Carbon monoxide stunning of Atlantic salmon (*Salmo salar* L.) modifies *rigor mortis* and sensory traits as revealed by NIRS and other instruments

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Abstract

BACKGROUND: Methods of stunning used in salmon slaughter are still the subject of research. Fish quality can be influenced by *pre*, *ante* and *post mortem* conditions, including handling before slaughter, slaughter methods and storage conditions. Carbon monoxide (CO) is known to improve colour stability in red muscle and to reduce microbial growth and lipid oxidation in live fish exposed to CO. Quality differences in Atlantic salmon, *Salmo salar* L., stunned by CO or percussion, were evaluated and compared by different techniques (Near Infrared Reflectance Spectroscopy NIRS, electronic nose EN, electronic tongue ET) and sensory analysis.

RESULTS: Thawed samples, freeze-dried preparates and NIRS devices proved to be the most efficient combinations for discriminating the treatments applied to salmon, i.e. first the stunning methods adopted, then the back-prediction of the maximum time to reach *rigor mortis* and finally to correlate some sensory attributes. A trained panel found significant differences between control and CO-stunned salmon: reduced tactile crumbliness, reduced odour and aroma intensities, and reduced tenderness of CO-treated fillets. CO stunning reduced radiation absorbance in spectra of thawed and freeze-dried fillets, but not fillet samples stored in ethanol where it may have interacted with myoglobin and myosin.

CONCLUSIONS: The good results in a rapid discrimination of thawed samples detected by NIRS suggest suitable applications in the fish industry. CO treatment could mitigate sensory perception, but consumer tests are needed to confirm our findings.

Key words: *Salmo salar* L., carbon monoxide stunning, electronic nose, electronic tongue, NIRS, fillet sample preparation, sensory scores, *rigor mortis*.

INTRODUCTION

Food safety and quality are perceived as an important concept in the EU.[1] Ethical aspects of food production, such as animal welfare and protection, are essential; in this context, retailers,[2] animal welfare associations and governments[3-4] are demanding more humane methods of fish slaughter. Humane slaughter procedures can improve *post mortem* quality of fish, as reported for warm-blooded animals.[5-6] Carbon monoxide (CO) has been successfully used as a fish sedative, avoiding visible stress responses in different species.[7-8] CO is also known for its ability to maintain colour stability of red flesh[9] and reduce microbial growth[10] in live fish exposed to CO.[8] These factors have driven the development of fast, efficient and reliable methods of food quality assessment. The fundamental vibrations of the C-, H-, O- and N- bonds in the infrared (IR) region rebound as overtones in the Near-IR region (800-2500 nm wavelengths). These vibrations may be deconvoluted and correlated with unique vibrations originating in the IR band when the incident radiation strikes organic molecules. Near Infrared Reflectance Spectroscopy (NIRS) capitalizes the overtones and combinations of sample constituents by appropriate chemometric methods. Vibrational spectroscopy in the NIR region is a versatile method with many applications in research, industry, farming, breeding and authentication.[11] NIRS has been used to attain good traceability of raw and cooked freeze-dried rainbow trout fillets.[12] Many at-line and offline methods have been destructive, as they require excision of large or small (4 cm^2) muscle samples for spectroscopy.[13] Although small samples conserve much of the material, they are subject to error due to non-representative sampling and interference of intramuscular fat.

NIRS has been used to discriminate categories and / or study quality traits in cattle meat,[14-16] as well as in chicken.[17] Ethanol preserved samples have been used for further NIRS scans of rabbit,[18] buffalo[19] and cattle [20] meat. The results are promising for discriminating genetic and ontogenic effects. In the meat industry, animal breeding programmes and consumer

expectations for meat-product labelling require fast screening techniques to determine meat quality. The best methods are in-line measurements in real time. In many non-automatic processes, at-line procedures that give results in 3-5 minutes are already available. As a first case, the Iberian pig production line has adopted a portable fast-NIRS device to analyse minced meat samples.[21] The method uses a database and algorithm based on standard laboratory offline methods.[22] Online examination of beef carcasses using Vis-NIR vibrational spectroscopy has been developed in the US for identification of select carcasses on the basis *Longissimus* muscle tenderness.[23]

Electronic noses (EN) exploit the electrochemical properties of low-weight molecules to excite complementary metal-oxide semiconductor sensors. They consist of an array of chemical sensors, each with partial specificity to a wide range of odorant molecules.[24] The signs of the sensory arrays produce flavour "fingerprints", which are then evaluated by chemometric methods.[25] Electronic noses are widely used for foodstuff analysis and show promise for industrial applications. In this particular field, EN can help define the freshness of products with limited shelf-life, such as fish.[26-27] EN has proven to be a valuable technique for product discrimination, classification, quality evaluation and control in the food and beverage industry.[28-30]

Electronic tongues (ET) are established as a rapid and easy-to-use tool for analysis of food quality in liquid media. They are still far from natural taste senses but have shown good correlations with sensory scores assigned by taste panels. Artificial senses are not subjective and can also be used for toxic samples.[31] Moreover, ETs have better sensitivity than the human tongue and can detect substances undetectable by their natural counterparts.[31] They can be used for process monitoring,[32] freshness evaluation, shelf-life investigation,[33] authenticity assessment,[34] foodstuff recognition/characterization,[35] quantitative analysis[36] and other quality control studies.[37-38]

The present paper deals with evaluation and comparison of offline analyses (NIRS, EN, ET and sensory analysis) to detect quality differences in Atlantic salmon, *Salmo salar*, stunned by CO or percussion.

MATERIAL AND METHODS

Fish and samples

Atlantic salmon (*Salmo salar* L.) were farmed at the facilities of the Institute of Marine Research (IMR), Matre, Norway. For the study, 30 salmon with a mean weight of 1.08 ± 0.09 kg were equally and randomly divided into 2 experimental tanks of 900 L seawater and maintained at a constant temperature of 7.3 ± 0.5 °C. Fish in tank 1 were used as controls (group C) and killed by percussion; the water in tank 2 was flushed with 100% food grade carbon monoxide (group CO) (Yara Praxair, Oslo, Norway) using a ceramic diffuser (wedge lock base unit, Point Four Systems Inc., Richmond, Canada) for 20 minutes at 2-3 bar. The CO-treated fish were then removed from the tanks and slaughtered by percussion. For personnel safety, CO concentrations in air were monitored and measured during the experiment with portable gas detectors (GasBadge Pro, Oakdale, PA, USA). The study was submitted to and approved by the Norwegian Animal Research Authority, application No. 4656.

Salmon from the C and CO groups were individually tagged, weighed, packed in polystyrene boxes with ice and stored in a cold-room at 2.5 ± 0.1 °C. Immediately after slaughter, *rigor mortis* was determined on 6 fish/treatment 0, 3, 9, 15, 24, 30, 40, 48 and 64 hours *post mortem* (interval T0-T1).[7] *Rigor mortis* was measured by tail drop and Rigor Index (RI) was calculated with the formula of Bito *et al.*:[39]

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

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 different time intervals. After the *rigor mortis* determinations (64 h *post mortem:* Time1) all fish were gutted, filleted and weighed. Right fillets were immediately vacuum packed in non-toxic polyethylene bags and stored at -20°C, whereas the left ones were stored for 14 days in a non-ventilated cold room at 2.5 ± 0.1 °C (Time2, 14 days) on polystyrene foam food trays carrying absorbent pads wrapped in plastic film. The left fillets were then vacuum packed and stored at -20°C until analysis. All right and left fillets of the 30 salmon (15 controls and 15 CO) were delivered in dry ice to the Department of Animal Medicine, Production and Health, Padova University, Padova (Italy). All right (15 C-64 h; 15 CO-64 h) and left (15 C-14 d; 15 CO-14 d) fillets were divided while frozen into three (R-cranial, R-central and R-caudal) and two (L-cranial and L-central-caudal) parts, respectively. The samples were then shipped in dried ice to Kaposvár University, Hungary (H; R- and L-caudal parts) and Torino (TO; R- and L-cranial parts). R-central parts remained in Padova (PD) for sensory analysis by trained panellists. The Hungarian laboratory required about 8 and 60 g of each sample to perform EN and ET analysis (EN-H; ET-H), respectively. NIRS scans (40 g material for each analysis) were first carried out on thawed material (THAW-H) and then on the same samples after freeze-drying (FD-H). The Hungarian laboratory freeze-dried the thawed samples and, after the NIRS scan, the same samples were NIRS scanned again in the Padova (FD-PD) and Torino (FD-TO) laboratories, in order to compare the prediction capacity of the three instruments. For sensory analysis, only thawed samples (C-64 h and CO-64 h) were tested using about 50 g from each thawed fillet. When the 60 fillet samples (C-64 h, C-14 d, CO-64 h, CO-14 d) arrived at the Hungarian lab, they were stored overnight in a chilled room at 4 ± 2 °C to ensure a slow and proper thawing process. The following morning, they were skinned, weighed (average weight 111.14 ± 21.51 g) and homogenized in a WARING 800 EG blender (Waring, Torrington, CT, USA). About 8 g was used for EN analysis. Approximately 60 g was sealed in bags and cooked in water at 75°C for 20 min prior to ET analysis. The rest of the homogenate (thawed

matrix) was used for the NIRS scan and then freeze-dried before the second NIRS analysis. When cranial samples arrived in Torino, they were thawed and 4 g was transferred to a 25 ml tube which was filled with 95% commercial ethanol solution. The samples were stored in the dark at 4°C for 24 h. Ethanol treatment causes rapid coagulation of muscle protein and substitution of tissue water with ethanol. The flesh acquires the appearance of cooked fish. NIRS scan of the specimens (ETOH-TO) was preceded by evaporation of the ethanol (1h) at room temperature in order to intensify the vibrational response of the salmon matrices.

Electronic nose

An αFox4000 (Alpha-MOS, Toulouse, France) EN with 18 metal oxide semiconductors (MOS) was used. Adsorption of volatile compounds onto the MOS surface generates a change in 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 via RS-232 ports. The EN sensor values of thawed salmon were saved as relative changes in resistance ($\Delta R/R_0$). According to the applied static headspace (HS) technique, samples were placed in 10-ml hermetically sealed vials. After equilibrium had established between the matrix and the gaseous phase, an ALPHA MOS HS 100 auto sampler was used to sample the HS. Synthetic air was used as permanent airflow. The acquisition time and time between subsequent analyses were 120 and 1080 s, respectively. Four parallel measurements were performed (no. = 4 x 4). Development of EN method indicated that the following parameters give acceptable signal intensity values: sample quantity 2 g, sample temperature 60°C, equilibration time 180 s, injection volume 3000 µl and flow rate 150 ml/min.

Electronic tongue

An αAstree II (Alpha-MOS, Toulouse, France) ET with a LS 48 auto sampler unit was used to measure the characteristics of liquid samples. The equipment consisted of an array of seven

cross-selective chemical modified field effect transistors (CHEMFET) based on 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 via RS-232 ports. The basic ET parameters were formed by averaging intensity values when sensors were in equilibrium. Three grams of each sample was diluted 1:20 in Millipore water, homogenized, centrifuged (12,000 rpm for 5 min) and filtered (Sartorius Stedim Biotech, grade: 1289, diameter: 125 mm). Finally, seven sub-samples from each treatment group were obtained, making a total of 28 ET measurements. Samples were placed in seven 25-ml glass tubes in which the chemical sensors, the reference electrode and a stirrer were inserted. Seven parallel measurements were performed (no. = 4 x 7). 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.

NIRS

Sixty thawed homogenized and freeze-dried fillet samples were scanned in four replicates at the Hungarian laboratory. NIRS spectra were collected in reflectance mode using a NIRSystems 6500 spectrometer (FOSS NIRSystems Inc., Silver Spring, MD, USA) equipped with a sample transport module and a small ring cup cuvette (IH-0307). Reflectance spectra were recorded from the 1100 to 2500 nm region and entered as log(1/R) at 2 nm intervals using WinISI II version 1.04 spectral analytical software (InfraSoft International LLC, State College, PA, USA). At the Padova laboratories, a FOSS NIRSystems 5000 (FOSS NIRSystem, Silver Spring, MD, USA) was used, and spectra were recorded in the same manner. At the Torino laboratory, vibrational examinations were conducted using a portable Model LabSpec® 4 spectrophotometer (ASD; Analytical Spectral Devices Inc., Boulder, CO, USA), equipped to collect four replicate spectra from 350 to 1250 nm.

Sensory analysis

For sensory analysis, the descriptive method was used to detect possible differences between the two stunning methods (Stun), C and CO. A total of 30 samples from the central part of the right fillets (15 from C-64 h, 15 from CO-64 h) were used. Each sample (with skin) was placed in an aluminium tray and cooked in a ventilated electric oven preheated to 200°C. Cooking was terminated when the internal temperature of the sample reached 75°C. The trays were then placed in an incubator at 50°C and served to the panel in random sequence. The trial involved 12 trained panellists, 5 women and 7 men, 30-58 years of age (mean 48 years). They were qualified as experts according to ISO 8586 and had experience with descriptive tests (ISO 13299) on various food matrices. They signed written informed consent before analysis. All judges who perform tests with accredited methods (ISO 13299, ISO 4120, ISO 8587) undergo training every three years. They underwent four pre-test training sessions of two hours each, in order to familiarize with the matrix and select appropriate descriptors, also drawn from the literature. They scored relative perceived intensity on a scale (Table 2). In two sessions, the panel received a list of descriptors to score on numerical and continuous scales from 0 to 10. The values of the reference sample used in evaluation of the study samples were extracted in this way.

Olfactory, tactile, gustative and textural aspects were evaluated. The descriptors were: overall odour (ortho-nasal perception) and aroma (retro-nasal perception) intensity (olfactory descriptors), crumbliness and tenderness (tactile descriptors), saltiness and sourness (taste descriptors), adhesiveness, fibrousness and tenderness (textural descriptors).

The panel was trained with fresh purchased salmon, portioned into pieces individually vacuum packed and stored in the freezer at a constant temperature of about -18°C. The evaluation sheet, distribution of samples to the judges and data acquisition were performed using FIZZ software (Biosystemes - France) installed in 12 terminals in the tasting booths of

the laboratory. For evaluation of the treatments, the purchased sample was the reference standard, and an arbitrary score was assigned for each descriptor: this sample was cooked together with the experimental samples. After cooking, each experimental fillet was divided into two parts of about 50 g each, in order to have two replicates. Each panellist was given three samples: the reference sample, the C sample and the CO sample, in sequence. The reference sample was evaluated first, to memorize the sensory perceptions of the standard sample as a benchmark for the experimental samples. The order of the C and CO samples was alternated. Sensory analysis was conducted over 3 days. During evaluation, the panellists ate unsalted crackers and drank natural water to neutralize any residual sensation between one sample and the next.

Statistical analysis

All the digital signals produced by the instruments, which were recorded in several native formats by specific software, were then imported into WinISI II version 1.04 for chemometric processing. The replicate spectra were averaged before any chemometric processing. Bi-factorial design with two levels produced four groups, which were considered dummy values (1-4) and compared with each other to build a distance matrix. The calibration process was performed in WinISI 1.04 using Modified Partial Least Squares (MPLS), with mathematical pre-treatment of standardization and 1st or 2nd derivatives to reduce noise and scattering effects, providing a cross-validation system to assess the optimal number of latent variables to enter in the equations, with one passage to eliminate outliers ($t>2$; $H>10$). The prediction capacity of the calibrated models was then evaluated with the 1-VR cross-validation parameter, where VR is the ratio of unexplained variance, which is routinely used by WinISI users and researchers,[40] and with the Relative Prediction Deviation (RPD)[41] that encapsulates the reliability of the predictive equation with new samples: values of 2.0 are considered useful for practical applications. The 1-VR coefficients did not give reclassification percentages, but the

fit to a linear regression model. A different approach, namely PLS-DA (Discriminant Analysis), supported by WinISI software, was applied to each set of "instrument-preparations" to classify observations into four subgroups. This directly linked the two methods, i.e. discriminant and cross-validated regression. Ward's Hierarchical Clustering Analysis (HCA) was performed via StatBox software version 6.5 (Grimmer Logiciel, Paris, France) on the distance matrix to compare the relative average dissimilarity patterns. HCA performs agglomerate hierarchical clustering of average objects based on distance measures of dissimilarity or similarity. In order to obtain objective judgments about the efficiency of instruments and preparations, a nonparametric paired Friedman test provided by the StatBox software was used, using the variables as keys to pair the observations. The observed value of Kruskal-Wallis H is distributed as a chi² (df = 1); since this test is one-sided, the *P*-value is compared at the significance limit: alpha = 0.05.

Rigor mortis test

For each salmon, a parabolic curve was fitted to establish the time of maximum *rigor*, in hours. A nonparametric Friedman test for independent samples was then applied to test for any differences between control and CO-treatment maximum *rigor mortis* times.

Panel test analysis

The 12 panellists were regarded as random effects in a mixed model (PROC MIXED by SAS/STAT software, release 9.1. Cary, NC: SAS Institute Inc. 2007). The fixed factor was Stun. The scores on fillet samples were considered for stun effect processing in a linear model. To fit the spectra (ET, EN, NIRS), the sensory scores were standardized as follows:

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

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

where C and CO are the scores estimated for C and CO salmon by the i^{th} panellist; S_c and S_co are standardized scores for C and CO salmon by the i^{th} panellist.

Correlation of the spectra with the rigor mortis and sensory variables

It is important to bear in mind that a spectrum is specifically linked to a sample, which was not analysed for *rigor mortis*; in fact the analyses were performed in other equivalent salmon of the same subgroup. It was therefore possible to assign every value pertinent to that subgroup to a spectrum, which implies multiplication of the spectral set as many times as the number of recorded cases of *rigor mortis* (i.e. 6 times). Similar reasoning holds for the sensory tests, where the spectral samples were multiplied by 12, i.e. the number of panellists. With reference to the panellist case, the first subset was coupled to the set-scores of the first panellist, the second subset of spectra was coupled to the second panellist's set-scores and merged below the previous one, and so on until the twelfth. Similarly, each of the 6 *rigor mortis* scores was applied to all the spectra recorded in the companion salmon, so the data set was multiplied by 6. The 1-VR coefficients of the regression equations calculated by the calibration and cross-validation software were considered in order to compare the different experimental factors (Stun and Time) as detected by the different devices (ET, EN, NIRS) as well as to perform a correlation study, and to feature the predictability of the sensory scores and the biological variable *rigor mortis*.

Comparison of the 1-VR coefficients in the fused all-spectra set, or separately for the two storage times, and ranking of instruments

If the spectra recorded at 64 h were different from the spectra recorded at 14 days, a single relationship between the two kinds of spectra and the measured values was unlikely. To

ascertain whether a Stun x Time interaction emerged, we compared the 1-VR values featured by two separate regressions in subgroups; for this purpose the z-score obtained by the Fisher transformation according to Preacher[42] was used to detect differences, with two-sided alpha <0.05 as limit. We also endeavoured to compare the different devices in the fused spectra and/or separately in the two Time categories. To do so, we considered the nonparametric Friedman tests (StatBox 1.5, Grimmer Logiciels, Paris, France) appropriate, using the groups and subgroups as key for coupling the observations; the observed value of Kruskal-Wallis H is distributed as a chi² (df = 1); since this test is one-sided, the *P*-value is compared at the limit of significance : alpha = 0.05.

Comparison of the 1-VR coefficients for the different sensory variables

By a similar process, it is possible to rank the nine sensory variables according their average 1-VR values detected with the different devices. In this case we compared each pair of the nine variables in order to rank the 1-VR coefficients. The z-score obtained by Fisher transformation was used as already described.

RESULTS AND DISCUSSION

Table 1 summarises the sample codes used for stunning, storage time, analytical techniques/instruments and laboratories.

Time and Stun factors as related to instrument-preparations

As an overall pattern, inclusive of all instruments and preparations, the Time factor was a greater source of variation for instrumental discrimination than the Stun factor. The average 1-VR coefficients are shown in Table 3: 0.84r *vs.* 0.49s values correspond to average RPD coefficients of 3.3 *vs.* 1.8, respectively. The two effects apparently did not interact because the 1-VR coefficients of the two Stun conditions are similar at 64 h and at 14 days (0.56t *vs.*

0.52t). However, there was an interaction for NIRS analyses of FD samples between Time and Stun. According to the Hungarian and Torino labs, the Stun effect was maximum after storage (14 days) while the Padova lab found a higher Stun effect before storage. Part of the low sensitivity appeared to be due to ET and EN. Using only NIRS, good separation of the Stun methods was obtained, and the effect of CO stunning emerged clearly in THAW (1-VR 0.96 at 64 h and 0.66 at 14 d) and freeze-dried samples in the Padova (0.94 and 0.89), Hungarian (0.71 and 0.81) and Torino labs (0.57 and 0.85). By contrast, Stun was only mildly perceived in ethanol-treated samples (0.49 and 0.37).

As regards sample preparation (Table 3), the maximum average efficiency was attained by the freeze-dried preparation examined in the Padova lab (average 1-VR = 0.90a), which was similar to the Torino result (0.85ab) but higher than the result of the Hungarian lab (0.84b). The THAW examination by the Hungarian lab was also at top level (0.87ab). The ethanol specimens (0.47c) and the EN instrument (0.57c) showed lower efficiency, and ET emerged as the least efficient instrument (0.18d). For the EN examination, it is important to note that the Stun effect was perceived better at Time 64 h (0.35t) than at 14 days (0.05u).

Figure 1 shows the average NIR spectrum (as Log (1/R)) of THAW, ETOH and FD specimens. A flat curve means low absorbance caused by high reflection of the signal. Obviously the thawed flesh has high absorbance due to water, giving a rising curve. The curves of FD specimens were flatter due to their very low water content. Note that the curves from the two FOSS instruments are almost superimposed. In the minor Vis-NIR band of the ASD instrument (Torino) the curves of ethanol-treated specimens were also higher than FD, because of the inherent ethanol-water absorbance of the signal.

With regard to the Stun effect, Figure 2 shows the percentage difference between the average CO and C spectra of all the preparations and measuring systems. In freeze-dried samples, this difference was negative because CO in stunned fillets absorbed less radiation in all ranges.

For THAW fillets these differences were slightly negative, whereas they were strong and positive for the ethanol preparations (average 0.49 *vs.* 0.43 = +9%).

In Figure 3, the second derivative of the THAW mean spectrum enhanced maximum absorbance at 1376-1408 and 1858-1892 nm: these are spectral bands associated with O-H stretch first overtone and O-H stretch second overtone, related to the water content of the samples.[43] However in THAW samples the correlation between the Stun and Time effects were not restricted to water properties but extended to the whole spectrum, especially the visible range. Figure 4 shows the highest correlations at 614-orange and 660-red for Stun effect and 542-green and 564-yellow for Time effects. The utility of visible spectroscopy for predicting oxidation of meat was confirmed by Cifuni *et al.*:[44] wavelengths from 380 to 600 nm were generally 82% accurate in discriminating daily ageing of rabbit meat, and this correlated with the TBARS trend.

In the present study, we observed several modifications in absorbance (Log 1/R) of the Vis-NIR spectra, which involved the visual appearance of the samples. The R and L* (sample lightness) parameters are related by a mathematical relationship: L* = -54.9 x R +91.6.[45] When the fillets were immersed in ethanol, protein coagulation gave them a cooked appearance, usually characterised by a decrease in L*. The absorbance of Vis-NIR radiation in these ethanol-treated samples increased sharply. Moreover, the Stun factor presumably modified tissue properties to Vis-NIR examination in both fresh and stored samples. This could mean that the effect was perceived equally at both time points, however it is not clear whether the effects were the same. Since the spectra at the two Times had the same discrimination after fusion, the answer is yes. In fact, the three PLS equations were homogeneous for each preparation and instrument, and involved similar radiation bands. Previous results by Mantilla *et al.*[8] showed that use of CO to improve fillet colour increased redness (a*) and lightness (L*) of white and red muscle of tilapia. Here we did not perform direct colour analysis, but the absorption spectra confirmed the previous study. Furthermore,

in the THAW and FD samples, CO Stun reduced absorbance, improving lightness. For the ethanol preparations, the absorbance was much higher in CO Stun samples, probably due to interaction of the CO gas with myoglobin and myosin. For some reason, ethanol appeared to intensify certain chromophobic properties of CO. It is interesting that NIRS of ETOH-TO specimens had lower predictability of Stun at 64 h than at 14 days. This was probably because the ethanol had less impact on fresh proteins (64 h). This is contrary to our observations with ET and EN, which reacted positively to fresh proteins. In general NIRS on FD samples was optimal, giving the best resolution of the groups. One reason is probably its greater sensitivity than ET and EN. The latter methods are based on an overall selectivity concept and only recognize a limited number of molecules.[25] Secondly, since removal of water through freeze-drying increases analytical accuracy, it is now the gold standard for various types of analysis.[25]

When looking at the high and very high 1-VR values for the Time factor, it may be argued that all the instruments detected storage time with a cross-validated error of much less than one day. In fact, the EN olfactometric method was used by Limbo *et al.*[46] to evaluate the freshness of European sea bass and was considered equivalent to the Thiobarbituric Acid Complex (TBA) and the Total Volatile Base-N (TVB-N) methods.

Distance matrix and PLS-DA

As shown in Table 3 the average distance matrices of THAW and FD preparations had very high 1-VR values (≥0.84); the ethanol preparation and the EN had a 1-VR of about 0.50, whereas the ET had a very low average distance matrix (0.18).

As highlighted in Figure 3, in four of the seven instrument-preparations the Time effect prevailed over Stun; interestingly, in the THAW like fresh samples NIRS showed an exaggerated Stun effect, and a similar result was found by the Padova lab for FD samples at both storage Times. In the ETOH samples the Time and Stun effects were interactive.

Table 4 shows the full reclassification square. The FD preparations examined in Hungary and Padova gave maximum efficiency (95% and 93%, respectively), the FD results from Torino and the THAW results from Hungary were less efficient (85%), followed by the EN (60%), with ETOH and ET below 50%. Pairing of the seven group records made it possible to calculate a relationship between the 1-VR coefficients and the reclassification percentage. As shown in Figure 4, the reclassification rate may be estimated as 0.98 + 0.78 * Log (1-VR), with R^2 = 0.81. The distance matrix and PLS-DA confirmed the findings reported above. NIRS scanning of THAW and FD samples gave the best separation of Time and Stun, Time being the most prominent factor. The strong relationship between 1-VR coefficients and PLS-DA indicates that the two criteria gave very similar results. In both cases NIRS on THAW and FD specimens (all laboratories) gave optimal separation of samples (Stun and Time).

Rigor mortis effects in the spectra

As highlighted in Table 5, the CO-Stun method had significant effects on the time to reach maximum *rigor mortis*: in fact onset was much earlier (9 h instead of 26 h) in CO-treated salmon, and individual response diversified (increase in standard deviation from 2.53 to 7.52 h). The unpaired Friedman test gave P = 0.0037. If we consider RI% values, the relationship with the Stun factor became weak (R^2 = 0.04 instead of 0.74; Table 5). In fact, CO treatment primarily affected the trend to time of maximum Rigor Index, with great individual differences in angular performance. The correlation of *rigor* values with the combined results of instrumental analysis is shown in Table 6, last three columns. Note that average 1-VR is quite high (0.61) and in four cases surpasses the 0.74 limit of discrimination based on the real maximum *rigor mortis* time. The Time factor in general did not affect back-prediction of *rigor mortis*. NIRS (all instruments except Padova) gave best back-prediction of actual fillet condition using FD, THAW and ETOH preparations (a, a, a for All spectra, and Times 64 h and 14 d, respectively). There was one exception: NIRS at the Padova lab detected less *rigor*

mortis effect than NIRS at the other two labs, when the spectra of the FD preparation at the two Times were fused (0.59b, 0.76a, 0.75a), hence the above-mentioned interaction Stun x Time. The EN had poor discrimination compared to NIRS for FD, THAW and ETOH preparations (0.47c, 0.63b, 0.47b), and the discrimination results for ET were even less efficient (0d, 0.43c, 0.25c). In conclusion, in spite of different preparations and different NIRS instruments, the effect of early onset of *rigor mortis* in fillets was predicted retrospectively in equivalent tissues, examined after different storage Times. It is noteworthy that the Time factor itself was also very apparent in the spectral composition (see previous results), but with a different vibrational signature.

When fish are killed, creatine phosphate is degraded before the breakdown of ATP.[47] When creatine phosphate and ATP reach similar concentrations, ATP content decreases and *rigor mortis* develops until full *rigor mortis* is reached with ATP at about 1 μmol/g. *Rigor mortis* occurs when myofibril cross-bridge cycling of myosin and actin ceases, and permanent links are formed. *Rigor mortis* slackens after some time. Possible causes of *post mortem* tenderization include weakening of myofibril Z-discs,[48] degradation of connective tissue and weakening of myosin-actin junctions.[49] Carbon monoxide caused early onset of *rigor mortis* and was efficiently back-predicted by NIRS of all exposed fish at both storage times. Rapid onset of *rigor mortis* is closely 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.[50] To our knowledge, there have been no previous studies in fish using NIRS to back-predict time to maximum *rigor mortis*. According to Lomiwes *et al*.,[51] NIRS is unsuitable as an online method to quantify glycogen in beef ($R^2 = 0.23$) and to predict ultimate pH (pH_u) ($R^2 = 0.20$) of *pre-rigor* in the *Longissimus dorsi* muscle. Notably in our trial, storage Time itself was a component in the spectra and the spectral signature of this factor generally did not interact with the Stun effect. In Atlantic salmon stunned/slaughtered with carbon dioxide (CO_2), electricity or percussion, *pre-mortem* stress during CO_2 application resulted in earlier

onset and resolution of *rigor* mortis,[52] as happened for the CO-treated group in the present study, because CO completely depletes ATP production, causing early onset of *rigor mortis*.

Sensory Test and predictability

As reported in Table 7, the significant consequences of CO treatment were reduction of aroma (-8%), odour (-10%), tactile-crumbliness (-13%) and tenderness (-15%), and enhancement of saltiness (+14%), although for this last parameter the effect was borderline significant (P = 0.0546). The reductions are not necessarily negative: acceptance trials with consumers are needed to determine whether these aspects can be reconciled with consumer sensitivity and expectations.

Table 8 shows the correlation between sensory scores and the different types of analysis. In general, many 1-VR coefficients were statistically significant, indicating an indirect relationship with the traits in the spectra. The tenderness score (Average 1-VR = 0.45a) and saltiness (0.41a) had the highest correlation score of all measurements. Odour intensity (0.38b) and tactile tenderness (0.36c) were quite similar. Lower correlations were found for tactile crumbliness (0.27d), aroma intensity (0.20e) and adhesiveness (0.17e). No correlation was established for sourness or fibrousness. Results on the ability of the different instruments to collimate with the panel can be found in the last three columns of Table 8. Note that average 1-VR is poor because it is derived from some insignificant sensory variables i.e. not affected by the Stun effect. The FD and THAW preparations gave the best fit. We observed general concordance of the spectral signature of the specimens with appreciation expressed by the panel for certain sensory traits. Not only rheological, but also flavour and taste properties are involved in this vibrational characterization. In the present study, CO-treated fish developed *rigor mortis* earlier and it was not followed by softening but rather by hardening of tissues, presumably related to lengthy (20 min) exposure to CO anaesthesia. Pre-slaughtering stress affected salmon firmness in relation to the severity and duration of stress: short-term

stress led to muscle softening, whereas long-term exhaustion led to increased muscle firmness, and CO did not cause the suffering of asphyxiation.[52] This is in line with reports on the influence of stress on mammal meat.[53] Moreover, CO seemed to reduce tactile crumbliness, probably due to higher drip loss during storage.[10,52] Odour and aroma intensity were less strong in the C group, maybe because CO delayed catabolic degradation processes.

In veal, sample preparation methods and NIRS methodology to predict sensory scores of two veal genotypes fed on different diets were first investigated by Brugiapaglia *et al.*[55] Three preparations of *longissimus thoracis* samples (thawed, ethanol-prepared and freeze-dried) were studied. The distance matrices reached different 1-VR levels: 0.65 (thawed and ethanol-treated samples), 0.42 (freeze-dried), and the panel was similarly distinctive (0.62). Prediction of panel scores was maximum for flavour (0.68) and texture (0.68), results appearing better than those of Andrés *et al.*[56] in lamb meat samples (1-VR 0.05 ÷ 0.29) in taste panel that increased to 0.10÷0.55 after exclusion of 78% of samples of intermediate tenderness.

It was concluded that NIRS scan of THAW samples anticipates results achieved by a wide set of laboratory analyses. NIRS analysis of ETOH samples was strongly predictive of panel scores. Regarding the ability of the instruments to collimate with the panel, FD and THAW preparations achieved the highest fit with all the different NIRS devices.

CONCLUSIONS

This study on Atlantic salmon fillets evaluated and compared the ability of near infrared reflectance spectroscopy, electronic nose and electronic tongue to detect the effects of two stunning methods (percussion and carbon monoxide). The observations made at two storage times (64 h and 14 days *post-mortem*) led to an increased response variability, but the Time and Stun factors were both clearly identified. Carbon monoxide stunning reduced radiation absorbance in spectra of thawed and freeze-dried fillets, but not in fillets stored in ethanol; the latter may have interacted with myoglobin and myosin. Stunning with CO was associated

with early onset of *rigor mortis* and some final aspects such as reduction of aroma and odour intensity (-8% and -10%, respectively), reduction in tactile-crumbliness (-13%) and tenderness (-15%), and a near significant increase in saltiness (+14%). CO treatment could cause sensory abatement, but acceptance trials are needed to confirm this trend and explain the sign of the change. Freeze-dried preparation and NIRS devices proved to be the combinations that best discriminated the treatments applied to Atlantic salmon, and also best back-predicted the maximum time to reach *rigor mortis*, as well as several sensory attributes. The good results achieved by thawed samples using NIRS indicate their suitability for rapid discrimination, until innovations in NIRS provide a common sustainable application layer.

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Fig 1. Average Vis-NIR spectrum (Log (1/R)) of thawed, ethanol-treated and freeze-dried preparates.

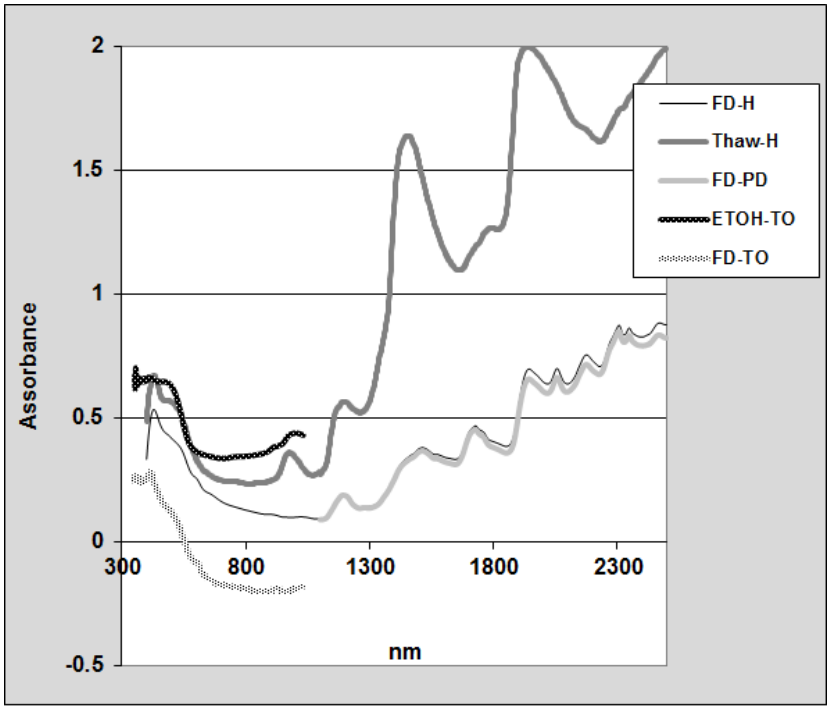

Fig 2. Percentage prevalence of average Vis-NIR spectra of Stun CO spectra on Control C spectra of Thawed, Ethanol and Freeze-dried preparates (CO/C-1).

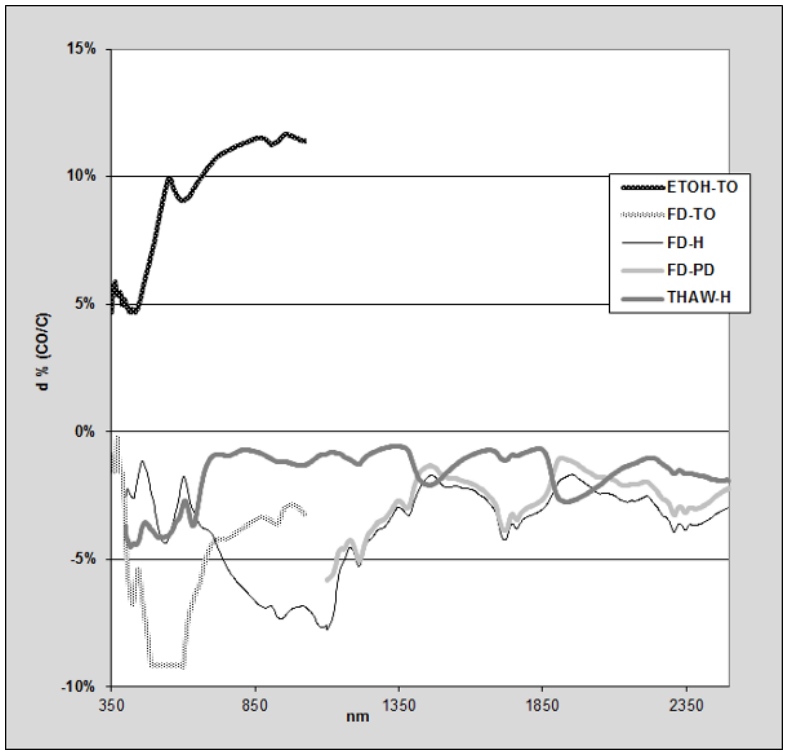

Fig 3. Second derivative of THAW mean spectrum. Ellipses indicate maximum absorbance at 1376-1408 and at 1858-1892 nm.

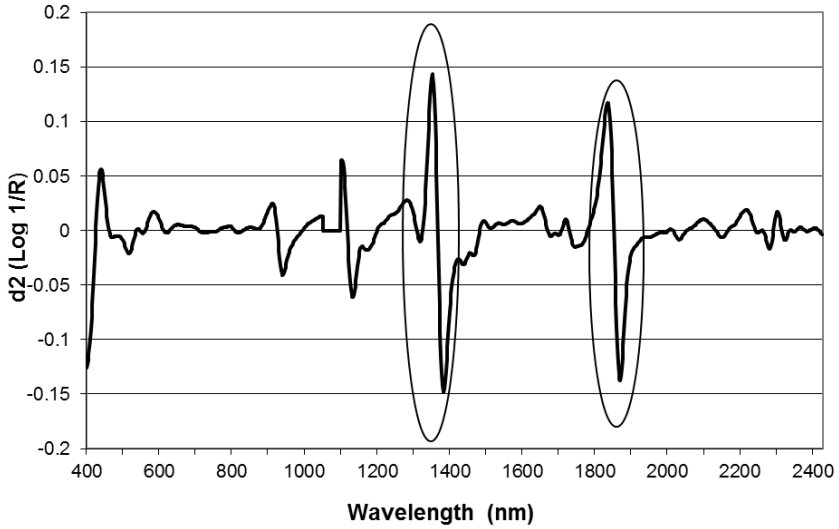

Fig 4. Correlation coefficients of the second derivative of THAW spectra with effects of Stun (C vs. CO) and Storage Time (64h vs. 14d) (right axis). Maximum correlations at 614 and 660 nm (Stun) and at 542 and 564 nm, respectively.

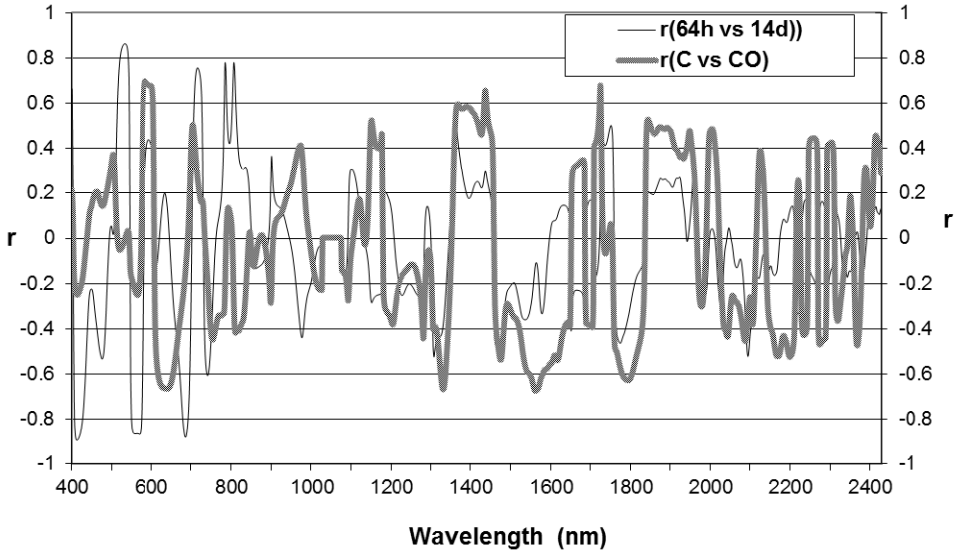

Fig 5. Cluster of four groups (C-64h, C-14d, CO-64h, CO-14d) based on distance matrix of mean spectra in cross-validation mode for the seven instrument-preparation samples (ET-H, EN-H, NIRS-FD-H, NIRS-FD-PD, NIRS-FD-TO, NIRS-THAW-H, NIRS-ETOH-TO). Circles and parallelograms highlight time effect and stun effect, respectively; hexagon shows interaction effects.

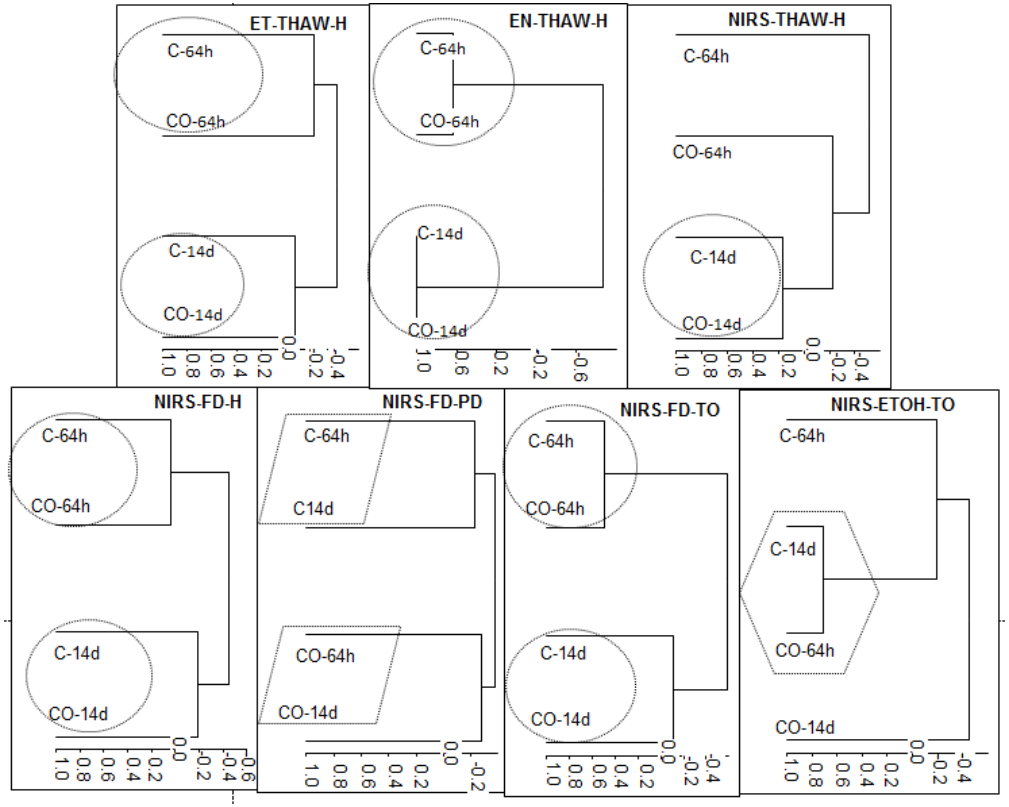

Table 1

Sample codes for stunning, storage time, analytical techniques/instruments and laboratories.

Factors	Description of levels	Codes	Norway	Hungary	Padova	Torino
Stunning method	Control	C	Execution			
	CO	CO				
Storage time	Time 1	64 h				
	Time 2	14 days				
Sample preparations	Freeze dried	FD		NIRS-FD-H	NIRS-FD-PD	NIRS-FD-TO
	Thawed	THAW		NIRS-THAW-H		
	Ethanol	ETOH				NIRS-ETOH-TO
Analytical techniques/instruments	Vibrational spectroscopy	NIRS				
	Electronic nose	EN		EN-THAW-H		
	Electronic tongue	ET		ET-THAW-H		
Research Unit	Norway		Rigor mortis examination			
	Hungary	H				
	Padova	PD			Sensory analysis	
	Torino	TO				

NIRS-H: 400-2498 nm, 1049 digits; instrument: FOSS 6500, Hungary
NIRS-PD: 1100-2492 nm, 700 digits; instrument: FOSS 5000, Padova
NIRS-TO: 350-1025 nm, 1049 digits; portable LabSpec 4 Standard-Res Lab UV-Vis-NIR Analyzer fiber optic diode array spectrophotometer, Torino.

Table 2

Panel test: Linear and continuous descriptors and definitions.

Sensory trait	Descriptors	Definitions
Olfactory (ortho/retro-nasal)	Global odor intensity	
	Global aroma intensity	
Tactile	Crumbliness	Degree of friability to touch
	Tenderness	Degree of tenderness to touch
Taste	Saltiness	Intensity of salty taste
	Sourness	Intensity of sour taste
Texture (in the mouth)	Adhesiveness	Adhesiveness of salmon to first few bites
	Fibrousness	Fibrousness of salmon to first few bites
	Tenderness	Tenderness of salmon to first few bites

Table 3

Chemometric analysis of the two main factors: stunning method (Stun) and storage time (Time) and their combinations for the seven instruments and preparations, according to the average (Avg.) values of 1-VR in cross-validation. Paired Friedman test compares the instruments (a>b>c>d) and Fisher's test compares the two main factor effects (r>s) and their combinations (Stun x Time) (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	0.49s	1.8
		Time	0.493	r	0.799	r	0.942	r	0.901	r	0.930	r	0.973	r	0.805	r	0.84r	3.3
		Group	0.000		0.596		0.845		0.787		0.825		0.749		0.236			
1_C_64 h	2_C_14 d	_12	0.310		0.662		0.944		0.844		0.865		0.968		0.663			
	3_CO_64 h	_13	0.000	t	0.354	t	0.959	t	0.717	u	0.937	t	0.565	u	0.402	t	0.56t	2.3
	4_CO_14 d	_14	0.000		0.596		0.945		0.946		0.946		0.983		0.804			
2_C_14 d	3_CO_64 h	_23	0.082		0.813		0.739		0.830		0.905		0.951		0.008			
	4_CO_14 d	_24	0.000	t	0.049	u	0.662	u	0.813	t	0.894	t	0.854	t	0.372	t	0.52t	1.8
3_CO_64 h	4_CO_14 d	_34	0.735		0.852		0.909		0.906		0.895		0.964		0.742			
		Avg.	0.18	d	0.57	c	0.87	ał	0.84	b	0.90	a	0.85	ab	0.47	c	0.66	2.5
[A] Reclassification %			46		60		85		95		93		85		38			

1-VR: 1- ratio of unexplained variance; RPD: Relative Prediction Deviation: standard deviation / standard error in cross validation.
a>b>c>d: Test of Friedman paired by columns, P<0.05; r>s: Test of Fisher for the main effects within instruments and preparations, P<0.05; t>u; z-Fisher's test for the effects of the Stun factor at the two conditions of Time, within instruments and preparations, P<0.05. [A] reclassification results from Table 4.

Table 4

Reclassification percentage (%R) in the four groups Stun x Time by the PLS-DA of the spectra from seven instrument-preparations.

			FD-H					FD-PD					FD-TO				
Stun	Time	Group	1	2	3	4	Misses	1	2	3	4	Misses	1	2	3	4	Misses
C	64 h	1	15	0	2	0	2	15	0	0	0	0	13	0	5	1	6
C	14 d	2	0	15	1	0	1	0	14	0	0	0	0	15	0	1	1
CO	64 h	3	0	0	12	0	0	0	0	13	1	1	2	0	10	0	2
CO	14 d	4	0	0	0	15	0	0	1	2	14	3	0	0	0	13	0
		60	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
		%R	0	0	20	0	5	0	7	13	7	7	13	0	33	13	15
			THAW-H					EN-H					ETOH-TO				
Stun	Time	Group	1	2	3	4	Misses	1	2	3	4	Misses	1	2	3	4	Misses
C	64 h	1	14	0	3	0	3	10	2	1	1	4	8	4	5	2	11
C	14 d	2	0	12	0	1	1	0	1	0	2	2	1	1	5	2	8
CO	64 h	3	1	0	11	0	1	5	2	13	0	7	4	4	4	1	9
CO	14 d	4	0	3	1	14	4	0	10	1	12	11	2	6	1	10	9
		60	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
		%R	7	20	27	7	15	33	93	13	20	40	47	93	73	33	62

			ET-H				
Stun	Time	Group	1	2	3	4	Misses
C	64 h	1	1	0	2	0	2
C	14 d	2	1	4	2	2	5
CO	64 h	3	3	1	4	0	4
CO	14 d	4	2	2	0	4	4
		28	7	7	8	6	28
		Misses	6	3	4	2	15
		%R	86	43	50	33	54

Table 5
Time at maximum *rigor mortis* (hours) in six selected *Atlantic salmon*.

	Maximum *rigor mortis* (hours)		RI%	
Salmon No.	Stun		Stun	
	C	CO	C	CO
1	24	1	98	100
2	24	3	76	81
3	24	6	76	94
4	26	10	100	87
5	28	11	52	70
6	30	22	85	90
Avg.	26	8.8	81	87
St.dev	2.5	7.5	18	11
Unpaired Friedman test *P*	*0.0037*		*0.57*	
PLS (1-VR) of C and CO groups	0.74		0.04	

Table 6

Time at maximum *rigor mortis* correlated with spectra of the different devices and Fisher's test of 1-VR values.

Instrument-Sample-Laboratory	Storage Time	No.	z-Fisher's test (1-VR) Max-Rigor		All spectra	64 h	14 d
ET-THAW-H	64h & 14d	168	0	b	d		
.	64h	84	0.43	a		c	
.	14d	84	0.25	a			c
EN-THAW-H	64h & 14d	360	0.47	b	c		
.	64h	180	0.63	a		b	
.	14d	180	0.47	b			b
NIRS-THAW-H	64h & 14d	360	0.74	b	a		
.	64h	180	0.81	a		a	
.	14d	180	0.66	b			a
NIRS-FD-H	64h & 14d	360	0.68		a		
.	64h	180	0.74			a	
.	14d	180	0.72				a
NIRS-FD-PD	64h & 14d	360	0.59	b	b		
.	64h	180	0.76	a		a	
.	14d	180	0.75	a			a
NIRS-FD-TO	64h & 14d	360	0.63	b	ab		
.	64h	180	0.56	b		b	
.	14d	180	0.74	a			a
NIRS-ETOH-TO	64h & 14d	720	0.70		a		
	64h	360	0.79			a	
.	14d	360	0.73				a
Average	.	.	0.61	.	.	.	.

1-VR: 1 minus ratio of unexplained variance; within column: a>b>c>d, z-Fisher's bi-lateral test: P<0.05.

Table 7
Results of mixed model analysis of sensory scores of significant variables by the 12 panellists for Atlantic salmon samples

Sensory variables	Stun		*P*	CO/C-1 (%)
	C	CO		
Tactile Crumbliness	4.68[a]	4.05[b]	*0.0384*	-13
Aroma Intensity	5.96[a]	5.48[b]	*0.0301*	-8
Odour Intensity	6.47[a]	5.82[b]	*0.0096*	-10
Saltiness	3.15	3.58	*0.0546*	+14
Tenderness	5.78[a]	4.92[b]	*0.002*	-15

Table 8
Sensory scores correlated with spectra of the different devices, and Friedman and Fisher tests of 1-VR values.

													[B] [C] Test of Friedman paired in rows			
Instrument-Sample-Laboratory	Storage time	No.	Sourness	Adhesiveness	Fibrousness	Tactile Crumbliness	Aroma Intensity	Odour Intensity	Saltiness	Tenderness	Tactile Tenderness	Average	[B] Within Instrument	[C] 64h & 14d	[C] 64h	[C] 14d
ET-THAW-H	64h & 14d	336	0.00	0.03	0.00	0.06	0.01	0.03	0.16	0.06	0.05	0.04	s	v		
	64h	168	0.00	0.10	0.00	0.19	0.18	0.20	0.23	0.36	0.21	0.15	r		u	
.	14d	168	0.00	0.09	0.00	0.05	0.13	0.15	0.19	0.34	0.16	0.11	r			u
EN-THAW-H	64h & 14d	1440	0.00	0.05	0.00	0.20	0.23	0.26	0.28	0.36	0.21	0.16	s	u		
	64h	720	0.00	0.20	0.00	0.41	0.32	0.39	0.41	0.51	0.40	0.26	r		t	
.	14d	720	0.00	0.12	0.00	0.36	0.33	0.42	0.43	0.53	0.36	0.25	r			t
NIRS-THAW-H	64h & 14d	720	0.00	0.16	0.00	0.32	0.31	0.41	0.47	0.57	0.37	0.26		t		
	64h	360	0.00	0.24	0.00	0.30	0.20	0.47	0.53	0.52	0.44	0.27			t	
	14d	360	0.00	0.23	0.00	0.27	0.14	0.46	0.52	0.48	0.43	0.25				t
NIRS-FD-H	64h & 14d	720	0.00	0.21	0.00	0.32	0.17	0.46	0.50	0.53	0.43	0.26		t		
	64h	360	0.00	0.27	0.00	0.34	0.20	0.49	0.53	0.49	0.47	0.28			t	
	14d	360	0.00	0.29	0.00	0.41	0.25	0.47	0.52	0.54	0.44	0.29				t
NIRS-FD-PD	64h & 14d	720	0.00	0.19	0.00	0.28	0.20	0.47	0.50	0.51	0.44	0.26		t		
	64h	360	0.00	0.25	0.00	0.44	0.27	0.47	0.52	0.53	0.44	0.29			t	
	14d	360	0.00	0.29	0.00	0.38	0.22	0.47	0.52	0.51	0.44	0.28				t
NIRS-FD-TO	64h & 14d	720	0.00	0.25	0.00	0.23	0.19	0.47	0.52	0.44	0.45	0.25		t		
	64h	360	0.00	0.26	0.00	0.23	0.16	0.46	0.52	0.44	0.45	0.25			t	
	14d	360	0.00	0.30	0.00	0.30	0.23	0.48	0.52	0.53	0.47	0.28				t
NIRS-ETOH-TO	64h & 14d	1320	0.00	0.02	0.00	0.02	0.00	0.06	0.02	0.07	0.04	0.02	s	v		
	64h	660	-0.01	0.00	0.00	0.29	0.25	0.43	0.36	0.54	0.41	0.23	r		t	
	14d	660	0.00	0.05	0.00	0.33	0.25	0.41	0.35	0.52	0.42	0.23	r			t
Average 1-VR of the variables, tested by z-Fisher [A]			0.00 f	0.17 e	0.00 f	0.27 d	0.20 e	0.38 b	0.41 a	0.45 a	0.36 c	0.22				

[A] a>b>c>d>e>f>g; P<0.05: z-Fisher's test to rank average sensory variable 1-VR instrumental correlations.
[B] r > s; P<0.05: Test of Friedman paired in rows for each instrument to highlight Time effects and interaction.
[C] t > u > v; P<0.05: Test of Friedman paired in rows across instruments to highlight instrument performance, whether in All spectra, Time1 or Time2 spectra.