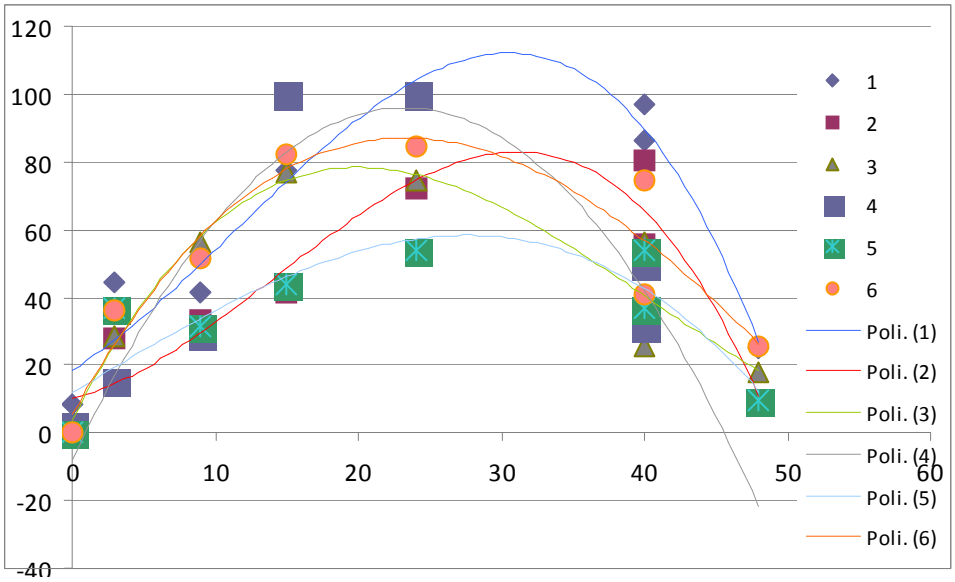


Figure 2. Fitting of the maximum *rigor mortis* time in the 6 CO Atlantic salmons.

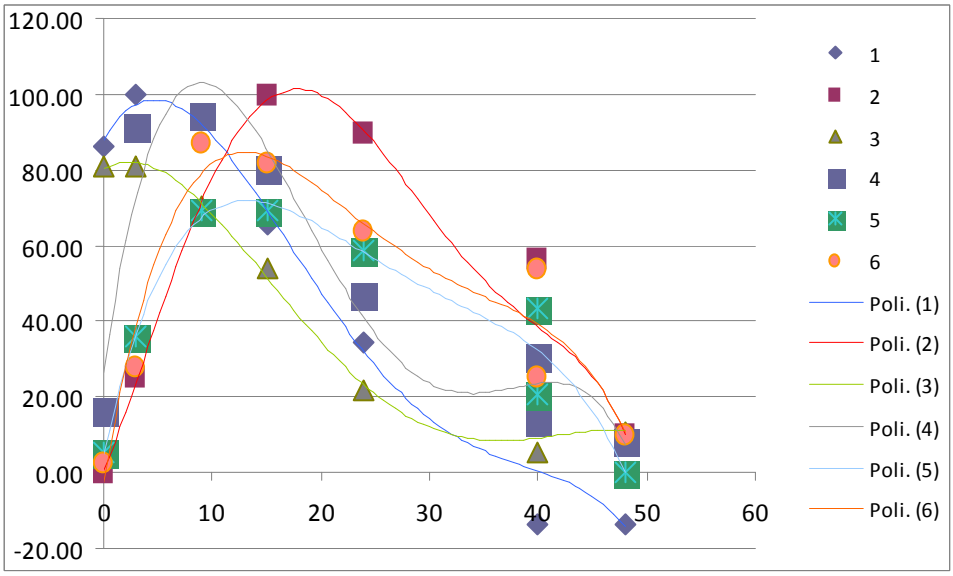


Figure 3. Cluster of the four groups based on the distances matrix of the mean spectra in cross-validation calibration mode for the seven instrument-preparation samples.

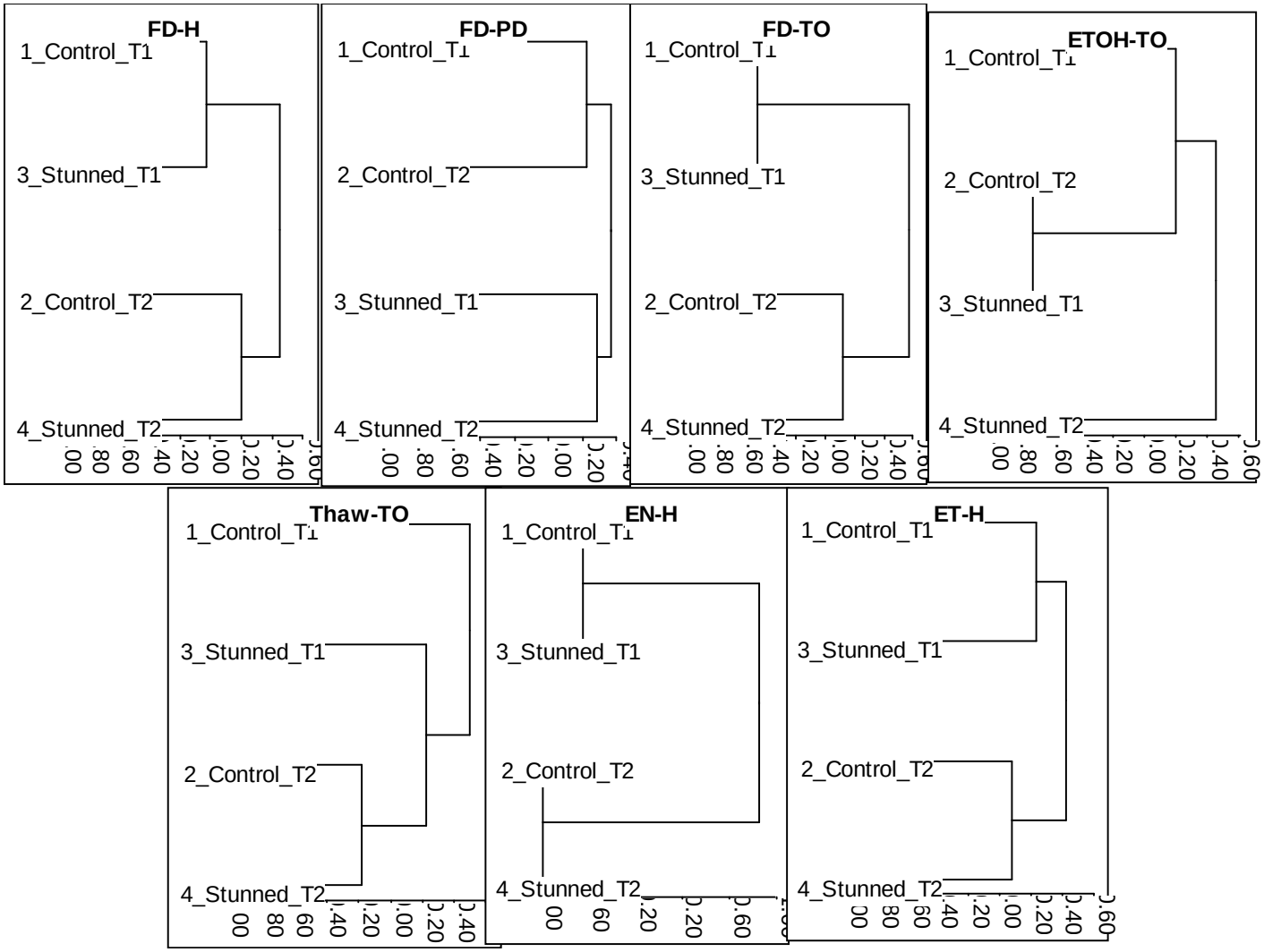


Figure 4. Relationships between the average 1-VR coefficients (X axis) of the PLS equations and the percentage of reclassification (Y axis).

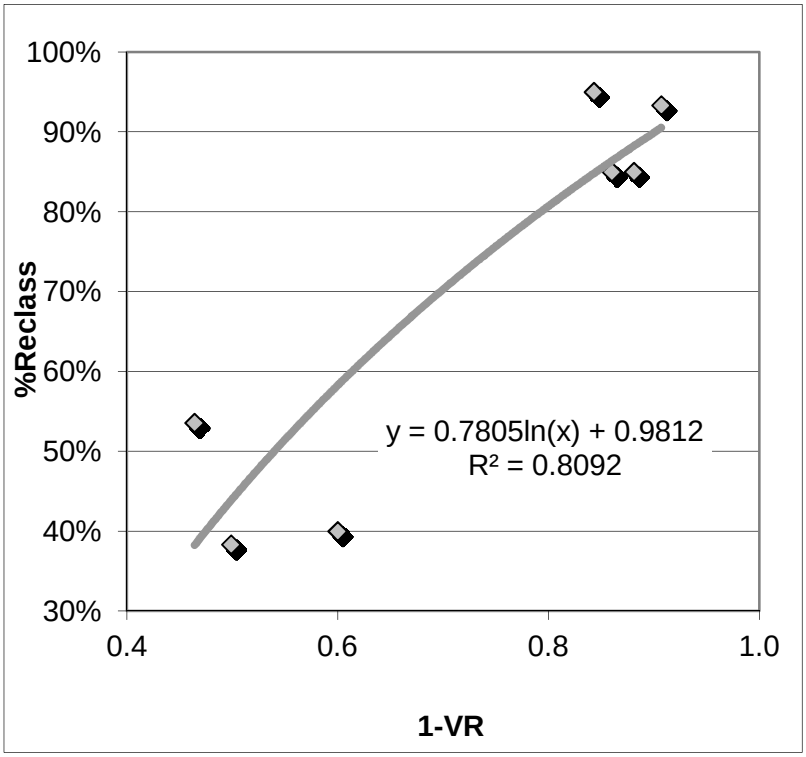


Figure 5. Average NIR spectrum (Log (1/R)) of Thawed, Ethanol and Freeze-dried specimen.

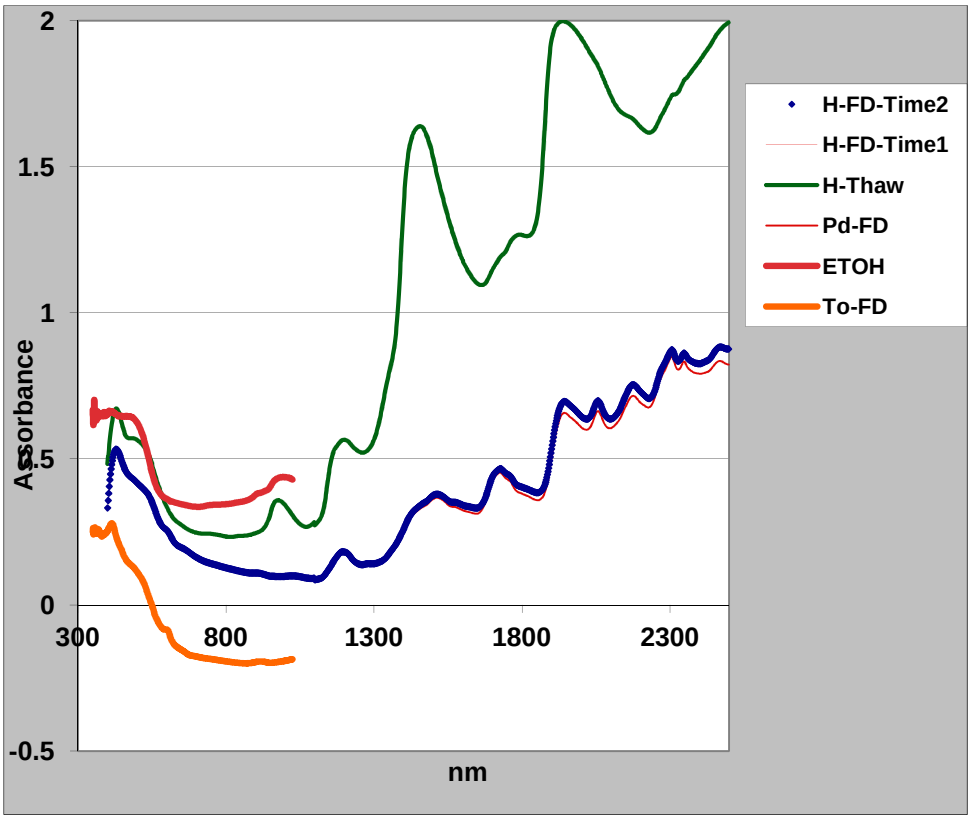


Figure 6. Plot of the PLS discriminant function for the C and the CO effects, based on the ten variables panellist scores.

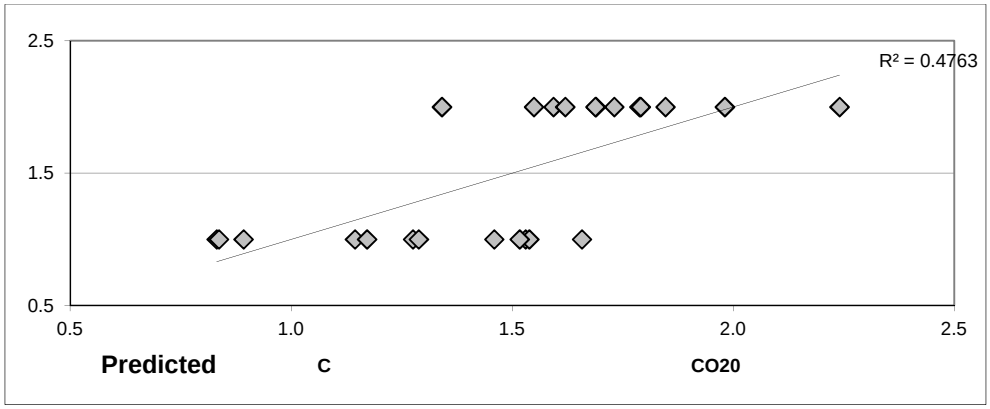
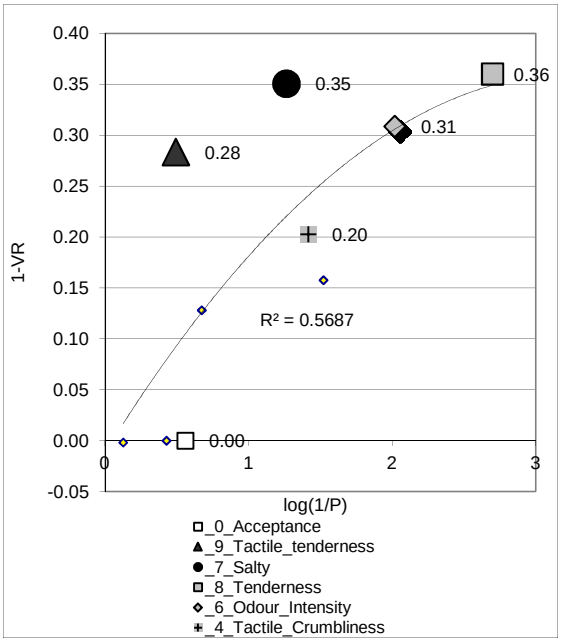


Figure 7. Plot of the average 1-VR values (Y) from all the devices *vs.* the log (1/P) (X) for the Stun factor in the ten sensory variables.



PAPER V

Effects of stunning/slaughtering methods on *pre rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions.

Abstract

The effects of stunning/slaughtering methods (carbon monoxide asphyxia, CO; electroshock, E; asphyxia in the air, A) on blood parameters, *rigor* evolution, fillets shape changes, ATP depletion and Adenylate Energy Charge (AEC) in muscle immediately after death were investigated in rainbow trout reared at two different temperature conditions (8 °C and 12 °C). Treatment A has proved to be the most stressful: cortisol concentration three times higher than baseline levels (153 ng /ml), high concentrations of lactate (5.58 mM) and glucose (6.08 mM), while treatment E resulted the most suitable method for slaughtering.

Water temperature influenced *rigor mortis* evolution: at 12 °C no significant differences among treatments emerged, while at 8 °C, the groups solved rigor in the order: A, CO and E. Fillets from treatment A exhibited both the strongest area and perimeter contractions (in the order A>CO>E and A>E>CO, respectively) and the most rapid length shrinkage and height increase, followed by E and CO. Globally CO treatments showed the highest ability in preserving muscle energy immediately after death, in fish reared at both temperatures.

Key words: *Oncorhynchus mykiss*;slaughtering methods; stress; blood indicators; ATP; *rigor mortis*

1. INTRODUCTION

Food quality is perceived as a global concept, which is unavoidable from animal's welfare. Conditions of anxiety, pain, suffering or fear above all have ethical implications, since the human being is considered to be responsible for the effective respect of the rights and welfare of other living animals, as stated in the declaration of UNESCO (1978). The respect for animal's welfare strongly affects consumer attitudes towards the product, influencing the choice to those products derived from animals that have not been subjected to ill treatment. Operations concerning stunning and slaughtering processes, as well as the immediately prior stages, can cause particular stress and disturbances that may affect meat quality. Humane slaughter procedures, therefore, can improve *post mortem* quality of fish, as reported for warm-blooded animals by many authors (Brown et al., 1998; Geesink et al., 2001). Fish slaughtering methods have been reviewed by Robb and Kestin (2002), and the most relevant identified are percussive stunning, CO_2 narcosis and electrical stunning. In addition to these methods, the use of carbon monoxide (CO) could present an attractive alternative to CO_2, as, contrarily to CO_2 (Poli et al., 2005),it does not produce aversive effects by animals. Substantial inhalation of CO can be fatal because of its high ability to bind to respiratory pigments, such as haemoglobin (Hb) (Davenport, 2002), for which it presents an affinity 210-270 times greater than that of oxygen (O_2) (Kalin, 1996). This strong binding is the major cause of CO stunning effectiveness, thanks to the exclusion of Hb in the O_2's transport, but also to the very slow reversibility of the carboxyhaemoglobin (COHb) complex at atmospheric pressure and oxygen saturation. Carbon monoxide is then able to form bonds with heme proteins like Hb, myoglobin (Mb) and neuroglobin (Ngb), the latter being a molecule that has the function of carrying oxygen to the brain and nerve tissues with a protective role during hypoxia (Brunori and Vallone, 2007; Liu et al., 2009; Sun et al., 2001), by replacing oxygen.

It is also believed that CO binds to proteins that retain the oxygen in *Saccus vasculosus*, a well vascularised organ of the caudal hypothalamus of elasmobranchs and most bony fish. This organ is well vascularized and many presumed functions like pressure regulation and reception, chemoreception, ionic regulation of the cerebrospinal fluid, storage and transport have been hypothesised (Sanson, 1998; Yáñez et al., 1997). When these proteins bind to the CO, the animal dies due to lack of O_2 without feeling its deficiency, and this is the reason why this gas is not considered harmful to the animals (Concollato et al., 2015). CO also influences cellular

respiration through the inhibition of many enzymes, such as cytochromes, which possess hem groups similar to those of Hb and Mb, causing the suppression of oxidative phosphorylation (Prescott et al., 1996).

Another important aspect to consider is CO ability to enhance the fish fillet's colour (Bjørlykke et al., 2011) and overall meat quality (Chow et al., 1998;Gee and Brown, 1981;Hsieh et al., 1998; Mantilla et al., 2008). Fillets of Atlantic salmon, herring and mackerel, anesthetized by injection of CO in seawater, had a more persistent red value (a*) and did not develop the characteristic odour of rancid after 6 days of refrigerated storagethan the groups not treated with CO (Concollato et al., 2015). Thus, the antioxidant ability of CO may be of considerable interest, mitigating the risk of lipid oxidation and thereby extending the shelf-life of products (Cornforth and Hunt, 2008).

At present, very little literature information about the practice of euthanizing fish with CO is available. Bjørlykke et al. (2011) and Concollato et al. (2014) studied CO effects on stress parameters and quality in Atlantic salmon. Bjørlykke et al. (2011) showed that CO positively affected fillet colour, resulted in an earlier onset of *rigor mortis* and a faster decrease in pH due to the lactate secretion. It was also highlighted that salmons exposed to CO did not express aversive reactions and were easily slaughtered by percussion. Concollato et al. (2014) found that CO treatment resulted in an increased level of catecholamines, enhancement of lightness (L*) and yellowness (b*) values, earlier onset of *rigor mortis*, as a consequence of a rapid pH decrease, and higher drip losses. Behaviour analysis showed that the observed aversive swimming could be elicited as a response to the loose of buoyancy or a biological response to hypoxia.

In tilapia (Mantilla et al., 2008), CO anaesthesia showed a significant increase in redness (a*) and L* on treated fillets in comparison with the control ones. It was observed that tilapia remained calm before dying, revealing that the process is not stressful, but that the use of CO has an anaesthetic effect on the animals since they stopped moving and remained calm until euthanasia was completed.

The aim of the study was to investigate the possibility to apply carbon monoxide and electricity in comparison with the asphyxiation in air, still widely used in some Italian farms, for stunning/killing rainbow trout reared at two different water temperatures (12 and 8 °C). Fish welfare and quality performances were evaluated.

2. MATERIAL AND METHODS

2.2 Experimental set-up

The study was performed at the experimental farm of Edmund Mach Foundation, in S. Michele all'Adige, Trento (Italy).

Five hundred rainbow trout (*Oncorhynchus mykiss*) were equally allocated in 5 tanks containing 3600 L of freshwater each. In tanks 1, 2, and 3 the water temperature was maintained at 12 °C whereas in tanks 4 and 5 the water temperature was maintained at 8 °C. Three stunning methods were applied: asphyxia in the air (A) lasting about 15 min (tank 2 and 5), electroshock (E) performed by the electronic teaser GOZLIN TEQ002 (GOZLIN, Modena, Italy) for 30s at 180 V (tank 1 and 5), and asphyxia with carbon monoxide (CO) until death (tank 3 and 4). Eighteen fish per experimental unit were sampled for the scheduled analyses.

Fish from tank 1 (mean weight 740 ± 105 g) were captured, hauled out of water and immediately treated by electricity (E_12 °C); fish from tank 2 (mean weight 684 ± 95 g) were used as control group and treated by asphyxia in the air (A_12 °C); fish from tank 3 (CO_12 °C) and tank 4 (CO_8 °C) (mean weight 737 ± 120 g and 773 ± 101 g, respectively), were flushed with 100% food grade CO(SIAD, Bergamo, Italy). From tank 5, 18 fish (mean weight 667 ± 97 g) were captured, hauled out of waterand then immediately treated by electricity (E_8 °C), afterwards other 18 fish (mean weight 760 ± 85 g) were sampled from the same tank and treated by asphyxia in the air (A_8 °C), due to the overall availability of only 5 tanks. All groups of fish were finally percussively slaughtered.

During the experiment, the CO concentration in the air was monitored and measured by the use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA) and by supplementary gas detectors in charge of the firemen of Trento province (Italy).

2.3 Plasma parameters

Immediately after percussive slaughtering, blood samples were collected from the caudal vein of 5 fish from each group. Blood was placed in heparinised tubes, centrifuged at 4000 rpm for 10 min; the resultant plasma was transferred into Eppendorf tubes and stored at -80 °C until analyses.

Plasma lactate and glucose were analysed using MaxMat PL (MaxMat S.A., Montpellier, France). Cortisol was determined using ELISA (RE52061, IBL International GmbH, Hamburg, Germany). Osmolality was measured using freeze

depression (Fiske® 210 Micro-Sample Osmometer, Advanced Instruments, Inc., Norwood, MA, USA) and ions (Na^+, K^+, and Cl^-) were analysed with selective ion electrodes (Cobas c 111, Roche Diagnostics Ltd., Rotkreuz, Switzerland).

2.4 Rigor Index and pH evolution, fillet shape changes during rigor mortis, ATP and related catabolites determination

After slaughtering the 5 fish per experimental group used for blood sampling were also considered for *Rigor Index* and pH evolution measurements. Three other fish were destined to the fillet shape change assessment, drip losses and ATP and its catabolites determination. The remaining 10 fish were used for further analyses, not included in this manuscript.

For *Rigor Index* and pH evaluation fish were individually tagged, weighed and stored in polystyrene boxes with ice, maintained in a cold room at a temperature ranging between 0 and +2 °C until *rigor-mortis* resolution, i.e. at about 76 hours *post mortem*. Measurements were done immediately after slaughter (T0) and at 4, 15, 24, 33, 39, 48, 57 and 76 hours *post mortem. Rigor mortis* was determined by the Rigor Index, calculated according to Bito et al. (1983) using the following formula:

$$RI\ (\%) = [(L_0 - L_t)/L_0] \times 100$$

where L_0 (cm) is the vertical distance between the base of the caudal fin and the table surface (used as a support base for the fish),measured immediately after the death, whereas L_t (cm) is the vertical distance between the base of the caudal fin and the table surface at the selected time intervals.

The pH was measured on the cranial part of epaxial fillet portion, using a Mettler Toledo FiveEasy™/FiveGo™ pH meter (Mettler-Toledo Ltd, Leicester, UK).

On the 3 fish per treatment mentioned above, manual filleting in *pre rigor* condition was carried out. Afterwards, left fillets, maintained in a cold room at a temperature ranging between 0 and +2 °C, were used to assess the shape changes during *rigor mortis*, by taking pictures at 0, 4, 9, 15, 24, 33, 48 and 60 hours *post mortem* with a NIKON D3000 camera with lens Nikkor 18-55. The photographed fillets were analysed by the Software Adobe Photoshop CS4 for the following parameters: area, perimeter, maximum length and maximum height.

From the cranial side of the epaxial portion of the right fillets of the same fish utilised for fillet shape changes analysis, maintained in a cold room at a temperature ranging

between 0 and +2 °C, 1 g of muscle was sampled immediately after death for adenosine 5'-triphosphate (ATP) and its catabolites. [i.e. adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx)] concentration analysis, determined by a HPLC based on Burns and Ke (1985) method. The HPLC apparatus comprised a pump system (Beckman mod. 125-S) equipped with a UV detector (Beckman mod. 166) with absorbance fixed at 254 nm, analogic interface (Beckman mod. 406), Ultrasphere ODS Reverse Phase column (Beckman; length 250 mm, internal diameter 4.6 mm; particle size 5 µm; pore size 80 Å), Ultrasphere ODS pre-column (4.6 mm ID, 45 mm length), and 20-µl fixed loop. The mobile phase was KH_2PO_4, 0.5 M, pH 7.0. Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA).

From ATP and related catabolites, Adenylate Energy Charge (AEC) = (0.5 ADP + ATP)/(AMP + ADP + ATP) (Atkinson, 1968) was also calculated.

Our main interest was to detect the amount of ATP and AEC left in the muscle immediately *post mortem* (T0), in relation with the stunning/slaughtering methods applied. These parameters are involved in the first period of freshness evolution.

2.5 Statistical analysis

Data were analysed using the General Linear Model procedures of the statistical analysis software SAS 9.1 (2004) for Windows. A two-ways ANOVA tested the stunning methods (three levels: A, CO and E) and the water temperatures (two levels: 8 and 12 °C) as fixed effects. The stunning method (S) x water temperature (T) interaction was also tested.

3. RESULTS

3.1 Plasma parameters

Fish subjected to A, E, and CO slaughtering methods, at the two temperature conditions (8 or 12 °C), showed significantly different ($P<0.001$) glucose levels (Table 1), with decreasing trend in the order CO>A>E.

The highest lactate levels were found in A and CO groups (5.58 and 5.36 mM, respectively), while the E group had the lowest lactate amount (3.23 mM).

Trout slaughtered by asphyxia had significantly higher cortisol level ($P<0.001$) compared to the CO and E groups (153 vs 60.4 and 40.4 ng/ml, respectively) with no differences between the CO and E groups.. However, cortisol showed a highly

significant (P<0.001) interaction between stunning method (S) and water temperature (T) (Table 2), indicating that trout reared at 8 °C and stunned by A, had the highest level of cortisol of all groups of treatment both at 8 °C -CO and E- that at 12 °C -A, CO and E- (231 *vs.* 49.5 and 40.4 ng/ml; 231 *vs.* 74.19, 31.26 and 80.45 ng/ml, respectively).

Stunning by A exhibited the lowest K^+ concentration (5.58 vs 6.59 and 6.06 mM for A, CO and E, respectively; P<0.05) and a significant interaction SxT between slaughter method and water temperature. As highlighted in Table 2, in trout maintained at 8 °C the K^+ level decreased in the order CO>E>A (P<0.001), whereas for those kept at 12 °C, the trend was A>CO (P<0.001), with the E group having intermediate values. When comparing stunning methods applied at the two different temperatures, CO_8 °C expressed the highest value of K^+ to all treatments, A_8 °C had significantly lower value only than A_12 °C (4.96 *vs.* 6.21mM, respectively); CO_8 °C significantly higher to all treatments applied at 12 °C (8.10 *vs.* 6.21 *vs.* 5.08 and5.38 mM, respectively), and E_8 °C significantly higher to CO_12 °C and E_12 °C (6.75 *vs.* 5.08 and 5.38 mM, respectively).

For plasma Chloride, no differences due to slaughtering method or rearing temperature were found.

3.2 Rigor Index and pH evolution, fillet shape changes during rigor mortis, ATP and related catabolites content

Trout maintained at 8 °C, and exposed to A, had an earlier onset and resolution of *rigor mortis* (Figure 1A), reaching Rigor Index (RI) = 0 only 48h post mortem, followed by CO and E groups, at about 75h after death. At 12 °C any significant difference was determined, even though the more rapid increase and resolution of rigor in A group was evident (Figure 1B). Considering pH trend, immediately after death, A group had both at 8 °C (2A) and 12 °C (2B) a significantly lower pH than E and CO groups, the latters not different between them. Later on, at 8 °C, no difference was attributed to the slaughtering methods till *rigor* resolution; while for trout reared at 12 °C, A group at 40 and 46 hours *post mortem* had the lowest pH, significantly different from that of CO group, while E showed intermediate values. At both temperatures, 8 °C and 12 °C, pH was not different among the three considered groups at *rigor* resolution (76h).

At 4h *post mortem* in A, CO and E fillets the perimeter values accounted for the ~92, 96 and 99%, respectively of the value recorded immediately after death (100%). Fillets from asphyxiated fish, had a significantly higher contraction both at 4h after death with respect to CO and E, that at 9, 15 and 24h with respect to E and CO (except at 24 h). Area contraction of A fillets at 4h after death was significantly higher than that of E fillets, with CO fillets having intermediate position (values accounted for the 92.5 *vs.* 99.5 *vs.* 95.7% of the initial value, respectively; Figure 3); at 9 and 15h *post mortem*, A continued to be the treatment with significantly higher area contraction. A group length contraction was of greater magnitude, which already at 4 hours *post mortem* reached a maximum value of 85% of the initial length of the fillet (as also observed for the previous parameters characterizing the fillet shape changes), while the other two groups reached later the maximum values of their contraction. Length contraction magnitude was smaller for CO fillets when compared to E fillets; at the end of the considered period (48h *post mortem*), the latters showed shrinkage values similar to that of A fillets, significantly higher with respect to that of CO. A more rapid increase in height was exhibited by A fillets, whereas the most intense was detected for E fillets and the lowest for CO fillets (12 and 7% more than the initial values, respectively), even if the differences did not result statistically significant. By averaging the values of the experimental treatments at the end of the monitoring period (48h *post mortem*), area, perimeter, length and height exhibited irreversible changes accounting for the 92.8, 92, 86.5, and +9.5% with respect to the initial values, respectively.

Results relating to the content in ATP and AEC immediately *post mortem* (T0) in muscle from rainbow trout subjected to three different stunning/slaughtering methods were compared (Table 3). Tissues samples from CO group exhibited significantly higher ($P<0.05$) amount both of ATP (2.27 µmol/g) that AEC value (0.83; $P<0.001$) when compared to A (1.20 µmol/g and 0.52, respectively) and E (1.13 µmol/g and 0.64, respectively). The AEC resulted affect by water temperature, indeed at 12 °C it increased significantly ($P<0.05$) with respect to 8 °C (Table 3). Significant ($P<0.01$) interaction SxT was found for ATP and AEC value (Table 4). As concerning ATP and AEC Index concentrations in the fillet, the lowest values were showed by E_8 °C and A_12 °C groups, within and between groups of treatment, respectively.

4. DISCUSSION

4.1 Blood parameters and cortisol

Both slaughtering methods and temperatures had significant effects on blood parameters. Fish exposed to A and E were hauled out of the tank and killed within a few minutes while CO fish were exposed to the treatment for a prolonged period of time. This means that CO fish would have more time to mobilize glycogen stores and increase plasma glucose levels when compared to A and E treated fish. Alterations in glucose metabolism are a common response to stress in captured fish (Barton and Iwama, 1991), which response provides extra energy resources enabling the animal to overcome the disturbance. This was evident in the present trial although the levels were increased in A group probably because of the intense muscle activity during the 15 min of asphyxia in the air.

Lactate increase is a consequence of anaerobic metabolism, and would be expected to increase in A fish in particular, which was the case (Thomas et al., 1999; Wood, 1991). It was also noticeable that the lactate level in CO fish increased following the treatment indicating that the latter part of the treatment was anaerobic and by confirming CO binding affinity towards O_2(Kalin, 1996; Davenport, 2002). The general increase in lactate production with temperature shows that the metabolism is higher at this temperature.

Trout slaughtered by asphyxia in the air had the highest level of cortisol, significantly different from those of CO and E groups. In general, fish resting levels of cortisol vary considerably (2-42 ng/ml), whereas post-stress levels are known to vary from 20 to 500 ng/ml (characteristically <300 ng/ml) (Barton and Iwama, 1991). Similar concentrations to that of A group were reported by Skjervold et al. (1999) in Atlantic salmon kept at low density (less than 50 kg/m^3), then slaughtered by live chilling (184.0 ± 62.66 ng/ml), and by Merkin et al. (2010) in rainbow trout after long term (4 hours) crowding (200-300 kg/m^3; 45.35 ± 35 ng/ml). A highly significant interaction SxT highlighted very high value for cortisol in A_8 °C group with respect to all the groups of treatment, both at 8 that 12 °C. This could be explained by the double use of tank 5, from which the fish slaughtered by electricity first, and then those by asphyxia in air were hauled out. The disturbance caused to the fish when removed from the tank itself was enough (beyond the killing method used) to generate an increase in cortisol in fish left in the tank (Pickering et al., 1982), this is what happened for A_8 °C group. This condition could have minimized the temperature effect.

Plasma K^+ levels were significantly higher in trout slaughtered with CO, when compared to the control, slaughtered by percussion. Ultsch et al. (1981) sustained that large increase in serum K^+ levels are reported in fish as consequence of strenuous exercise and intracellular acidification, and as an effect of acute stress and haemoconcentration (Mc Donald and Milligan, 1997). This could explain the highest values found in CO group mainly due to CO action in displacing O_2 and favouring cellular acidification, which was probably more intense in CO group since exposition was prolonged in time if compared to A one. The significant interaction SxT obtained revealed an effect of the different slaughtering methods, depending on the temperature of the water in which trout were maintained: the K^+ concentration in plasma of CO groups was the highest and the lowest in rainbow trout reared at 8 °C and at 12 °C, respectively. Waring et al. (1996) proposed multiple and cumulative effects to explain serum K^+ variations in Atlantic salmon under stress conditions. No difference was found for chlorides level in the plasma, nor between the groups slaughtered by different methods or among groups maintained at different temperature.

4.2 Rigor Index and pH evolution, fillet shape changes during rigor mortis, ATP and related catabolites content

At 8 °C, asphyxiated group showed an earlier onset and resolution of *rigor mortis* followed by CO and E groups, resulting significantly different from them. This trend was detected also at 12 °C but without any significant difference. The different behaviour observed in A groups when compared to the others, could be the result of the procedure adopted for the slaughtering of fish kept in tank 5 at 8 °C. It is therefore likely that on these fish, two cumulative stressor effects were added, the first associated to the collection of E group from the same tank, and the second to the slaughtering method. In species subjected to capture or handling stress, it was observed an earlier onset and resolution of *rigor mortis*(Berg et al., 1997; Jerret et al., 1998; Robb, 2001). The intense activity before slaughter caused considerable muscular glycogen consumption and thus lactate production, resulting in a rapid onset and resolution of *rigor* in A group when compared to CO and E groups. This condition could explain the lower strength with which *rigor* was expressed, and its faster resolution. E group entered in rigor after A and CO respectively since, being electricity a very rapid slaughtering method (30s *vs.* 15 min. for asphyxia *vs.* ~30 min. for CO), fish suffered a minor stress immediately before death, resulting in the saving of the

muscle glycogen stores, postponing its degradation to lactate and thus delaying in the time of the *rigor mortis* resolution.

It is knew the high CO affinity towards hem proteins and its blocking effects on O_2 utilisation at high concentrations; it is more likely that this property resulted less stressing for the fish with respect to the asphyxiation in air, but more than the electrical stunning, especially at lower temperatures.

pH values showed that both at 8 °C and 12 °C, A group significantly differed, at least immediately after death, from CO and E groups, which exhibited a similar trend through all the monitoring time (76h) (Figure 2). This means that the double disturbance caused to A_8 °C group and the different water temperatures, had no influence, at least on the pH evolution, since in both cases for all the respective stunning methods applied, pH evolution followed the same pattern, ending up with not different values.

Maximum area and length contraction values similar to that of A group were found, with different timing, by Misimi et al. (2008) on both stressed (chased to exhaustion for 30 min.) and unstressed (anesthetised with AQUI-S™) Atlantic salmon (~14% and ~9%, respectively); whereas Mørkøre et al. (2006) found for cod anesthetized by metacaine (MS222), a length contraction value of 21%. Further, it has been reported that an earlier onset of *rigor mor*tis, caused by stress, can provide greater muscle contractile tensions and shortening than those observed in unstressed fish (Nakayama et al., 1999), by confirming what happened for A group. It is interesting to note that CO seemed to reduce fillets length contraction at the end of the monitoring period (48 h), with respect to electroshock and asphyxia slaughtering methods. During *rigor*, A and E fillets' height increased as a consequence of the major length decrease: height increased up to 9 and 12% at about 4 and 9h after death, respectively, corresponding with maximal rigor of whole fish, to reach again at 48h *post mortem* the values observed at 4 and 9h. On Atlantic cod fillets Misimi et al. (2008) found a significant effect of stress on *perimortem* changes in the height.

The recorded behaviour seems to confirm that fillets obtained from the most stressed animals, because of the adopted slaughtering method by asphyxiation in air, begin to contract and change their shape earlier than fillets obtained from slaughtered animals with the other two techniques. This result, which is in line with the values registered for the blood parameters and with the pH value, must be related with the greater

depletion of muscle energy in the case of fish that have suffered greater stress condition at the time of death.

Results obtained from chemical analysis, confirmed data from plasma, fillet shape changes, rigor (only at 8 °C) and pH (both at 8 °C that 12 °C) evolution. Asphyxia in the air resulted as the most stressing stunning/slaughtering method applied in this trial, because of the prolonged (15 min.) exposition, which resulted in an intense stress and muscle activity, accompanied by marked ATP depletion immediately after death, together with E treatment (Lowe et al., 1993; Ruff et al., 2002; Thomas et al., 1999). Lower muscle pH immediately *post mortem* and a more rapid decline during storage in A group are also confirmed with observations in other species of fish (Izquierdo-Pulido et al., 1992; Nakayama et al., 1992). The lower pH levels have generally been attributed to H^+ generation associated with lactate production and ATP breakdown (Hochachka and Mommsen, 1983; Wood and Perry, 1985). Thomas et al. (1999) detected similar ATP concentrations to CO group in stressed and stressed and exercised Atlantic salmon immediately after death (2.22 and 2.17 µmol/g, respectively), and to A and E groups (1.20 and 1.13 µmol/g, respectively) after 12h of storage (1.23 µmol/g) of the same fish. Mishima et al. (2005) in horse mackerel (*Trachurus japonicus*) slaughtered by temperature shock, at 8h *post mortem* found similar values (~ 2.10 µmol/g) to those of CO group, whereas 12h *post mortem*, when slaughtered by cutting the brain, to those of A and E (~ 1.20 µmol/g).

The different degree of stress sustained by all the groups of treatment and the variation in the actual time of death affected *post mortem* AEC (Adenylic Energetic Charge) values, significantly higher for CO followed by E and A groups.

Berg et al. (1997) reported for stressed (stunning with CO_2) Atlantic salmon AEC values similar to those of E group only 3 hours after slaughter (0.66 ± 0.07 *vs.* 0.64 ± 0.08, respectively), which were comparable to values of unstressed group from the same trial (netted individually and killed within 25s by a blow to the head) at about 20 hours *post mortem*. Similar AEC values to CO group (0.83 ± 0.08) were found by Erikson et al. (1999) in Atlantic salmon been chased for 1h before slaughter (0.88 ± 0.04) and by Schulte et al. (1992) in rainbow trout been exercised to exhaustion for 30 min before slaughter (0.84 ± 0.011).

Interactions showed that water temperature exerted an important effect. Reduction of fish muscle temperature, removes substantial thermal energy accessible for the muscle degradation that starts within hours after slaughter (Skjervold et al., 2001). At 8 °C

electroshock seemed to be the treatment which deplete more energy, at 12 °C, instead it was asphyxia, whereas CO seemed not been affected by water temperature. This seems to show an evident effect of the temperature since the treatments applied were always the same, but it must be keep in mind that while at 12 °C electroshock and asphyxia in the air where applied in fish from two different tanks, whilst at 8 °C first E and than A were applied in fish from the same tank because of last minute contingencies which deprived us of one tank. Only groups treated with CO did not differed in energy content both at 8 and 12 °C.

5. CONCLUSIONS

From this study it resulted that electroshock was the most suitable slaughtering method, able of limiting stress in rainbow trout, asphyxia in air seemed to be the most stressing, as confirmed also by *rigor mortis* and pH evolution, whereas CO was placed in the middle. Fillets from asphyxiated fish had the strongest area and perimeter contractions followed by CO and E on one side, and the most rapid length shrinkage and height increase, followed by E and CO, on the other. CO_8 °C and CO_12 °C were able to preserve the higher amount of muscle's ATP immediately after death.

It must be born in mind that, in this trial, the fish asphyxiated in the air reared at 8 °C was strongly stressed by the double sampling. In this preliminary study on the CO application for slaughtering rainbow trout some critical points with regard to the procedure of gas release into water were highlighted, thus it would be interesting to perform a pressurize release of CO in a closed circuit, with subsequent injection into the water, to improve gas efficiency and personnel safety. On the other side, the application of electrodes on animals removed from the water, and therefore in compromised conditions from a welfare point of view, is also considered a critical point.

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Table 1. Plasma parameters and cortisol levels in rainbow trout reared at 8 °C or 12 °C and stunned/slaughtered by asphyxia (A), CO (CO) or electricity (E) (No. = 5 fish/group).

Parameters	Stunning (S)			Temperature (T)		*P*-value			RSD [(1)]
	A	CO	E	8 °C	12 °C	S	T	SxT	
Glucose (mM)	6.08^{a}	7.47^{a}	5.00^{c}	6.03	6.34	<0.001	NS	NS	0.79
Lactate (mM)	5.58^{a}	5.36^{a}	3.23^{b}	4.26^{b}	5.19^{a}	<0.001	<0.01	NS	0.84
Cortisol (ng/ml)	153^{a}	60.44^{b}	40.4^{b}	107^{a}	62^{b}	<0.001	<0.01	<0.001	50.317
K^{+} (mM)	5.58^{b}	6.59^{a}	6.06^{ab}	6.60^{a}	5.56^{b}	<0.05	<0.001	<0.001	0.69

[(1)] Residual Standard Deviation

a, b: Within each criterion, means in the same row having different superscripts are significant at P ≤ 0.05 level.

NS: not significant.

Table 2. Plasma parameters and cortisol levels in rainbow trout reared at 8 °C or 12 °C and stunned/slaughtered by asphyxia (A), CO (CO) or electricity (E): interaction temperature x stunning/killing methods (No. = 5 fish/group).

Parameters	8 °C			12 °C		
	A	CO	E	A	CO	E
Cortisol (ng/ml)	230.90^{A}	49.50^{B}	40.44^{B}	74.19^{B}	31.26^{B}	80.45^{B}
K (mM)	4.96^{D}	8.10^{A}	6.75^{B}	6.21^{BC}	5.08^{D}	5.38^{CD}

A, B: means at the same time after death having different superscripts are significantly different at P ≤ 0.01 level.

Table 3. ATP and AEC values immediately *post mortem* (T0) in muscle of rainbow trout reared at 8 °C or 12 °C and stunned/slaughtered by asphyxia (A), CO (CO) or electricity (E) (No. = 3 fish/group).

Parameters	Stunning (S)			Temperature (T)		*P*-Value			RSD[(1)]
	A	CO	E	8 °C	12 °C	S	T	SxT	
ATP	1.20[b]	2.27[a]	1.13[b]	1.14	1.93	<0.05	NS	<0.05	0.77
AEC	0.52[b]	0.83[a]	0.64[c]	0.62[b]	0.71[a]	<0.001	<0.05	<0.001	0.08

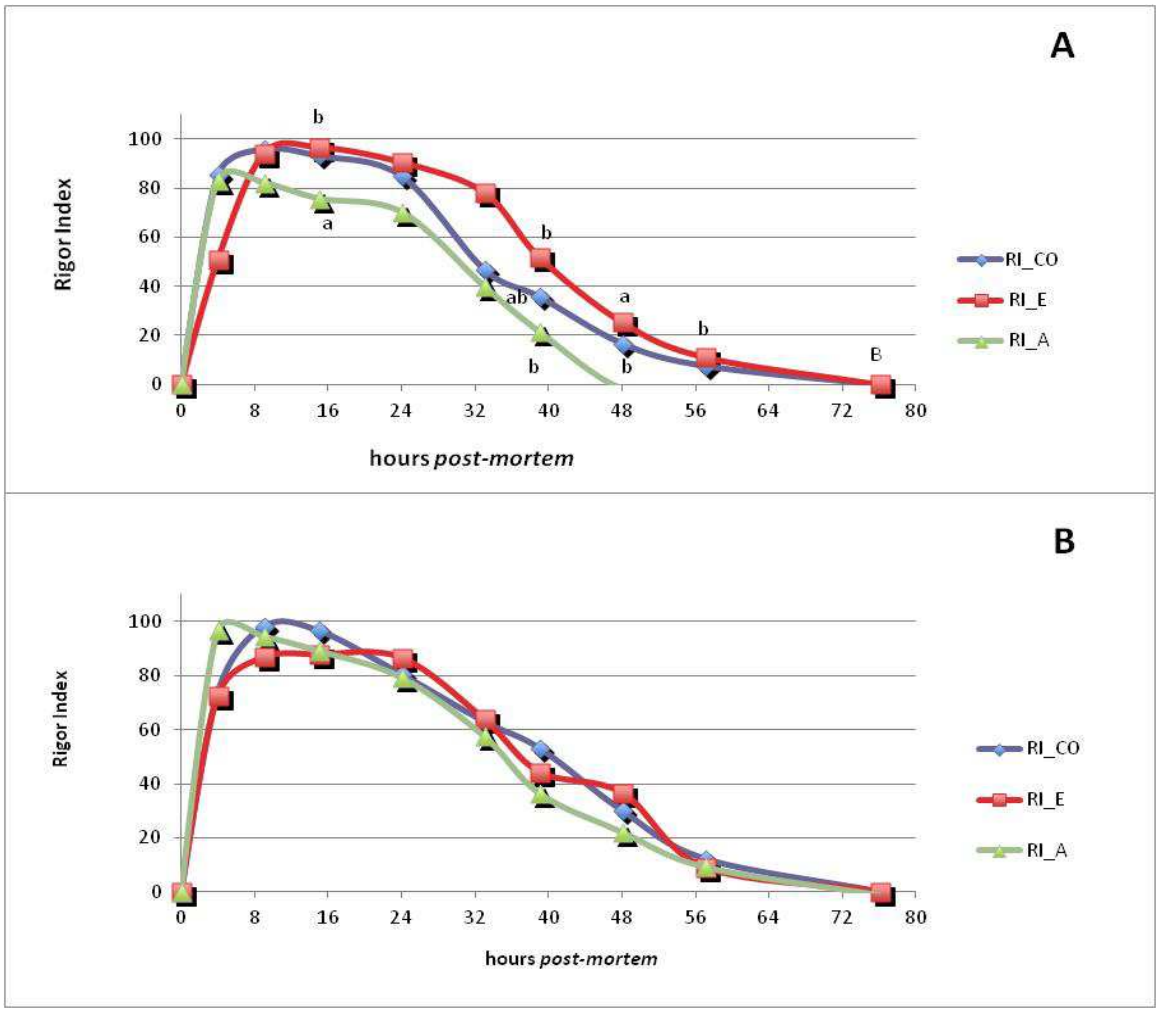
[(1)] Residual Standard Deviation
a, b: Within each criterion, means in the same raw having different superscripts are significant at P ≤ 0.05 level;
NS: Not significant.
.

Table 4. ATP and AEC values immediately *post mortem* (T0) in muscle of rainbow trout reared at 8 °C or 12 °C and stunned/slaughtered by asphyxia (A), CO (CO) or electricity (E): interaction temperature x stunning/slaughtering methods (No. = 3 fish/group).

Parameters	8 °C			12 °C		
	A	CO	E	A	CO	E
ATP	1.39[at]	2.02[ab]	0.0031[c]	1.00[bc]	2.51[a]	2.26[ab]
AEC	0.63[b]	0.81[a]	0.41[c]	0.42[c]	0.85[a]	0.86[a]

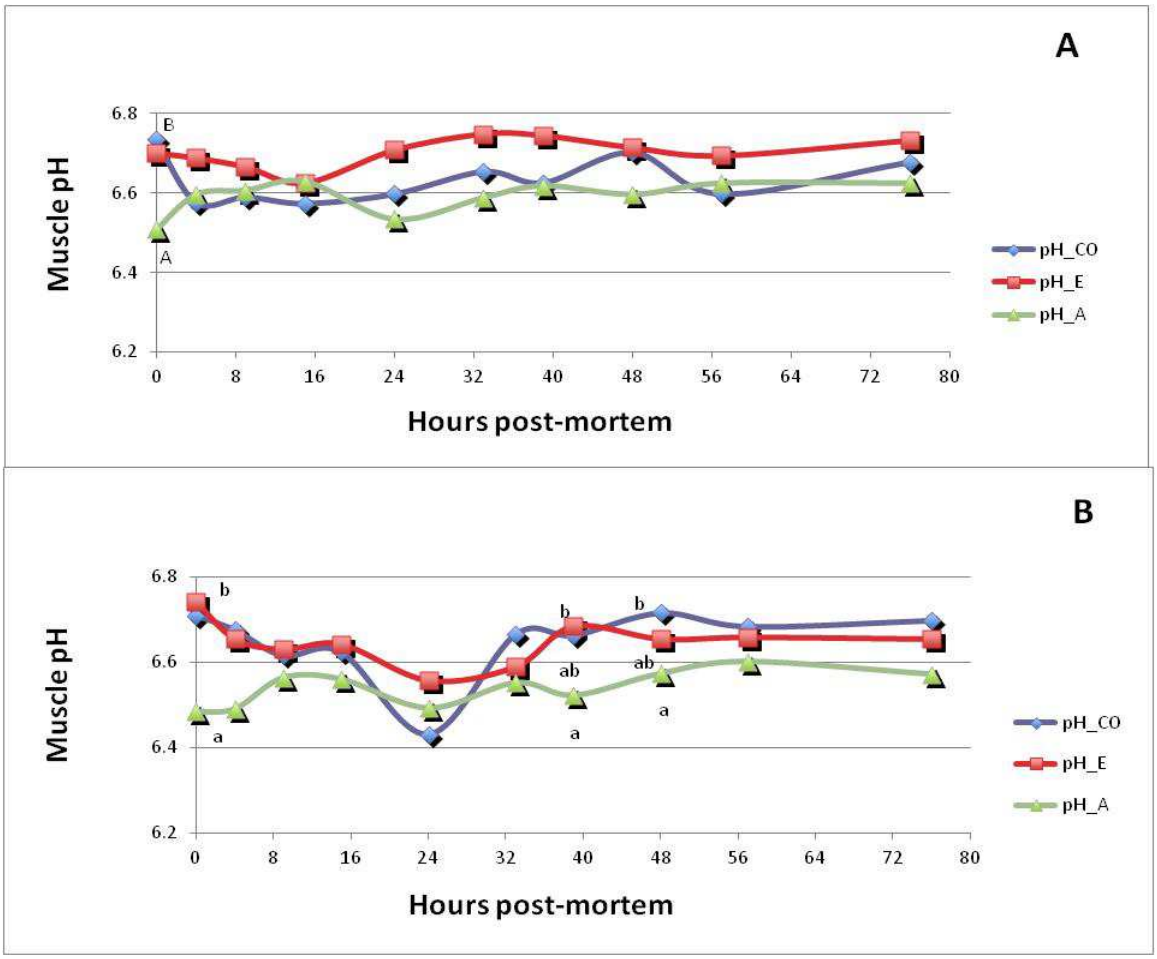
a, b: Within each criterion, means in the same row having different superscripts are significant atP ≤ 0.05 level.

Figure 1.*Rigor* Index (RI) evolution in rainbow trout reared at 8 °C (A) and 12 °C (B) stunned/slaughtered by A, E or CO. Value are presented as means (No. = 5 fish/group). Uppercase and lowercase denotes significant differences (A, B = P<0.01; a, b = P<0.05, respectively).



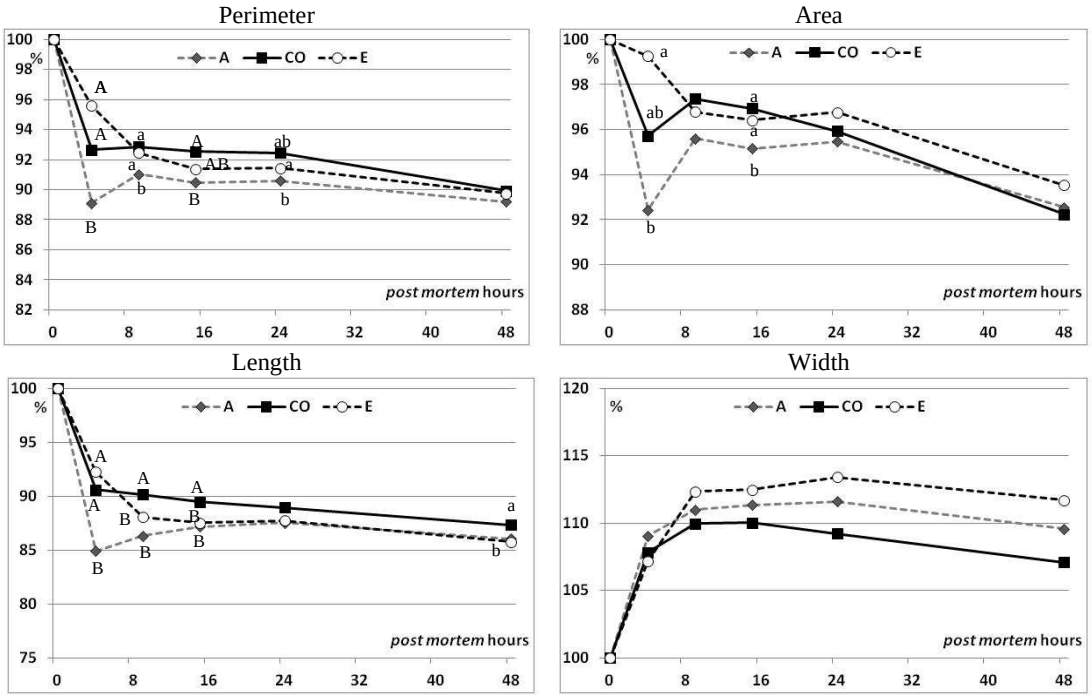
A, B: means at the same time after death having different superscripts are significantly different at P ≤ 0.01 level;
a, b: means at the same time after death having different superscripts are significantly different at P ≤ 0.05 level.

Figure 2. pH evolution in rainbow trout reared at 8 °C (A) and 12 °C (B) stunned/slaughtered by A, E or CO. Value are presented as means (No. = 5 fish/group). Uppercase and lowercase denotes significant differences (A, B = P<0.01; a, b = P<0.05, respectively).



A, B: means at the same time after death having different superscripts are significantly different at P ≤ 0.01 level;
a, b: means at the same time after death having different superscripts are significantly different at P ≤ 0.05 level.

Figure 3. Perimeter, area, maximum length and maximum height of fillet in rainbow trout stunned/slaughtered by A, E or CO, measured at different times after death, expressed as a percentage of the value measured immediately after death (No. = 3 fish/group).



A, B: means at the same time after death having different superscripts are significantly different at $P \leq 0.01$ level;
a, b: means at the same time after death having different superscripts are significantly different at $P \leq 0.05$ level.

PAPER VI

Effects of stunning/slaughtering methods on *post rigor mortis* changes in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions.

Abstract

Post rigor mortis changes related to texture, chemical and sensory properties in rainbow trout (*Oncorhynchus mykiss*) reared at two different temperature conditions (8 and 12 °C) were investigated to better understand to what extent different stunning/slaughtering methods, *i.e.* carbon monoxide asphyxia (CO), electroshock (E) and asphyxia in the air (A), can influence their evolution in the course of storage time.In long terms ($T_{RR}7$ = 10 days *post mortem*), considering both K and K_1-values, freshness results well preserved irrespective of the stunning/slaughtering method applied, and water temperature. At *rigor* resolution ($T_{RR}0$)CO fillets showed higher pH than A fillets ($P<0.01$), whereas seven days after *rigor* resolution ($T_{RR}7$) also E fillets pH resulted significantly higher than A fillets. CO treatment ensured higher a* and C* colour values, and intermediate b* value, whereas electroshock provided the lowest a*, b* and C* values in fillets. Texture profile analysis revealed an effect of the stunning method and of the temperature for the cohesiveness parameter. Fish slaughtered by CO presented significantly lower ($P<0.001$) malondialdehyde content in fillets when compared to the other two groups at $T_{RR}0$, whereas at $T_{RR}7$ no differences were detected. Canonical Discriminant Analysis of sensory attributes, instrumental texture and physico-chemical measurementsresulted as an accurate tool in discriminating and classifying the three groups of treatments at the two considered rearing water temperatures.

Key words: rainbow trout; stunning methods; shelf-life; colour; TBARS; texture profile analysis; sensory analysis

1. INTRODUCTION

Freshness is the most important attribute when assessing fish quality. Microbial, biochemical and sensory changes are associated with deterioration of fish quality during handling and storage (Ehira & Uchiyama, 1986; Gregory, 1994). It is fundamental to reduce muscle activity during transport and netting and to ensure stunning/slaughtering methods able to minimize *pre mortem* stress of fish since it may result in detrimental effects on fillets texture (Nakayama, 1996; Ando, Toyohara &Sakaguchi, 1992), colour perception (Robb, Kestin, & Warriss, 2000; Robb & Warris, 1997; Jittinandana et al., 2003), shelf-life (Lowe et al., 1993) and K-value (defined as a later indicator of fish freshness; Izquierdo-Pulido, Hatae, & Haard, 1992).

Stress can provide greater muscle contractile tensions and shortening than observed in unstressed fish (Nakayama, Ooguchi, & Ooi, 1999). As reported by Robb (2001) in rainbow trout, a very rapid drop in muscle pH due to stress can affect colour parameters (higher L*, H°, C* and lower Roche card score) making fish flesh appearance lighter and more opaque. The use of absolute or relative amounts of particular degradation products as indicators of freshness and spoilage is very common in scientific literature (Ehira & Uchiyama, 1986; Jones, Murray, Livingston, & Murray, 1964). During the capture/harvesting process and the struggling associated with the death of the fish, much of the ATP (adenosine triphosphate) is converted to AMP (adenosine monophosphate) and sometimes further to IMP (inosine monophosphate), and the sequence ATP to IMP is generally complete within two days of storage in the ice after death. Over the first few days of storage in ice, loss of IMP occur, by affecting the flavour in fresh fish. IMP is recognized as a flavour enhancer of meaty foods, especially of the umami flavour (Kawai et al., 2002), and it is likely the IMP contributes to the sweet, creamy, meaty flavours of fresh fish (Bremner, Olley, Statham, & Vail, 1988; Fletcher, Bremner, Olley, & Statham, 1990;Fraser, Pitts, & Dyer, 1968;Fuke & Konosu, 1991; Hashimoto, 1965). The K-value has been much used as an Index of freshness (Ehira &Uchiyama, 1986), defined as the ratio of the sum of the non-phosphorylated compounds, Ino (Inosine) and Hx (Hypoxanthine), to the sum of all ATP-derived degradation products. Generally an upper K-value limit of 70 to 80% is for good quality and, lower than 40-50% for excellent quality large commercial-size Atlantic salmon at 14 and 7 days *post mortem*,respectively (Erikson et al., 1997).In almost all storage trials described in literature, concentrations of the

adenine nucleotides are very low and a revised K-value, often designated as K_1-value, is calculated as the ratio of the sum of Ino and Hx to the sum of IMP, Ino and Hx. In this case, K_1-value monitors loss of IMP (Howgate, 2005). Animal welfare and product quality are linked aspects of the total quality of fish; therefore requirements of fish welfare and efficient aquaculture should be guarantee. To maintain the best original quality, fish should be stunned until death and killed by avoiding any kind of stress (Poli et al., 2005). Most relevant fish stunning/slaughtering methods are mechanical percussion, CO_2 narcosis and electrical stunning (Robb & Kestin, 2002). Percussive stunning is mainly used for salmon and others large fish. The fish is hit in front of the brain and instantly rendered insensible. For the fish is still possible to recover, if the destruction of the brain is partial, so it is important that the method is followed by bleeding or by another slaughtering practice (Wall, 2001). CO_2 narcosis is commonly used in some salmonid farms. Fish are placed in a bath with CO_2 gas saturated water (> 400 mg / l with a pH of 5.0-5.5). The CO_2 dissolves in water to form an acid, fish blood's pH is lowered and consequently the fall cause the destruction of brain activity, narcosis and eventually death (Kestin, Wotton, & Adam, 1995; Robb, 2001) in about 3-4 minutes (in Salmonid),then fish are slaughtered by cutting the gills and bleeding. Researches have shown that several species of fish exhibit aversive behaviour towards CO_2 narcosis and loss of sensation may occur after few minutes, depending on the species, resulting in the total exhaustion of the fish at the time of death (Erikson, Hultmann, & Erik Steen, 2006; Marx, Brunner, Weinzierl, Hoffmann, & Stolle, 1997;Robb, 2001), which reach the condition of *rigor mortis* during the processing line, approximately two hours after death (Berg et al., 1997). Electro-narcosis (typically 50-70 V) is used as routine for laboratory purposes or in some farms, especially in the case of trout and salmon (Lambooij et al., 2002c). It is considered "humane" because, if properly applied, the animal is rendered immediately insensible, as the electric current stops brain activity (Kestin et al., 1995). Electrical stunning is immediate, easy to control, efficient (Wall, 2001), it makes possible the anaesthesia of many fish all together (Roth &Moeller, 1999). On the other side, the strong contraction of the muscles causes tetany rather than anaesthesia (Close et al., 1996), intense electrical currents can damage the carcass (Kestin et al., 1997), causing hematoma, blood clots, spinal and vertebrae fractures (Kestin et al., 1995; Roth & Moeller, 1999; Wall, 2001). The use of carbon monoxide (CO) presents itself as an attractive alternative to the use of CO_2 for the slaughter of the fish, as it does not

produce aversive effects by animals, as happens with CO_2(Poli et al., 2005). CO forms bonds with hem proteins -for which presents an affinity 210-270 times greater than O_2(Kalin, 1996)- like haemoglobin (Hb), myoglobin (Mb) and neuroglobin (Ngb), by replacing O_2; thus substantial inhalation of CO can be fatal. When fish are exposed to CO, microbial growth, lipid oxidation and browning may possibly be reduced, therefore the shelf-life of the product is prolonged (Cornforth & Hunt, 2008;Prescott, Harley, & Klein, 1996). This would be preferable in fatty fish like salmon and trout, which are highly vulnerable to lipid oxidation due to the high level of unsaturated fatty acids and the hem containing proteins. CO acts as a reducing agent in which it forms complexes with iron or copper in enzymes (White et al., 1973), and therefore the hem-catalysed lipid oxidation is reduced when CO is bound to the hem. Meat and fish exposed to CO at low levels show desirable bright red, stable colour of the muscle (Cornforth & Hunt, 2008; El-Badawi, Cain, Samuels, & Angelmeier, 1964; Lanier et al., 1978; Sørheim, Nissen, & Nesbakken, 1999). Colour of fish fillets is also affected when live fish is exposed to CO (Concollato et al., 2014; Concollato et al., 2015; Mantilla et al., 2008). CO is shown to enhance the colour and quality of fish (Chow, Hsieh, Tsai, & Chu, 1998; Gee & Brown, 1981;Hsieh, Chow, Chu, & Chen, 1998). CO promotes MMb reduction and thereby it has anti-oxidative capacity (Lanier et al., 1978). The use of CO in fish slaughtering may therefore contribute to a more stable product (Bjørlikke et al., 2012).

The aim of the present study was to investigate the effects on *post rigor* fillet characteristics of different stunning/slaughtering methods, *i.e.* electroshock (E), carbon monoxide asphyxia (CO), asphyxia in the air (A), being the latter a traditional method widely used in Trentino Alto Adige (Italy) rainbow trout farms.

2.MATERIAL AND METHODS

2.1 Experimental set-up

The study was performed at the experimental farm of Edmund Mach Foundation, in S. Michele all'Adige, Trento (Italy).

Five hundred rainbow trout (*Oncorhynchus mykiss*) were equally allocated in 5 tanks, containing 3600 L of freshwater each. In tanks 1, 2, and 3 the water temperature was maintained at 12 °C whereas in tanks 4 and 5 at 8 °C. Three stunning methods were applied: asphyxia in the air (A) lasting about 15 min. (tanks 2 and 5), electroshock (E) performed by the electronic teaser GOZLIN TEQ002 (GOZLIN, Modena, Italy) for

30s at 180 V (tanks 1 and 5), and asphyxia with carbon monoxide (CO) until death (tanks 3 and 4). Eighteen fish per experimental unit were sampled.

Fish from tank 1, with mean weight 0.740 ± 0.105 kg, were captured and immediately killed by electricity (E_12 °C); fish from tank 2 (mean weight 0.684 ± 0.095 kg) were used as control group and killed by asphyxia in the air (A_12 °C); fish from tank 3 (CO_12 °C) and tank 4 (CO_8 °C) (mean weight 0.737 ± 0.120 kg and 0.773 ± 0.101 kg, respectively), were flushed with 100% food grade CO(SIAD, Bergamo, Italy). From tank 5, 18 fish (mean weight 0.667 ± 0.097 kg) were hauled out and then immediately killed by electricity (E_8 °C), afterwards other 18 fish (mean weight 0.760 ± 0.085 kg) were sampled from the same tank and slaughtered by asphyxia in the air (A_8 °C), due to the overall availability of only 5 tanks.

All groups of fish were finally percussively slaughtered. During the experiment, the CO concentration in the air was monitored and measured by the use of portable gas detectors (GasBadge Pro, Oakdale, PA, USA) and by supplementary gas detectors in charge of the firemen of Trento province (Italy).

2.2 Energy metabolism, freshness indexes, drip losses, pH, colour and texture profile analysis

After slaughter, all the animals (No.=108) were individually tagged and weighed. Fifteen fish per treatment (on overall 90 fish) were stored whole in polystyrene boxes with ice in a cold room ranging between zero and +2 °C until *rigor mortis* resolution ($T_{RR}0$), i.e. 76 hours *post mortem.* Other 3 fish per treatment (on overall 18 fish) were immediately manually filleted in *pre rigor* condition.

At 0, 2, 7, 10 days *post mortem* (T0, T2, $T_{RR}4$, $T_{RR}7$, respectively), from the cranial side of the right fillet epaxial part, 1 g of muscle was sampled. The concentrations of adenosine 5'-triphosphate (ATP) and related catabolites, i.e. adenosine 5'-diphosphate (ADP), adenosine 5'-monophosphate (AMP), inosine 5'-monophosphate (IMP), inosine (Ino) and hypoxanthine (Hx) were determined by a HPLC with the analysis method based on Burns &Ke (1985) (results reported in PART II, PAPER V). The HPLC apparatus comprised a pump system (Beckman mod.125-S) equipped with a UV detector (Beckman mod. 166) with absorbance fixed at 254 nm, analogic interface (Beckman mod. 406), Ultrasphere ODS Reverse Phase column (Beckman, length 250 mm, internal diameter 4.6 mm; particle size 5 µm; pore size 80 Å), Ultrasphere ODS

pre-column (4.6 mm ID, 45 mm length), and 20-µl fixed loop. The mobile phase was KH_2PO_4, 0.5 M, pH 7.0. Standards were purchased from Sigma (St. Louis, USA).

Adenylate Energy Charge (AEC) = (0.5 ADP + ATP)/(AMP + ADP + ATP) (Atkinson, 1968), ATP/IMP ratio (Erikson et al., 1997) (results reported in PART II, PAPER V), K-value (%) = [(Ino + Hx) / (ATP + ADP + AMP + IMP + Ino + Hx)] * 100 (Saito et al., 1959), and K_1-value (%) = [(Hx + Ino) / (Hx + Ino + IMP)] * 100 (Karube et al., 1984) were also calculated. Our main interest was to detect K and K_1-values in the muscle at the end of the storage period considered ($T_{RR}7$), in relation with the stunning/slaughtering methods applied, since are involved in an advanced period of freshness evolution.

Drip loss was also determined, by weighing the left fillets immediately after death (T0), at time of the *rigor* resolution ($T_{RR}0$) and 7 days after *rigor* resolution (corresponding to the end of the trial; $T_{RR}7$). Drip loss was calculated by the formula:

$$\text{Drip loss (t)} = [(D0 - D_{RR}0 \text{ or } D_{RR}7)/D0] \times 100$$

where D0is the fillet weight immediately after filleting, $D_{RR}0$ and $D_{RR}7$ correspond to the fillet weight at *rigor mortis* resolution and after 7 more days of storage, respectively.

Three values of drip loss were then calculated: from T0 till $T_{RR}0$, from $T_{RR}0$ till $T_{RR}7$, and the cumulative value from T0 till $T_{RR}7$.

During the *rigor mortis* process, *i.e.* 24 h *post mortem*, pH of the fillets was measured by using a pH-meter (Mettler Toledo FiveEasy™/FiveGo™ pH meter (Mettler-Toledo Ltd, Leicester, UK).

After *rigor mortis* resolution (76 h *post mortem*, time $T_{RR}0$), all the 90 fish were transferred to the processing plant (ASTRO, San Michele all'Adige, Trento, Italy), where they were mechanically filleted and weighed. Afterwards, right fillets were *vacuum* packed and stored at -80 °C for further analyses ($T_{RR}0$ samples), whereas the left fillets were stored for 7 days ($T_{RR}7$ samples), in polyester trays with absorbent pads on the bottom, in a cold room at +2.5 °C, for the analyses scheduled during the shelf-life. Daily, from $T_{RR}0$ until $T_{RR}7$, L*a*b* colour values (CIELab) and pH were measured, by using a spectrocolorimeter (X-Rite, RM200QC; X-Rite, Incorporated, Neu-Isenburg, Germany) and the pH-meter above mentioned.

A Texture Profile Analysis (TPA) was carried out eight days after the *rigor* resolution ($T_{RR}8$), using a Zwick Roell® 109 texturometer (software: Text Expert II, version 3)

equipped with a 1kN load cell. Kramer cell test and Warner-Bratzler shear force test were performed on the caudal region of each fillet.

The Warner-Bratzler shear force test was performed using a straight blade that moved down at a constant speed of 15 mm/s to 100% of the total deformation. Maximum shear force, defined as the maximum resistance of the sample to shearing (Veland &Torrissen, 1999) was determined.

The Kramer cell test was performed on a 80 mm x 80 mm sample. The Kramer cell was composed of 5 linear blades moving down at a crosshead constant speed of 10 mm/s and withdrawing at a speed of 15 mm/s. The force *vs.* deformation curve was registered until the 50% of the total deformation. The test was repeated for 5 cycles simulating the chewing. Five texture parameters were calculated, as suggested by Veland & Torrissen (1999) and Ayala et al. (2010): hardness (peak force of the first compression cycle), energy of shear (the sum of the area of the first upstroke and the area of the first downstroke), cohesiveness (ratio of positive force area during the second compression compared to that obtained during the first compression), resilience (ratio of the area of the upstroke compared to the area of the first downstroke during the first compression cycle) and gumminess (hardness multiplied by cohesiveness).

All measurements were done at room temperature.

2.3 Lipid oxidation (TBARS Index)

Lipid oxidation was determined on 90 fillets (15 per treatment) both at the *rigor* resolution ($T_{RR}0$) and at the end of the storage period considered ($T_{RR}7$) by determination of thiobarbituric acid reactive substances (TBARS), according to the method described by Siu & Draper (1978) and modified by Luciano et al. (2013). Oxidation products were quantified as malondialdehyde (MDA) equivalents (mg MDA/kg fillet).

2.4 Sensory evaluation

The descriptive method has been used. Twelve panelists with experience in determination of sensory profile of different food matrices were subjected to training sessions with the purpose to familiarize with the matrix of interest, to select the appropriate descriptors and to define on a scale of measure the relative perceived intensity. Olfactory, tactile, gustative and textural sensory aspects were evaluated and

for each of them different descriptors were chosen after an accurate literature research. Global odour and aroma intensity (olfactory descriptors); friability and tenderness (tactile descriptors); saltiness and sourness (taste descriptors); adhesiveness, fibrousiness and tenderness (textural descriptors) were evaluated by scored, linear and continuous scale of measure (0-10). Brackish/marine, animal feed, cardboard, stale, boiled potatoes, salmon and others (olfactory descriptors); bitter, astringent and metallic (taste descriptors); stringy, unctuous and low solubility (textural descriptors) were evaluated by categories (presence/absence).

Sensory analysis was performed in duplicates on 90 fillets (15 for each group of treatment), at time of *rigor* resolution ($T_{RR}0$) in two consecutive days. Fillets were placed in aluminium box previously drilled on the bottom so as to prevent the cooking of the same in their own liquids (cooking loss). An aluminium foil was placed on the top of each box. Cooking process was carried out in an electric oven, pre-heated at 200 °C and the cooking time has been set up to the achievement of a core temperature of 75-85 °C. Each panellist received 50 g fillet sample, and evaluated one at a time the six samples, corresponding to the six treatments. The presentation order for all samples in both sessions was randomised to prevent first order and carry over effects (Macfie, Bratchell, Greenhoff, & Vallis, 1989). Data acquisition was performed by FIZZ software (Biosystemes - France) installed in the 12 terminals provided in laboratory's tasting booths.

2.5 Statistical analysis

Data were analysed using the General Linear Model procedures of the statistical analysis software SAS 9.1 (2004) for Windows. A two-ways ANOVA tested the stunning/slaughtering methods (three levels: A, CO and E) and the water temperatures (two levels: 8 and 12 °C) as fixed effects. The interaction stunning/slaughtering method (S) x water temperature (T) was also tested. Multivariate discriminant analysis was performed on sensory data, instrumental texture and physicochemical measurements by considering treatments as discriminant variable (SAS 9.1, 2004).

3. RESULTS

3.1 Energy metabolism, freshness indexes, drip loss, pH, colour and texture profile analysis

Information relating to K and K_1 values at the end of the storage period ($T_{RR}7$), from rainbow trout subjected to three different stunning/slaughtering methods were compared. The mean K-value of rainbow trout slaughtered by asphyxia in the air in this study was significantly lower than that of trout slaughtered by electroshock (45.5 *vs.* 57.0%; $P<0.05$), whereas CO group exhibited an intermediate value (50.2%). Fish reared at 12 °C showed significantly higher K-value with respect to that at 8 °C (55.9 *vs.* 45.9%; $P<0.05$), at $T_{RR}7$.K_1-value resulted not affected, neither by the stunning/slaughtering methods applied, nor by the water temperature at $T_{RR}7$ (Table 1).

Table 2 reports cumulative drip loss (DL%) calculated at different time *post mortem*. Results showed that the experimental treatments did not affect fish drip loss.

Effects on pH and fillets colour during the shelf-life period ($T_{RR}0$-$T_{RR}7$) are shown in Table 3. Slaughtering methods did not affect colour indexes systematically: at $T_{RR}0$,only redness (a*) was affected, with CO group showing significantly higher ($P<0.01$) values with respect to E group (16.3 *vs.* 14.4, respectively); the same pattern was shown at $T_{RR}7$, with a* and Chroma values significantly higher ($P<0.05$) for CO with respect to E (18.1 *vs.* 16.6 and 25.5 *vs.*23.5,respectively),whereas yellowness (b*) resulted significantly ($P<0.05$) higher for A compared to E (18 *vs.* 16.5, respectively).

Water temperature instead, resulted in more important and constant differences on fillet's chromatic characteristics. Lightness (L*), a*, b* and Chroma resulted always higher for trout reared at 8 °C, with significant differences at the different considered times, except for $T_{RR}3$. A significant effect of stunning/slaughtering methods and water temperatures on pH at $T_{RR}0$ and $T_{RR}7$ emerged. At the first day of *rigor* resolution ($T_{RR}0$), pH of CO group was clearly higher than that of A group, whereas E presented a mean value; low water temperature (8 °C) significantly increased pH values. At the end of the storage ($T_{RR}7$), higher water temperature favoured a slight increase in pH; also a general lowering in pH was observed, but CO and E groups still reported the highest values.

About the texture characteristics, slaughter conditions affected only cohesiveness, which presented significantly higher values in A and E fillets compared to the CO ones (0.52 *vs.* 0.41, $P<0.01$; Table 4).The other texture parameters showed a marked

similitude between A and E groups, while the CO one differed numerically for its structure, presenting higher hardness and shear stress values, but lower resilience and gumminess. Water temperature had a major effect on the texture of the fillet than the slaughtering method: at 12 °C, it resulted in increased hardness and shear stress, as well as lower values of cohesiveness and resilience were registered.

3.2 Lipid oxidation

Stunning/slaughtering method significantly affected malondialdehyde (MDA) content (Table 5). Fillets of fish slaughtered by CO showed lower MDA values ($P<0.001$) than the other two groups (0.66 *vs.* 1.22 and 1.10 mg/kg; $P<0.01$), confirming CO ability to reduce and delay over time lipid oxidation of the product with respect to the other two slaughter methods, within 76h *post mortem*. At the end of the storage ($T_{RR}7$), instead, no differences were detected concerning MDA content among the different treatments.

3.3 Sensory analysis

Canonical Discriminant Analysis (CDA) was used to achieve the most discriminative variables for the three-stunning/slaughtering methods. Variables significantly responsible for the discrimination are reported in increasing order of discriminating power in Table 6 (8 °C) and 7 (12 °C), according to Wilks λ significance ($P<0.01$). The CDA showed up that fillets from A, CO and E groups are well discriminated within fish reared at 8 and 12 °C for the same variables: adhesiveness, pH_IR24, tactile tenderness and acidity; this means that the different water temperatures did not have any effect on them. Stunning methods significantly affected cohesiveness, a*, b*, resilience and shear stress of fish reared at 8 °C, whereas they affected juiciness, saltiness and odour intensity of fish reared at 12 °C.

The relative positions of the treatments in the graphical representations reflect the high discriminant ability of the considered variables (Figures 1 and 2). CO_8 °C, A_8 °C and E_8 °C groups resulted totally separated in Figure 1 (0% error), whereas in Figure 2 groups CO_12 °C, A_12 °C and E_12 °C were mostly separated (20% error).

In Figure 1 the first axis (Can-1) accounted for the 88% of the total variability of the measured variables (cohesiveness, adhesiveness, pH_IR24, tactile tenderness, acidity, a*, b*, resilience and shear stress), whereas the second axis (Can-2) accounted for the 12%. Fifteen out of fifteen samples were correctly classified, with no errors (Table 8). In Figure 2, the first axis (Can-1) account for the 63% of the total variability of the

measured variables (saltiness, juiciness, odour intensity, pH_IR24, acidity, tactile tenderness and adhesiveness), whereas the second axis (Can-2) account for the 36%. One out of fifteen samples was misclassified, involving E treatment which is mixed with A in the cross validation (Table 9).

4. DISCUSSION

4.1 Energy metabolism, freshness indexes, drip losses, pH, colour and texture profile analysis

The rapid depletion of ATP ($T_{RR}0$) (see PART II, PAPER V) and faster loss of freshness (higher K-value) presented by E group at $T_{RR}7$ (10 days *post mortem*), is probably associated with the tetanus and higher level of muscle activity during electrical current exposure (Chiba et al., 1990).K-values similar to those of E and CO groups were found by Erikson, Beyer, & Sigholt (1997) in unstressed (~56%) -individually netted and killed by a blow on the head- and baseline (~ 52%) -first anesthetized and then killed by blow on the head- salmons, 10 days *post mortem* during storage in ice, respectively. Ozogul & Ozogul (2004), reported K-value of ~50% and ~60% (as CO and E groups, respectively) after 10 days of MAP or ice-storage in rainbow trout slaughtered by a blow on the head, respectively. In grass carp slaughtered by electricity, Scherer et al. (2005) found after 10 days of ice storage a K-value (~60%) similar to that of E group.

Electroshock resulted unexpectedly the stunning method less able in preserving freshness of the fillet, with respect to asphyxia in the air, if only considering K-value information. Taking into account some other parameters like cortisol levels, *rigor mortis* evolution, ATP and AEC values immediately *post mortem* (T0) of the three groups of treatments (see PART II, PAPER V), it was likely to be A the worse treatment. In salmonids the K-value seems to increase sharply during the first days of storage before levelling off at about 7 days*post mortem*. However, the variation in reported values for salmonids seems to be large, with K-value after 7 days of storage ranging between 40 and 80% (Erikson et al., 1997). Haitula, Kiesvaara, & Moran (1993) proposed for whitefish (*Coregonus wartmanni*) a K-value upper limit of 80% as criteria for good quality fish, and the same criteria was also used for trout. Considering K-value limits proposed by Erikson et al.(1997), trouts in this study presents an excellent quality range. K_1-values resulted not different among the three groups, but showed a global higher value with respect to K-value, as expected,because

of the increase ATP degradation to IMP over time (Karube et al., 1984). In long terms ($T_{RR}7$ = 10 days *post mortem*), considering both K and K_1-value, freshness results well preserved irrespective of the stunning/slaughtering method applied, and water temperature.

Any significant effect was attributed to stunning/slaughtering method or water temperature for drip losses, measured along the whole study.

Considering the storage period ($T_{RR}0$-$T_{RR}7$), it resulted a significant effect of stunning/slaughtering method and water temperature on pH measured at $T_{RR}0$ and at $T_{RR}7$. At the day of *rigor* resolution ($T_{RR}0$), the pH of fillets of CO group was clearly higher than that of A group, with E fillets at intermediate values. Earlier studies on stress of relatively short duration have demonstrated a faster drop of muscle pH after slaughter and a lower final pH (Sigholt et al., 1997; Thomas, Pankhurst, & Bremner, 1999). This confirmed that probably the higher pH presented by CO group, is due to the pre-slaughter prolonged stress condition suffered,so that the most of the glycogen was consumed before death, resulting in a lower lactate production and pH decrease. At $T_{RR}7$, pH presented a general lowering, because of the natural degradation processes and breakdown products formation, but CO and E groups still reported the highest values. Low water temperature (8 °C) maintained significantly higher the pH at $T_{RR}0$, probably because contributed to reduction in the activity of the enzymes taking part in glycogenolysis and further breakdown of glucose (Skjervold et al., 2001). At the end of the storage, instead, 8 °C-reared fish showed significantly lower pH.

Like the aforementioned parameters, also fillet colour seemed to be influenced by the stunning/slaughtering methods applied in the present study. In rainbow trout(Robb, 2001) the decrease in pH resulted in significantly higher L*, Hue and Chroma values (more yellow and brighter meat), or lower scores in the subjective evaluation by Roche colorimetric cards. As regards the stunning methods, anesthetized rainbow trout showed darker (lower L*) and redder (lower Hue) meat, and lower Chroma than electro-narcotized fish (Robb, Kestin, & Warriss, 2000). In the present study, exposure to CO resulted in a significant increase in redness both at $T_{RR}0$ that $T_{RR}7$ when compared to E, but it was not different from A, even if the value was numerically higher. It is known that CO binds easily to oxymyoglobin/oxyhemoglobin (OMb/OHb), displacing oxygen, producing COMb/COHb that has a cherry red colour. The latters are stable compounds and the degradation to meth-forms MMb/MHb takes

longer time and will thus prevent discoloration. The significant difference in redness at $T_{RR}0$ (4 days *post mortem*) and $T_{RR}7$ is thus mainly due to COMb/COHb production. In Atlantic salmon, herring and mackerel anaesthetized by injecting CO in seawater, a* value was more persistent than in the control group even after 6 days of cold storage (Concollato et al., 2015). A slight increase of a* value was detected by Bjørlykke et al. (2011) both on the fillets that on gills of Atlantic salmon slaughtered by CO compared to control (percussion), similarly to what was found in this study. Furthermore, several other studies show that CO enhances colour and quality of the fish (Chow, Hsieh, Tsai, & Chu, 1998; Hsieh et al., 1998). Yellowness (b*) and Chroma were affected at the end of the storage period ($T_{RR}7$) only, with a general loss in colour in E fillets.

After 7 days of cold storage, as expected, b* value increased in all stunning/slaughtering methods applied, and it was significantly higher in A than E, CO presenting intermediate values. This is likely attributed to both lipid and heme proteins oxidation process: it has been demonstrated that the use of CO can reduce lipid oxidation (Cornforth & Hunt, 2008; Hsieh et al., 1998)even when live fish is exposed to CO (Mantilla et al., 2008). Heme proteins, once oxidized to MHb/MMb, can give a brown-yellowish appearance to the red muscle, thus explaining the increase in yellowness value (Kristinsson and Demir, 2003). It is important to take into account that colour is also dependent on astaxanthin and cantaxanthin amount in the flesh, which depends on its inclusion level in feed stuffs (Nickell and Springate, 2001) and that the high fat content in farmed salmonids causes dilution of astaxanthin and interferes with colour perception (Christiansen et al., 1995), by minimizing treatment differences.

Water temperature affected fillet's chromatic characteristics. At $T_{RR}0$L*, a* and Chroma resulted always higher for rainbow trout reared at 8 °C, but at $T_{RR}7$ only L* value was significantly higher in 8 °C group.Our results support those found by other authors in Arctic charr (*Salvelinus alpinus*).Olsen & Mortensen (1997) found that Arctic charr reared at 8 °C had a stronger fillet pigmentation than fish reared at 12 °C. Later, Ginés, Valdimarsdottir, Sveinsdottir, & Thorarensen (2004) showed that flesh from Arctic charr reared at 10 °C had a more intense red/orange colour than flesh from Arctic charr reared at 15 °C, regardless of the strain.

Regarding texture profile analysis (TPA) parameters, stunning methods affected cohesiveness only; in particular, CO fillets resulted as the less able to fully recover the

original structure during the break between two successive compressions (cohesiveness), behaviour supported by the not significant but higher shear stress and hardness, and the lower resilience and gumminess. As demonstrated by Nakayama (1996) and Roth, Moeller, Veland, Imsland, & Slinde (2002), our results confirmed that *pre mortem* muscle activity (asphyxia) in fish contributes to a softer texture. Water temperature had a major effect than the stunning/slaughtering method on fillet's texture: it seems like that low temperatures favoured a lower shear stress and hardness explaining thus the greater cohesiveness, resilience (which give a measure of the springiness) and gumminess, resulting in lower TPA values.Ginés, Valdimarsdottir, Sveinsdottir, & Thorarensen (2004) found that Arctic charr reared at 15 °C had a lower (7.95 ± 1.75 g) but not significant cohesiveness than that of those reared at 10 °C (8.28 ± 1.60 g). Our studysupports that of Ginés, Valdimarsdottir, Sveinsdottir, & Thorarensen (2004) but it's likely thatit could be the result of low temperature and the double intense stress event (catching and stunning). Due to this unexpected fish response, what impact has water temperature on fillet texture is still unclear. Skjervold et al. (2001) have shown that crowding stress before slaughtering increased firmness of meat, although this effect was not significant ($P<0.057$). From a previous study of Atlantic salmon by Sigholt et al. (1997) it resulted a less firm texture in fish stressed by crowding for less than 1h. The stressed fish in our study (A) probably reduced the glycogen stores before slaughtering, showing a small reduction in *post-rigor* pH, but still significantly lower (6.52) with respect to the other CO (6.57) and E (6.60) groups at $T_{RR}7$ (day before the texture analysis was performed). It seems that pre-slaughter stress affected salmon firmness depending on the severity and duration of stress: short term stress leads to muscle softening, while long term exhaustion leads to increase muscle firmness (Skjervold et al., 2001). This is in accordance with patterns of stress influence in mammal meat (Hedrik et al., 1994).

4.2 Lipid oxidation (TBARS Index)

Results obtained from this study confirmed CO capability in reducing/delaying over time lipid oxidation of the product when compared to the other two-stunning/slaughtering methods. It is known that lipid oxidation is affected by many factors: oxygen, temperature, Mb content, metal catalysts and enzymes, pH, NaCl, etc. Slaughtering methods and *pre mortem* stress had no effects on lipid's oxidation in the study of Huidobro et al. (2014) on gilthead sea bream (*Sparus aurata*) slaughtered

with ice plus water or with liquid ice. In eels (*Anguilla anguilla*), Morzel and Van de Vis (2003) found that lipids were significantly more susceptible to oxidation in fish slaughtered by the commercial method (salt baths) with respect to those by gas combined with electricity. Salt baths cause physical damage to the muscle, thus making cells easily accessible to catalytic enzymes and oxidative substrates. The strong affinity of CO towards Mb, prevents O_2 binding to Mb making difficult its oxidation and the consequent production of superoxide radicals, responsible for the initiation of lipid peroxidation (Cornforth & Hunt, 2008;Hsieh, Chow, Chu, & Chen, 1998). This may explain why the group stunned/slaughtered by using CO presents, even after only 76h from death, a significantly lower MDA content than that of A and E groups, for which instead the process of lipid oxidation seemed to be more intense and rapid. Furthermore, no differences were detected in MDA content among the experimental groups at the end of the storage ($T_{RR}7$, *i.e.* 10 days after death), in agreement with the Mantilla et al. (2008) findings.However, if fish have been treated with CO, the COcontent in their flesh is expected to decline overtime, and this could explain the similar lipid oxidation for the considered treatments. Ishiwata et al. (1996) reported that the increase in CO concentration on extended storage is one of the indicators used by the Japanese health authorities to discriminate fish treated or not with CO.

4.3 Canonical Discriminant Analysis (CDA) of sensory, instrumental texture and physico-chemical measurements

Sensory differences in colour and texture are especially important for consumer appreciation and preference for salmonids (Sylvia et al., 1995). Results from the CDAs relating to sensory analysis, instrumental texture and physico-chemical measurements for the three stunning/slaughtering methods showed that the most predictive variables at lower (8 °C) rearing water temperature were mostly related to texture and colour, whereas for higher (12 °C) temperature to few sensory variables. These findings confirm results obtained from Table 3 and 4. With regard to fillet pigmentation, it has been demonstrated that a reduced food stay in the gut due either to high water temperature or to increased feed intake may affect the digestibility of carotenoids negatively (Ytrestøyl et al., 2005): in Arctic charr, lowering of environmental temperature has been shown to increase pigment deposition (Ginés et al., 2004; Olsen & Mortensen, 1997). Azevedo, Cho, Leeson, & Bureau (1998),

Choubert, Fauconneau, & Luquet (1982) and Elliott(1976)shown that with salmonids the digestibility of dry matter, energy and protein is positively related with temperature, and this aspect could have had partly influenced juiciness, saltiness and odour intensity in trout reared at 12 °C. Cross validation and CDA scattegram approaches resulted as good discriminating tools both at 8 °C that 12 °C.

5. CONCLUSIONS

In long terms ($T_{RR}7$ = 10 days *post mortem*), considering both K and K_1-values, freshness results well preserved irrespective of the stunning/slaughtering method applied, and water temperature. During refrigerated storage fish fillets from CO and E groups showed a higher pH than A group, both at time of *rigor* resolution ($T_{RR}0$), and at the end at the period considered ($T_{RR}7$). CO treatment was effective in ensuring a more intense red colour to the fillet and high Chroma, whereas E treatment exhibited lowest a*, b* and Chroma values.

Texture profile analysis revealed an effect of the stunning/slaughtering (S) method, of the water temperature (T) and an interaction SxT, related to cohesiveness. TBARS value resulted slightly significantly lower in fish stunned by CO, when compared to A and E groups, in the first 76h *post mortem*. At the end of the storage period ($T_{RR}7$), no TBARS differences were detected among treatments. Canonical Discriminant Analysis resulted as an accurate tool in discriminating and classifying the three treatments, at the two considered rearing water temperature.

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Table 1. K and K_1 content in rainbow trout reared at 8 °C or 12 °C and stunned/killed by asphyxia in air (A), asphyxia with CO (CO) or electricity (E).

Time (day)	Parameters	Stunning (S)			Temperature (T)		*P*-value		RSD [(1)]
		A	CO	E	8 °C	12 °C	S	T	
$T_{RR}7$	K_1 (%)	53.22	59.76	63.21	55.65	61.81	NS	NS	7.44
$T_{RR}7$	K (%)	45.50^{b}	50.23^{ab}	57.03^{a}	45.94^{b}	55.90^{a}	<0.05	<0.05	7.16

(1) Residual Standard Deviation

A, B: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.01$ level;
a, b: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.05$ level.
NS: not significant.

Table 2. Cumulative drip losses (DL,%) during storage in refrigerated room (+ 2.5 °C) of rainbow trout fillets belonging to groups exposed to asphyxia (A), CO (CO) or electricity (E). T0 (day of slaughter), $T_{RR}0$ (day of *rigor mortis* resolution), $T_{RR}7$ (last day of storage).

	Days *post mortem*	Stunning (S)			Temperature (T)		*P*-value		RSD[(1)]
		A	CO	E	8 °C	12 °C	S	T	
DL T0-$T_{RR}0$	3	0.8	0.7	0.6	0.6	0.8	NS	NS	0.3
DL $T_{RR}0$-$T_{RR}7$	7	3.3	3.2	2.8	3.3	3	NS	NS	0.7
DL T0-$T_{RR}7$	10	4.1	3.9	3.4	3.8	3.8	NS	NS	0.8

(1) Residual Standard Deviation.
NS: not significant.

Table 3. pH and colour parameters measured during storage in refrigerated room (+2.5 °C), at $T_{RR}0$ (day of the resolution of *rigor mortis*) and $T_{RR}7$ (last day of storage), of farmed rainbow trout reared at two different temperature conditions (8 or 12 °C) and subjected to three different methods of stunning/killing (A, CO or E).

T_{RR}	Parameters	Stunning (S)			Temperature (T)		*P*-value		RSD[(1)]
		A	CO	E	8 °C	12 °C	S	T	
0	pH	6.70^{B}	6.79^{A}	6.76^{AB}	7.0^{a}	6.46^{b}	<0.01	<0.001	0.092
	L*	42.1	42.0	43.0	42.8^{A}	41.9^{B}	NS	<0.01	1.7
	a*	15.2^{AB}	16.3^{A}	14.4^{B}	15.8^{a}	14.9^{b}	<0.01	<0.05	2.1
	b*	14.7	15.0	14.0	15.0	14.2	NS	NS	2.4
	Chroma	21.2	22.1	20.2	21.8^{a}	20.5^{b}	NS	<0.05	3.0
	Hue	44.1	42.9	44.2	43.5	43.7	NS	NS	3.6
7	pH	6.52^{B}	6.57^{A}	6.60^{A}	6.54^{B}	6.58^{A}	<0.001	<0.01	0.070
	L*	41.0	40.4	40.9	41.5^{A}	39.9^{B}	NS	<0.001	1.7
	a*	17.6^{ab}	18.1^{a}	16.6^{b}	17.8	17.2	<0.05	NS	2.0
	b*	18^{a}	17.9^{ab}	16.5^{b}	17.7	17.3	<0.05	NS	2.3
	Chroma	25.3^{ab}	25.5^{a}	23.5^{b}	25.1	24.1	<0.05	NS	2.9
	Hue	45.5	44.6	44.7	44.7	45.2	NS	NS	2.8

(1) Residual Standard Deviation.

A, B: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.01$ level;
a, b: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.05$ level.
NS: not significant.

Table 4. Texture Profile Analysis parameters of rainbow trout fillets belonging to groups reared at 8 or 12 °C, and exposed to asphyxia in air (A), CO (CO) or electricity (E), measured at day 8 after *rigor* resolution ($T_{RR}8$), after the storage in refrigerated conditions (+2.5 °C).

Parameters	Stunning (S)			Temperature (T)		*P*-value		RSD[(1)]
	A	CO	E	8 °C	12 °C	S	T	
Shear Stress (N)	56.17	63.63	57	52.61^{b}	65.25^{a}	NS	<0.05	12.83
Hardness (N)	254.79	287.74	271.97	240.14^{B}	302.86^{A}	NS	<0.001	38.86
Cohesiveness	0.52^{a}	0.41^{b}	0.52^{a}	0.56^{A}	0.41^{B}	<0.01	<0.001	0.07
Resilience	0.11	0.08	0.13	0.13^{a}	0.09^{b}	NS	<0.05	0.04
Gumminess	129.09	115.58	137.96	132.62	122.47	NS	NS	24.17

(1) Residual Standard Deviation.
A, B: Within criterium, means in the same row having different superscripts are significant at $P \leq 0.01$ level;
a, b: Within criterium, means in the same row having different superscripts are significant at $P \leq 0.05$ level
NS: not significant.

Table 5. Malondialdehyde content (MDA) in rainbow trout fillets from groups reared at 8 or 12 °C and exposed to asphyxia in air (A), CO (CO) or electricity (E), stored in cold room (+2.5 °C) and analyzed at $T_{RR}0$ (day of rigor resolution, 76h *post mortem*).

Parameters	Stunning (S)			Temperature (T)		*P*-value		RSD[(1)]
	A	CO	E	8 °C	12 °C	S	T	
MDA (mg/kg)	1.22^{A}	0.66^{B}	1.10^{A}	1.02	0.97	<0.01	NS	0.73

(1) Residual Standard Deviation.
A, B: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.01$ level;
a, b: Within criterion, means in the same row having different superscripts are significant at $P \leq 0.05$ level
NS: not significant.

Table 6. Summary of the most significant variables extracted by the two CDAs (Canonical Discriminant Analysis) for the three-stunning/slaughtering methods applied at 8 °C.

Variables	CDA for the three stunning methods				
	Partial R-Square	F value	Pr < F	Wilks' λ[a]	*P* <λ
Cohesiveness	0.42	3.6	0.066	0.29	0.01**
Adhesiveness	0.37	2.59	0.13	0.18	0.009**
pH_IR24	0.56	5.04	0.04	0.08	0.003**
Tactile Tenderness	0.55	4.9	0.04	0.04	0.0002***
Acidity	0.47	3.17	0.10	0.02	0.0002***
a*	0.58	4.07	0.08	0.008	0.0002***
b*	0.70	5.79	0.05	0.002	0.0001***
Resilience	0.73	5.47	0.07	0.0007	0.0001***
Shear Stress	0.87	10.34	0.05	0.00009	0.0001***

[a]Descriptors are sorted according to their Wilks λ significance.
**: P< 0.01; ***: P< 0.001

Table 7. Summary of the most significant variables extracted by the two CDAs (Canonical Discriminant Analysis) for the three-stunning/slaughtering methods applied at 12 °C.

Variables	CDA for the three stunning methods				
	Partial R-Square	F value	Pr < F	Wilks' λ[a]	*P* <λ
Saltiness	0.60	7.52	0.01	0.40	0.01**
Juiciness	0.48	4.19	0.05	0.21	0.004**
Odour Intensity	0.46	3.44	0.08	0.11	0.0034**
pH_IR24	0.50	3.5	0.09	0.06	0.0024**
Acidity	0.50	3.04	0.12	0.03	0.0024**
Tactile tenderness	0.61	3.87	0.10	0.01	0.002**
Adhesiveness	0.77	6.6	0.05	0.003	0.001**

[a]Descriptors are sorted according to their Wilks λ significance.
**: P< 0.01; ***: P< 0.001

Table 8. Cross validation table of the CDA of rainbow trout fillets according to three stunning/slaughtering methods, at 8 °C.

Treatment	Classified by CDA			Total	Errors (%)
	A	CO	E		
A	5	0	0	5	0
CO	0	5	0	5	0
E	0	0	5	5	0
Total	5	5	5	15	0

Table 9. Cross validation table of the CDA of rainbow trout fillets according to three stunning/slaughtering methods, at 12 °C.

Treatment	Classified by CDA			Total	Errors (%)
	A	CO	E		
A	5	0	0	5	0
CO	0	5	0	5	0
E	1	0	4	5	20
Total	6	5	4	15	6.67

Figure 1. CDA scattergram of the three stunning/slaughtering methods in fish reared at 8 °C. The axes (Can-1 = 88% and Can-2 = 12%) account for the total variability of the measured variables (cohesiveness, adhesiveness, pH_IR24, tactile tenderness, acidity, a*, b*, resilience and shear stress). Ninety-five percent ellipses are drawn around each centroid of groupings in such a way that leaves outside the misclassified animals.

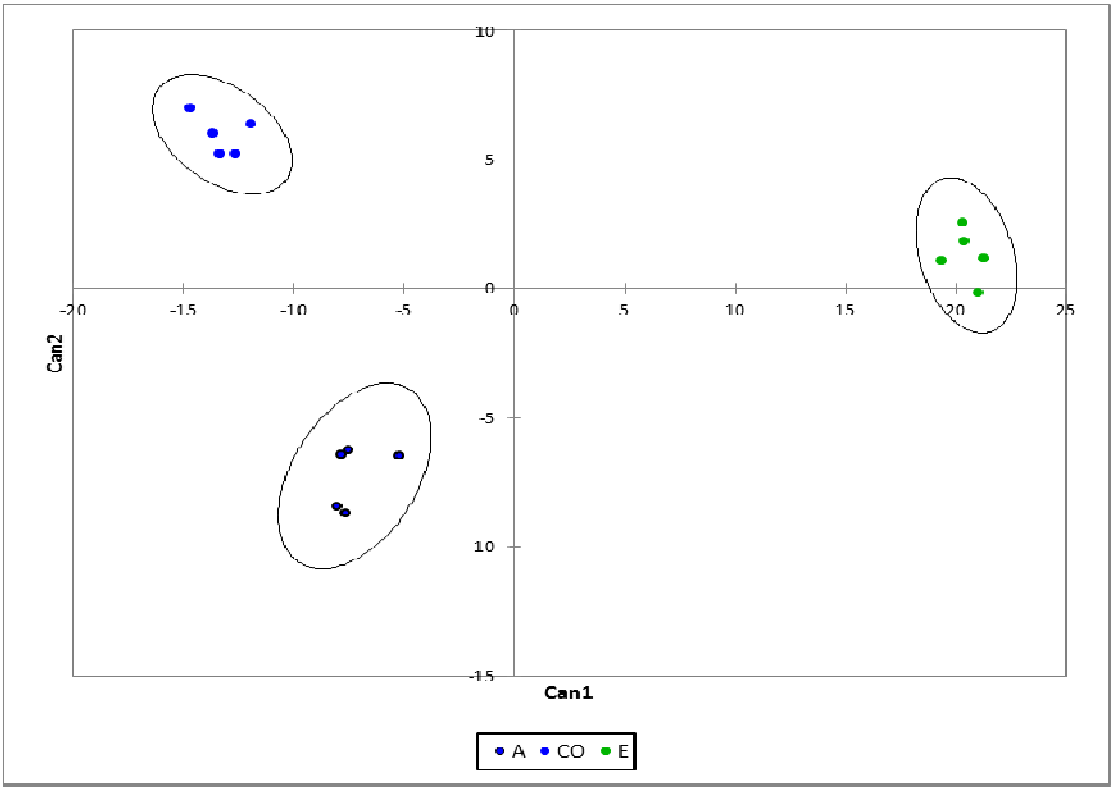
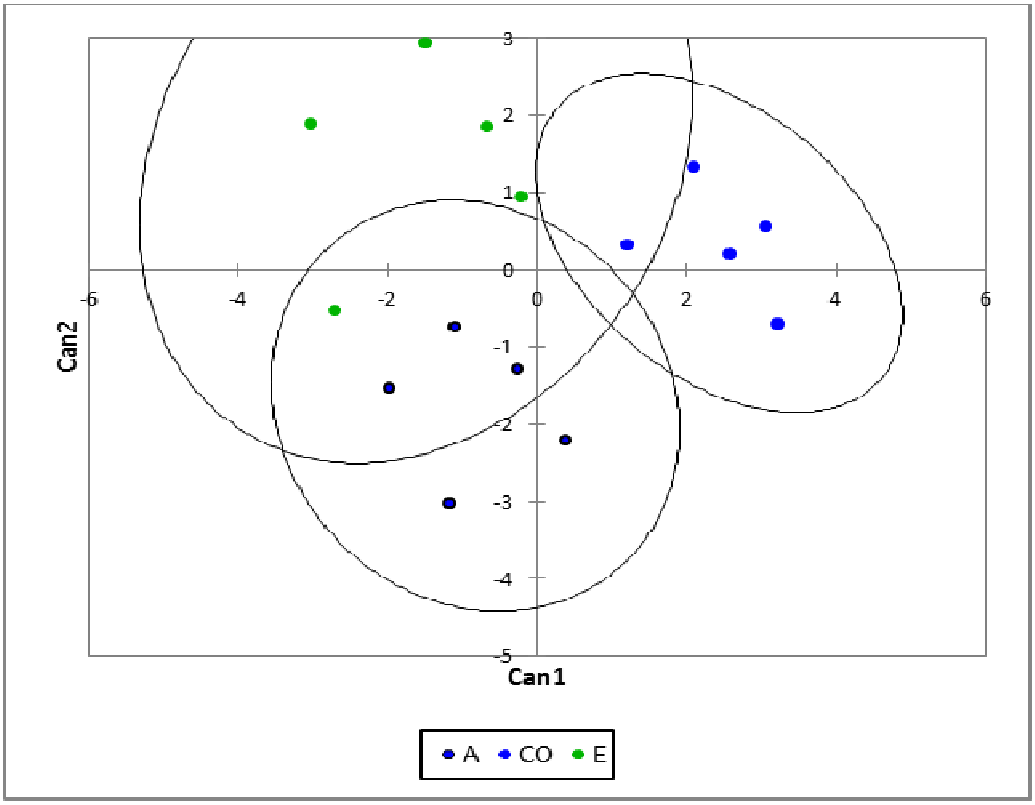


Figure 2. CDA scattergram of the three stunning/slaughtering methods in fish reared at 12 °C. The axes (Can-1 = 63% and Can-2 = 36%) account for the total variability of the measured variables (saltiness, juiciness, odour intensity, pH_IR24, acidity, tactile tenderness and adhesiveness). Ninety-five percent ellipses are drawn around each centroid of groupings in such a way that leaves outside the misclassified animals.



6. CONCLUSIONS

The application of rapid/non-destructive methodologies can successfully discriminate fish fillets and provide information about their quality. Specifically:

- ◊ on rainbow trout NIRS, supported by appropriate chemometric tools, has proven its ability and accuracy in estimating proximate composition, fillet yield, cooking loos and fatty acid profile; and in classifying samples by rearing farm and genetic strain with no relevant differences between raw and cooked freeze-dried fillets in both cases
- ◊ On Atlantic salmon NIRS performed on differently prepared samples and scanned from three different instrumentation resulted the best methodology in discriminating samples and in evaluating the presence and incidence of slaughtering method applied and storage time, respectively, when compared to sensory analysis, NIR, *e*-nose and *e*-tongue methodologies

The study of different stunning/slaughtering methods, demonstrated that is possible to reduce stress condition immediately prior to slaughter and thus improve fillets quality. Specifically:

- ◊ Asphyxia in the air (A) resulted the most stressing method by increasing cortisol secretion three times over the basal levels, resulting in the earlier onset and resolution of *rigor mortis* and most intense fillet shape changes and showing the highest lipid oxidation rate. It must be born in mind that the group reared at 8 °C undergone to a double sampling that for sure influenced the results
- ◊ Carbon monoxide asphyxia (CO) resultedthe most stressing when only compared to percussion slaughtering in Atlantic salmon; whereas was placed in the middle when compared to A and electroshock (E) in rainbow trout. In rainbow trout CO showed its ability in preserving ATP immediately after death, improving red colour of the fillet and in delaying in time lipid oxidation when compared to A and E
- ◊ Electroshock (E), globally, resulted the best stunning/slaughtering method showing the lowest levels of cortisol in the blood, later onset of *rigor mortis* and less intense fillet shape changes,butit did not improve the overall filletcolour.

Research efforts in the field of infrared spectroscopy, sensors and instrumental techniques are addressing some of the challenges of food product measurements, and of the physico-chemical changesnot well understood by using traditional chemistry responsible for modifications of food products'stability. Further studies on CO application as stunning/slaughtering method in fish, considering its positive effects on fillets quality,should include reliable measurements of CO dissolved in the water and possible improvements of delivery systems, so as to minimize stress perception immediately before slaughtering.