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Detection of cold chain abuse in frozen and chilled salmon using the comet assay

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ABSTRACT

DNA damage in frozen and chilled salmon (*Salmo salar*) was investigated using the comet assay. Frozen salmon was thawed at 10 °C and chilled salmon was kept in flake ice, mimicking a thermal abuse and an extended storage, respectively. Electrophoresis conditions were as follows: a second layer of low-melting-point agarose at 1 g/100 mL; a voltage of 2.5 V cm^{-1}; and running time of 1.0 min. Control cells showed relatively round shaped comets with a large and intense head, and a short tail. Their olive tail moment (OTM) profiles showed a predominance of low values that were narrowly distributed (between 8 and 25). As thawing or chill storage progressed, comets showed an increase in tail size, a shift of intensity from the head to the tail and a clear separation between the two. This was associated with a shift of OTMs toward higher values and a wider distribution of these values: between 17 and 43 after 42 h of thawing; and between 12 and 46 after 12 days of chill storage. Based on OTM values and their distribution, the DNA damage in salmon cells was visible for frozen salmon after 3 h of thawing at 10 °C and for chilled salmon after 9 days of storage.

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1. Introduction

Today, refrigerated storage is one of the most widely used processes for the preservation perishable foods and the reduction of risks linked to microbial growth. Among the industries involved, fisheries, a key economic sector in most countries, rely heavily on the cold chain to ensure the commercial viability of its products. The cold chain refers to all operations in the production, distribution, storage and retailing of refrigerated and frozen foods (Likar & Jevsnik, 2006). It extends from the raw material supplier (e.g. the fisherman at sea) through to the consumers' refrigerator or freezer, and all the steps in between. Fish and seafoods are among the most sensitive products with regard to food poisoning. Indigenous pathogens include *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Vibrio cholerae*, *Clostridium botulinum* Type E (Balebona, Moriñigo, & Borrego, 1995; DePaola, Hopkins, Peeler, Wentz, & McPhearson, 1990; Huss, Ababouch, & Gram, 2003; Lalitha & Surendran, 2002), and enteric microorganisms such as *Salmonella* spp. and *Shigella* spp. (David, Wandili, Kakai, & Waindi, 2009). Other non-indigenous pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* can be present as a result of processing and post-processing handling operations (Huss et al., 2003).

Control of the storage temperature is vital in maintaining the safety of the food, its taste and appearance throughout the food continuum (gate to plate). This is why there are prescribed maximum temperatures associated with each type of refrigerated storage and with each type of food. For fish, molluscs and shellfish, the Agreement on the International Carriage of Perishable Foodstuffs (ATP, 1970) specifies 0–2 °C (ice slurry) and −18 °C as maxima for chilling and freezing, respectively. As a consequence, it is important that good chill/freeze storage procedures are in place to ensure effective control of the cold chain in a continuous operation (no stopping or delays) between temperature-controlled areas, e.g. delivery trucks to holding stores; or storage areas to retail display units. With this regard, the transport and distribution sections of the chain are especially critical, since they are the most likely to be associated with thermal abuses. Storage conditions are also an important authenticity issue in the fisheries sector as they relate to adulteration and improper description of fish (Arvanitoyannis, Tsitsika, & Panagiotaki, 2005). Frozen fish can for instance be

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passed for fresh, or it can be thawed for filleting or other operations and refrozen (Uddin & Okazaki, 2004). For the benefit of the consumer and the prevention of unfair competition in the trade of fishery products, correct labeling of frozen-thawed fish or fillets is desirable.

Conducting frequent and systematic temperature checks on chilled and frozen fishery products using appropriate and calibrated instrumentation, is the major tool at the industry's disposal. However, this does not constitute a guarantee of fish quality and safety. In fact, although low temperatures dramatically extend lag and generation times of most microorganisms, the major food spoilage and food poisoning bacteria are psychrotrophic bacteria that are able to grow at chill temperatures (Russell, 2002). Therefore, the storage time is also important since extended storage can give the opportunity for some pathogens to gradually multiply, and also for detrimental reactions to occur. Time Temperature Indicators (TTIs) are labels attached to food packages that are commonly used to trace the history of a product through a change in color that is dependent on time and temperature (Sahin, Babaï, Dallery, & Vaillant, 2007). The principle of these devices is based on enzymatic, chemical or microbiological reactions which cause a color change in the labels in dependence on time and temperature conditions. They can detect a break in the cold chain or an over-extented storage when they are in direct and constant contact with the food. This means that a temperature rise that would occur before the implementation of the indicators or after their withdrawal would remain undetected. The efficiency of these indicators in demonstrating 'due diligence' in accordance with food safety legislation remains therefore limited.

In addition to the storage conditions and duration which allow deterioration prior to consumption, the quality and shelf-life of fish is also related to the load of bacteria initially present and to the efficiency of processing. Accordingly, the edibility of fish can be determined by microbiological analysis, where the total quantity of living bacteria is the significant factor, as well as the presence of any pathogenic bacteria. Microbial analysis remains however often cost-prohibitive and time-consuming. The need remains for rapid and cost-effective technologies, devices and approaches that would allow the continuous monitoring and recording of the relevant data throughout the entire supply chain.

DNA degradation is a natural process which takes place in the cells after death, and which is considerably slower at low temperatures; and practically absent at freezing temperatures (Cerda & Koppen, 1998). This phenomenon is taken advantage of in the comet assay or single-cell gel electrophoresis (Ostling & Johanson, 1984). In this assay, a cell suspension is mixed with an agarose solution and layered on a microscope slide. The trapped cells are treated with a detergent to disrupt the membranes and free the nuclear DNA, and then subjected to electrophoresis, causing DNA to migrate in the gel, producing a characteristic "comet" pattern that is revealed by the use of an appropriate dye. The length and shape (relative amounts of DNA in the head and the tail) of the comets are indicative of the extent of DNA damage undergone by the cell. The comet assay has been used over the years as a method of measuring DNA damage in applications such as genotoxicity, cancerogenocity and ecogenotoxicology testing (Bonisoli-Alquati et al., 2010; Collins, 2004; Dhawan, Bajpayee, & Parmar, 2009; Fairbairn, Olive, & O'Neill, 1995; Jha, 2008; Kumaravel & Bristow, 2005; Liao, McNutt, & Zhu, 2009; Luo et al., 1998; Speit, Vasquez, & Hartmann, 2009). Food applications were in the vast majority related to the detection of irradiated products such as meats and seafood (Khan, Khan, & Delincée, 2002a, 2002b), fresh fruits (Marin-Huachac, Lamy-Freund, Mancini-Filho, Delincée, & Villavicencio, 2002), spices and beans (Khan et al., 2002a, 2002b), and poultry (Villavicencio, Araujo, Marin-Huachaca, Mancini-Filho, & Delincée, 2004). As for the determination of food freshness, the applicability of the comet test has been demonstrated on poultry (Cerda & Koppen, 1998; Faullimel, Ennahar, Aoudé-Werner, Guterl, & Marchioni, 2005).

Since fish is one of the most sensitive products with regard to food poisoning, the chilled and frozen fish supply chains were selected as a test case in the present study. The use of the comet assay as a freshness test for salmon was investigated by monitoring the DNA damage occurring during the thawing of frozen samples, as well as during the extended storage of chilled samples.

2. Materials and methods

2.1. Samples

Salmon (*Salmo salar*) samples were provided by Haliomer (Boulogne-sur-Mer, France). Fish was put in flake ice or frozen immediately after catch and evisceration, and was conveyed accordingly either in ice or under temperatures not exceeding −20 °C. Control chilled salmon corresponded to fish in flake ice caught less than 24 h prior to the start of the test. Control frozen salmon corresponded to unthawed fish that was put at −20 °C immediately after being caught and eviscerated.

2.2. Chemicals

All chemicals used in this study were of analytical grade. Low melting point (LMP) agarose was purchased from Bio-Rad (Marnes-la-Coquette, France). Boric acid and sodium dodecyl sulfate (SDS) were supplied by Merck Chemicals (Darmstadt, Germany). Tris base, hydrochloric acid, Tris HCl, ethylene-diamine tetraacetic acid disodium salt dihydrate (EDTA) were purchased from Sigma–Aldrich (Schnellendorf, Germany). 0.5 g/100 mL Trypsin–EDTA (10×), Sybr Green I (10,000×) staining solutions were purchased from Invitrogen (Cergy-Pontoise, France). Ultra-pure water (18.2 MΩ) was produced by a Milli-Q Plus filtration system from Millipore (Molsheim, France).

2.3. Cold chain experiments

Thawing experiments were performed on frozen salmon samples in temperature-controlled chambers at 10 °C in the dark to avoid UV radiation incidence. Accelerated thawing, at 22 °C, was used for setting up experimental conditions prior to cold chain experiments. The temperature was closely monitored using an electronic thermometer. Prior to thawing, salmon fillets were cut into about 1 cm^3 pieces while maintained on an ice-cold flat tray. For fresh unfrozen salmon, experiments consisted in keeping samples for several days in flake ice (0 °C). Samples were analyzed rapidly and handled while kept cold.

2.4. Comet assay

2.4.1. Slide preparation

Partially frosted microscopic slides (26 × 76 mm) (Marienfeld Glassware, Lauda Koenigshofen, Germany), prewashed overnight in methanol, were dried and covered with 50 μl of 0.5 g LMP agarose in 100 mL ultra-pure water at 40 °C. They were immediately covered with a 24 × 60-mm size coverslip (Menzel-Gläser, Braunschweig, Germany) while avoiding air bubbles and kept on an ice-cold flat tray for 10 min to allow the agarose to solidify, before the coverslip was gently removed. This pre-coating step improves adherence of the second LMP agarose layer containing the cells.

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