

MEASURES TO IMPROVE THE SAFETY AND QUALITY OF SCOMBROID FISH IN MOZAMBIQUE: THE METHODOLOGY OF QUALITY INDEX METHOD AND HISTAMINE MEASUREMENTS

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ABSTRACT

Safety and freshness are important attributes of the overall quality of seafood. The main aim of this study was to learn about sensory methods for seafood, with focus on freshness evaluation and how sensory evaluation tools may be used to improve the sensory quality of Mozambique exported seafood products. Further, the aim was to deepen the knowledge about histamine formation and receive training in histamine measurements. Quality Index Method (QIM) is a sensory method that can be applied to evaluate the freshness of seafood. QIM was adapted and evaluated for whole Senegalese sole within this project. Whole Senegalese sole was stored in ice for up to 21 days and changes in quality attributes over storage time were observed. Parallel, changes in odour and flavour were monitored. A new 26-point QIM scheme developed for Senegalese sole during this project was suggested. A preliminary shelf life study showed a strong linear relationship ($R^2= 0,969$) between the average QI for each storage day and storage time in ice. During shelf life experiments with Atlantic mackerel, five groups were kept and stored at different temperatures and sampled after 26, 50 and 74 hours for biogenic amines measurements. One group was dipped in an inoculum of *Photobacterium phosphoreum* (10^3 CFU/mL) and a second group in an inoculum of *Lactobacillus casei* (10^3 CFU/mL) followed by storage at 21 °C. The other three groups were not inoculated but stored at 1,5 °C, 12 °C and 21 °C. Higher storage temperature resulted in higher BA/histamine formed, especially in samples dipped in an inoculum of *Photobacterium phosphoreum* (554 ppm) or *Lactobacillus casei* (256 ppm).

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1 INTRODUCTION

Fish production is one of the major industries in Mozambique, on average, the annual catch from marine resources is about 120.000 tonnes. Officially, marine capture fisheries account for more than 90% of Mozambique's total fish catch and coastal communities depend on the sea and its resources for survival, with fish accounting for 50% of the population's protein intake (Doherty et al., 2015). In Mozambique, fishing contributes to food security, providing fish for consumption (supplying proteins) and improving the living conditions of fishing communities, as well as earning hard currency for the country. The main marine commercial species are lobster, crabs, shallow water prawn, deep-water prawn (gamba), crayfish and squid (Ministério das Pescas, 2013).

The Ministry of Sea, Inland waters and Fisheries (MIMAIP) is the main authority in Mozambique for control of the fish quality for export and is also responsible for imports of seafood. A subgroup under the Ministry, the National Institute of Fish Inspection (INIP) acts as the competent authority for control of the quality of fish and fisheries products.

Food safety remains important and has become increasingly stringent for exported products (Lokuruka, 2009). Consumers today expect that food is convenient and palatable, however they also demand that their food is safe to eat (Olajos, 2015). The distribution of marine fish to the export markets, principally to inland areas, distant from the coast, is a problem for many countries, like Mozambique, with regard to quality and safety of the fish. This is often a reason for developing good handling procedures as well as application of regulations to the whole production chain, from handling fish on board fishing vessels to the retailing of fish (Lokuruka, 2009). Food businesses that participate in the global food trade are responsible for ensuring that their food products are safe and the competent authorities must implement food safety measures according to international standards in order to ensure consumer protection (Olajos, 2015). For this it is necessary to develop forms of control and monitoring that aim to confirm the application of good handling procedures in fish species, like scombroid fish, that may form histamine during storage.

Currently the principal problem in fish inspection is insufficient knowledge in governments on the sanitary inspection of fish belonging to the family Scombridae, principally larger fish, such as tuna. It is important to know how to determine the histamine in the fish belonging to the family Scombridae using HPLC (High Performance Liquid Chromatography) method, because

recently, INIP bought two machines of HPLC and they are not being used, because knowledge is lacking. Solving this main problem, it will be possible to make a complete sanitary inspection of the fish belonging to the family Scombridae. This will help Mozambique to export, as well as import fish with high quality.

Sensory evaluation is an important method for the assessment of freshness and quality, and is commonly used in the fisheries sector and fish inspection services (Ólafsdóttir et al., 1997). Fish inspectors are charged with the responsibility of ensuring safety and quality of locally and internationally traded fish and fish products. This involves inspection of fish and fish handling methods in the production sector, transport, markets and fish processing factories in order to ensure compliance with national and international regulations.

In Mozambique, Fish Inspection Laboratories use sensory tests for evaluation of the raw material and final products, but improvements are needed, and the current methods and procedures need to be replaced by new and more efficient methods, as well as to develop schemes for sensory evaluation of important species. The development of reliable freshness evaluation method such as the Quality Index Method (QIM), for fish species found in the Mozambican waters, specially tuna, and its application in the fishery sector is essential.

The QIM can be useful in quality control, processing industries and for fish inspectors because of how specific the method is, as well as being an important tool in training of fish inspectors (Martinsdóttir et al., 2001).

The overall objective of this project is to contribute to the improvement of the safety and quality of fish in Mozambique, by applying the methodology of Quality Index Method and Histamine measurements.

The main aim of this project was to learn about sensory methods for seafood, with focus on freshness evaluation and how to apply sensory evaluation tools to Mozambique export and import of seafood products. This was done through the development and evaluation of a QIM scheme to measure the freshness of farmed Senegalese sole. Further, the aim was to deepen the knowledge about histamine formation and receive training in histamine measurements. This was done by shelf life experiments with Atlantic mackerel, where histamine formation was monitored by contamination of Atlantic mackerel with bacteria known to facilitate histamine formation (*Lactobacillus casei* and *Photobacterium phosphoreum*) and storage of Atlantic

mackerel at different temperatures without contamination. More specifically, the objectives of the project were achieved by:

- 1) Development of QIM scheme for farmed Senegalese sole.
- 2) Training of panellists for the sensory evaluation of QIM and Torry scheme of farmed Senegalese sole.
- 3) A study of the procedures of a shelf life study with whole farmed Senegalese sole stored on ice using developed QIM scheme.
- 4) Using High Performance Liquid Chromatography method to determine the histamine formation in Atlantic mackerel.
- 5) Learning the procedures of how the samples of Atlantic mackerel are taken and the laboratory procedure to evaluate histamine formation.

2 LITERATURE REVIEW

2.1 Senegalese sole (*Solea senegalensis*)

Solea senegalensis is a benthonic marine species very common in Mediterranean and Southern Atlantic waters, living in sandy or muddy bottoms, off coastal areas up to 100 m depth, in brackish lakes and estuaries (Cabrita et al., 2006; Oliva et al., 2010). Senegalese sole is a flatfish with oval and asymmetric body (eyes on the right side) (Figure 1) that feeds basically on benthonic invertebrate, such as larvae from polychaets, bivalve molluscs and on small crustaceans (Oliva et al., 2010).

Sexual maturity is reached when the size is 30 cm (Oliva et al., 2010). Presently, Senegalese sole reproduction is obtained using breeders captured from the wild, since reproduction of F1 broodstocks has failed (Cabrita et al., 2006). Spawning happens between the months of March until June. Senegalese sole is a well-adapted species to warm climates and is commonly exploited in extensive aquaculture production in Spain and Portugal and have been used in field and laboratory toxicity assays being a sensitive species to pollutants (Oliva et al., 2010).

As an aquaculture species, Senegalese sole is of high commercial importance in Southern Europe that has been a promising development in diversifying the offer of cultured fish. The optimal rearing temperature of Senegalese sole is around 20 °C to 25 °C. Senegalese sole displays clearly nocturnal self-feeding patterns with 77% to 85% of feed demands occurring at night, preferentially on polychaetes, crustaceans and bivalve molluscs (Fernández, 2012).

Senegalese sole is highly regarded in the south of Europe and industrially cultured on the Mediterranean and Atlantic coasts, with most farms located along the Atlantic coast in Portugal, Spain, France, Italy and the Canary Islands. A maximum production of 571 tonnes was registered in 2013, but mostly in Europe. In 2012 the total value was 360 670 USD (Filcun, 2012; FAO, 2014).

As a wild species, Senegalese sole is considered as a lean fish, with total fat content in edible tissue (fillet) of 2,9% fat up to 3,5%. As a cultured species it is a medium fat fish, with total fat content in edible tissue of 3,5% fat up to 7,2% (Filcun, 2012).

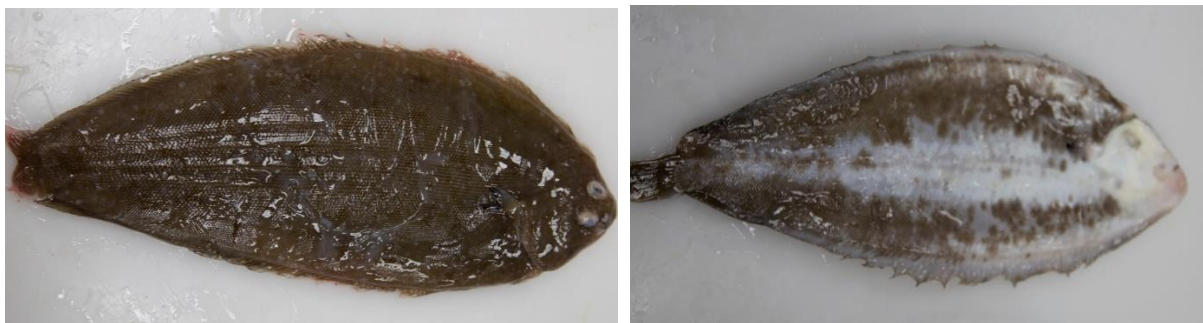


Figure 1. Senegalese sole (*Solea senegalensis*) used in this project.

2.2 Atlantic mackerel (*Scomber scombrus*)

Atlantic mackerel (Figure 2) are distributed widely on both sides of the North Atlantic. On the eastern side, their distribution extends from the coasts of Morocco in the south to northern Norway and also into the Black Sea, Mediterranean, Kattegat, Skagerrak, and the Baltic. In the Northwest Atlantic, the distribution extends from off North Carolina in the south to Labrador in the north. The Northeast Atlantic mackerel stock is considered to consist of three spawning units; south, west, and North Sea, of which the west is currently the largest. The spawning season for mackerel is from February to July, and after spawning, the south and west units migrate into the Norwegian Sea and North Sea to feed (Astthorsson et al., 2012).

Mackerel were first reported with certainty as a single fish from a southwest coast fjord near Reykjavík in 1895 and during the same year as single fish from two fjords on the east coast. Since 1996, the waters around Iceland have been relatively warm, yielding a temperature increase of ~1–2 °C in the upper 50 m off the south and west coasts of the country (Astthorsson et al., 2012).

The distribution and abundance of mackerel in Icelandic waters have both gradually increased and, since ~2007, they have been observed in the area in large numbers, which has led to an extensive fishery within Iceland's Exclusive Economic Zone, EEZ (Astthorsson et al., 2012). The Atlantic mackerel is considered as fatty fish species because its edible portion is composed of 11.4% fat and 18.7% protein (FAO, 1989). Mackerel is abundant in cold and temperate shelf areas, forms large schools near the surface. It is mainly diurnal and feeds on zooplankton and small fish (Astthorsson et al., 2012).

The Namibian horse mackerel is the smallest of all the horse mackerels fished around the globe and the lowest in fat content. Horse mackerel is a basic food product that competes with the price of chicken pieces, e.g. producers are very much restricted in their price policy (Lee, 2013). Mozambique imports mainly Horse mackerel from Namibia. Table 1 below shows the evolution of the import of the horse mackerel by Mozambique in tonnes in the period between 2014 until 1st semester 2017.

Table 1. Evolution import of Horse mackerel by Mozambique in tonnes in the period between 2014 until 1st semester 2017.

	2014	2015	2016	1 st Semester 2017	Total 2014 - 1 st Semester 2017	Specific weight%
SADC						
Import from Namibia	51,985	52,182	49,518	27,975	181,660	75.3
Import from RSA	7,070	6,420	3,763	566	17,819	7.4
SADC Total Import	59,055	58,602	53,281	28,541	199,479	82.7
Rest of the world						
Impor from the rest of the world	8,850	9,663	16,147	6,957	41,617	17.3
Total Import	67, 905	68,265	69,428	35,498	241,096	100.0

Analysing table 1 above of the cumulative imports of horse mackerel in Mozambique, the SADC (Southern African Development Community) region appears with a weight of 82.7%, and Namibia with a greater weight of about 75% followed by South Africa with 7.4% import. The other imports in the order of 17% come from the rest of the world outside the SADC region.



Figure 2. Atlantic mackerel (*Scomber scombrus*) used in this project.

According to Oucif et al. (2012) Atlantic mackerel is sensory acceptable, less than 24 hours at ambient temperature (26 ± 3 °C) for up to 3 days at 4 °C and 3 months at -18 °C. A few hours after capture, initial histamine content in Atlantic mackerel is already high (6,39 mg/100 g). It exceeds the toxic level of histamine of 10 mg/100 g muscle rapidly after 24 hours of storage at ambient temperature, due to a high amount of histidine in red muscle of fish, a large proliferation of mesophilic bacteria capable of histidine decarboxylation, a high temperature and a suitable pH for the synthesis and activity of histidine decarboxylase, reached rapidly in the flesh of mackerel (Oucif et al., 2012).

2.3 Sensory analysis

Sensory evaluation is the scientific discipline that evokes, measures, analyses and interprets human reactions to characteristics of food perceived through the senses of sight, smell, taste, touch and hearing. Scientific sensory evaluation methods must be performed under carefully controlled conditions in order to reduce the effects of test environment, personal bias, and so on (Martinsdóttir et al., 2009a). Sensory methods relying on trained assessors, i.e., objective sensory methods are required for use in quality control for evaluation of freshness and for determination of remaining shelf life of seafoods (Martinsdóttir et al., 2001).

For Martinsdóttir et al. (2009a) in the problem to be solved it is extremely important to define what is to be measured. Sensory evaluation is quantitative: numerical data are collected to establish the relationship between product characteristics and human perception. It is critical to use proper analysis of the sensory data and interpret the results. The sampling system, methods and procedures for sensory evaluation must be very well defined to serve its purpose. In sensory evaluation, a sensory panel is established, and panellists or inspectors trained to perform sensory analysis with clear and descriptive guidelines. During perception, most or all

the sensory attributes overlap. However, with training, independent evaluation of each attribute is obtained (Martinsdóttir et al., 2009a).

There are different types of sensory evaluation methods of seafood quality. However, the choice of the method depends on the purpose of the application and whether it is used for product development, quality control, consumer studies or research (Ólafsdóttir et al., 1997). The most used are ranking, scaling methods and grading e.g. EU scheme, Quality Index Method (QIM), Torry scale, raw fillets grading method and Quantitative Descriptive Analysis (QDA) (Martinsdóttir et al., 2009b).

According to Ólafsdóttir et al. (1997) and Sveinsdóttir et al. (2003), QIM can be defined as a grading system for freshness estimation of fishery products in which the descriptions of the individual grades are precise, objective and independent. It is usually based on quality attributes of raw fish, which are given scores according to descriptions of sensory attributes, and the method needs to be developed or adjusted to each fish product. Martinsdóttir et al. (2009b) suggested QIM as a freshness grading system for seafood and it is widely accepted as a reference method in research and it is, also, important that the fish sector implements the method.

A relatively large number of fish quality attributes are evaluated in sequence by sight, smell and touch. Each attribute is scored from 0 to 3 (typically) by sensory assessors with low scores indicating freshness. The sum of all attribute scores is called demerit points, or QIM index points, and this value increases linearly with storage time of a given fish (Martinsdóttir et al., 2001). In quality management it is important to be able to apply a sensory system that reflects the different quality levels in a simple, reliable and documented way. QIM has those advantages, in addition to being rapid, cheap to use, non-destructive and objective compared with other sensory methods. Further, it is easy to work with as it includes instructions. It is a convenient method to teach inexperienced people to evaluate fish, and to train and monitor performance of panellists. Different types of seafood have different characteristics and spoilage patterns, and QIM schemes must be adapted to each species incorporating their respective characteristics (Martinsdóttir et al., 2009b).

For sensory evaluation of fish fillets, it is common to cook the fillets and evaluate their odour and flavour. The Torry scale is the most frequently used industry scale for evaluating the freshness of cooked fish. It is used both by producers and buyers. It is a descriptive 10-point

scale that has been developed for lean, medium fat and fat fish species. Scores are given from 10 (very fresh in taste and odour) to 3 (spoiled). It is considered unnecessary to have descriptions below 3, as the fish is then not fit for human consumption. The average score of 5,5 has been used as the limit for “fit for consumption” (Martinsdóttir et al., 2009b).

2.4 Histamine content in fish

Histamine is a biogenic amine produced in fish tissue through the decarboxylation of free histidine by exogenous decarboxylases released by microorganisms (Maia, 2015). Biogenic amines (BAs) are organic, basic, nitrogenous compounds of low molecular weight, mainly formed by the decarboxylation of amino acids and with biological activity (EFSA, 2011). Scombroids fish, such as tuna, mackerel, skipjack, bonito, marlin, are particularly susceptible to the formation of histamine, since they naturally contain high levels of free histidine in muscle tissue that, in certain situations, may undergo decarboxylation and transform to histamine by natural bacteria (Olajos, 2015; Evangelista, 2015). The microorganisms that cause histamine formation naturally occur in the environment of mackerel and are thus present on the surface of the fish and gills. The microorganisms therefore grow as soon as the fish is harvested and the conditions are favourable (Mensah, 2013). If the temperature is above 4 °C during or after the catch, enzymatic bacterial decarboxylation can cause histidine degradation into histamine (Olajos, 2015).

A number of the histamine-forming bacteria are facultative anaerobes, such as *Photobacterium phosphoreum*, which can grow in reduced oxygen environments. This bacterium is a well-known light organ symbiont of several fish species. The most common habitat of this organism is the intestine of marine fish (Dalgaard et al., 1997b; James et al., 2013). *Photobacterium phosphoreum* is a specific spoilage organism (SSO) that limits shelf-life of fresh marine fish when stored in modified atmosphere packaging (MAP). In aerobically stored fish products *P. phosphoreum* was found to produce trimethylamine (TMA) from trimethylamine oxide (TMAO) along with participating in histamine production (Dalgaard et al., 1997a; Dalgaard et al., 1997b).

Lactobacillus casei is a lactic acid bacterium (LAB) that forms part of the facultatively heterofermentative species cluster that produce lactic acid from hexose sugars via the Embden-Meyerhof pathway and from pentoses by the 6-phosphogluconate/phosphoketolase pathway. *Lb. casei*, form a group of common indigenous microorganisms, which are natural inhabitants

of the gastrointestinal tract of animals, where biofilms form in the gut microbiota to allow them to persist during harsh environmental conditions and maintain ample populations. *Lactobacillus* exhibits a mutualistic relationship with the host body to protect the host against potential invasions by pathogens, and in turn, the host provides a source of nutrients (Hosseini-Nezhad, Hussain & Britz, 2015; Khan, 2018).

Levels of free histidine differ between at-risk fish species and there is evidence of a correlation between levels of histidine and the formation of histamine in different species. The minimum histidine concentration required for bacterial histidine decarboxylase activity is estimated to be 1.000 to 2.000 mg/kg (Lee et al., 2012).

Scombrototoxin is a toxin produced in fish tissue when certain spoilage microorganisms containing the enzyme histidine decarboxylase cause the breakdown (decarboxylation) of the amino acids histidine to amines (histamine) (Mensah, 2013). Scombrototoxin fish poisoning (SFP) (often called “histamine poisoning”) is caused by ingestion of certain species of marine fish that contain high levels of histamine and possibly other biogenic amines (FAO-WHO, 2012).

Histamine formation in fish is both temperature and time dependent and immediate freezing or cooling below 4 °C is necessary to avoid histamine accumulation above acceptable levels (typically 100 mg/kg, according to European Commission Regulation, 2005). In instances where the cold chain is poorly maintained and proper hygienic conditions are lacking such as may pertain in smaller coastal vessels or during inconsistent energy supply in cold stores, histamine development can be accelerated (Olajos, 2015).

EU, EC regulation 2073/2005 stipulates that for a batch to be acceptable 9 independent samples from each batch should result in: i) an average histamine concentration lower than 100 ppm. ii) no more than 2 samples out of the 9 with a concentration of between 100 and 200 ppm. iii) no sample with a histamine content higher than 200 ppm.

Once the fish may contain toxic levels of histamine and, sensorially, do not present significant differences, chemical analysis of foods for traces of this potential food allergen is necessary, and for the determination of histamine in fish and its derivatives there is a wide variety of methods among them the HPLC which is important because it can quantify and differentiate all the BAs, being precise and exact (Maia, 2015; Olajos, 2015).

As shown in the schematic diagram in Figure 3, HPLC instrumentation includes solvent reservoirs, a pump, a degasser, injector, column, detector and integrator or acquisition. The heart of the system is the column where separation occurs.

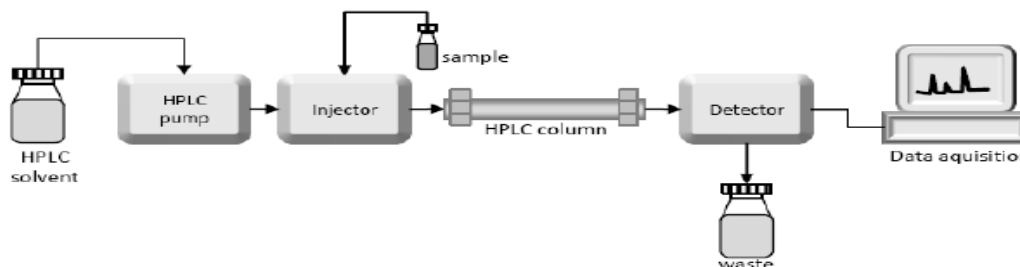


Figure 3. Schematic of HPLC (<https://laboratoryinfo.com/hplc/>)

3 MATERIALS AND METHODS

3.1 Experimental design

The experiments were conducted at MATIS laboratories in Reykjavik, Iceland, 6 December 2018 to 15 February 2019. The project was carried out in two parts: i) sensory evaluation of Senegalese sole and ii) determination of histamine in Atlantic mackerel using HPLC.

- i) For sensory evaluation of Senegalese sole primarily focused on pre-observation of fresh Senegalese sole during iced storage, carried out by 4 assessors, which ended with a design of preliminary QIM scheme. Secondly, the QIM scheme was further developed parallel to the training of 10 panellists from MATÍS in sensory evaluation of Senegalese sole. At the same time, Senegalese sole of different freshness was cooked and tasted to monitor changes in odour and flavour in line with the Torry scheme for lean or medium fat fish (Figure 4).
- ii) For the determination of histamine thawed Atlantic mackerel was split into five groups; While the first group (control) was stored at 1,5 °C. The second one at 12 °C and the third group at 21 °C. The rest was contaminated respectively with *Lactobacillus casei* and with *Photobacterium phosphoreum* and stored at 21 °C. For all groups, samples were collected after 26, 50 and 74 hours and histamine content analysed (Figure 4).

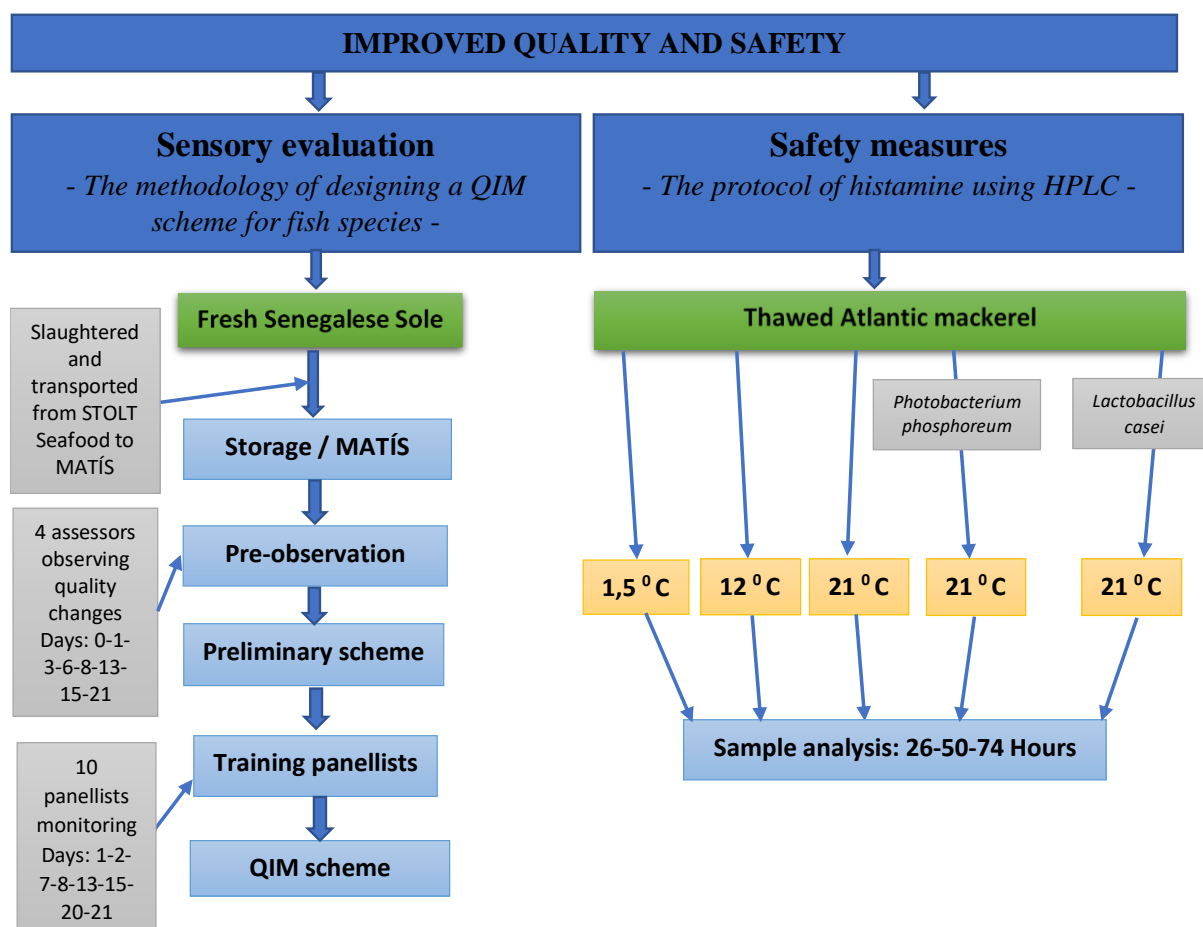


Figure 4. Chart showing experimental design for the sensory evaluation of fresh Senegalese sole and safety measures using thawed Atlantic mackerel.

3.2 Raw material

3.2.1 Senegalese sole

Different batches of thirty-seven Senegalese sole were obtained from Icelandic fish farm “STOLT Seafood”. The fish were packed in styrofoam boxes (Figure 5), covered by a thin plastic film, with crushed ice in the box, and transported to the MATÍS laboratory on 6, 8, 11, 13, 18, 20, 21, 27 December 2018, and 3 and 9 January 2019. Average weight was 350 ± 50 g.

At MATÍS, fish were conditioned in styrofoam boxes with perforated bottoms to drop water, covered by a thin plastic film with crushed ice in the box. The boxes were stored under refrigerated conditions at $2,5 \pm 0,5$ °C, and the ice was replenished on alternating days. Samples

were taken for sensory evaluation after 0, 1, 3, 6, 8, 13, 15 and 21 storage days in ice, for pre-observation, and 1, 2, 7, 8, 13, 15, 20 and 21 for training of panellists.

3.2.2 *Atlantic mackerel*

Twenty-six Atlantic mackerel used in this project, were caught on 6 August 2018 in Icelandic sea waters, - 1,5 °C after catching. From Neskaupsstaður to Reykjavik (MATÍS) the fish was transported by refrigerator truck. At MATÍS laboratory the fish was kept at - 25 °C until 18 January 2019. Fish was then thawed at ambient temperature (21±1 °C) and split in five sample groups.

3.3 Development of Quality Index Method

Sensory evaluation of whole Senegalese sole was carried out in a special room equipped with big inox table, where the white trays were placed on the stainless table (Figure 5), under white light, in order to facilitate visual sensory evaluation.

A preliminary QIM scheme for farmed Senegalese sole was developed during the pre-observation sessions by four assessors. The scheme was further tested during panellist training by ten panellists. The QIM scheme for farmed Senegalese sole was developed during panellist training sessions. Ten panellists, all employees of MATÍS ohf (Icelandic Food and Biotech), participated in the sensory evaluation of raw (QIM) and cooked (Torry) Senegalese sole fillets. They had all been trained according to international standards (ISO 8586: 2014), including detection and recognition of tastes and odours, training in the use of scales, and in the development and use of descriptors. The members of the panel were familiar and experienced in using QIM and Torry scale for other fish species.

Pre-observation, development of preliminary QIM scheme

The objective of the pre-observation of Senegalese sole was to get an idea about the changes in quality parameters with storage time in ice. Before the pre-observation, quality parameters and descriptors in a QIM scheme for sole (Martinsdóttir et al. 2001) and QIM scheme for Senegalese sole (Gonçalves et al. 2007) were used as a basis for the evaluation.

The attributes analysed on the ocular side were spots on skin, appearance, mucus, skin odour and texture, the form and clarity/brightness of eyes, and finally the colour and odour of the gills but on the blind side only appearance (Table 7). Four assessors evaluated the Senegalese sole

during three sessions. During this period, two persons observed and registered all changes occurring in Senegalese sole until spoiled. Based on these observations, each description received a score in which 0 corresponded to fresh fish and the score increased according to spoilage with a maximum score of 3 for each parameter. This information was used to make a draft of a modified QIM scheme, where the total sum of score was 28 points (Table 7).



Figure 5. Table prepared for QIM evaluation of whole Senegalese sole (left) and styrofoam box used to condition the fish (right).

Training panellists, development of the QIM scheme

A total of twenty-one fish were analysed with a preliminary QIM scheme during the training panellists sessions. Ten sensory panellists, were trained during three sessions using the preliminary QIM scheme developed during pre-observation sessions. In each session, 8-12 Senegalese sole of different storage days (ranging from 1 to 21 days in ice) were observed.

The procedure of evaluation was introduced to the panellists and each parameter evaluated was discussed. During the evaluation the panellists had opportunities to ask questions concerning the evaluation at any time. The panellists were informed of the plan to develop a QIM scheme for Senegalese sole stored in ice and were asked to provide comments and suggestions regarding parameters and sensory descriptors in the new scheme. Suggestions provided by the panel were considered and the scheme was slightly modified. After each session, the suggestions given by the panellists were recorded and improvements of the scheme were made accordingly.

Before each session, the fish were placed on a table, 25-30 minutes before the evaluation. At the third session, each fish was coded with a number consisting of three digits that did not indicate the storage time or condition of the fish.

All observations of the fish were carried out under standardised conditions at room temperature using electric light and with as little distraction as possible.

The panelists evaluated the fish individually and registered their evaluation for each quality parameter in the scheme. The quality index (QI) was calculated as a sum of the score for evaluated parameters. The average score of each panellist for each fish was calculated. After each sampling day the average QI of fish was calculated. The evaluation took 25-30 minutes.

3.3.1 Sensory evaluation of cooked fish

Senegalese sole fillets of different freshness, weighing about 40–50 g, were taken from the fillet loins and placed in aluminium boxes closed with plastic covers, coded and cooked in pre-warmed oven (Convotherm Elektrogeräte GmbH, Eglfng, Germany) with air circulation and steam (Figure 6), without any additives for 6 minutes at 150 °C.

Care was taken in serving the assessors and panellists the samples at that temperature. Each assessor/ panellist was served a similar a part of the fish and evaluated all the fish samples and gave an average score. The amount of samples tested was at least three bites each. Assessors/ panellists received each sample separately and began with the odour and then the taste. The number of samples tasted at a time was not more than three samples. Water was served, and palate cleaners were used.

Pre-observation of cooked fish

During pre-observation of cooked fillets, four assessors evaluated the odour and flavour on day 1, 8, 13, 15 and 21 of sampling, using the Torry scoresheet for freshness evaluation of cooked medium fat fish such as redfish (Table 2) and Torry scoresheet for freshness evaluation of cooked fat fish such as plaice (Table 3), both described by Shewan et al., 1953; Martinsdóttir et al., 2001. Each assessor gave their opinion about the odour and flavour of the fish. This information was used to decide on which Torry scheme was used for Senegalese sole (Table 9). The shelf life of cooked farmed Senegalese sole storage in ice was estimated (approximately).

Table 2. Torry scoresheet for freshness evaluation of cooked medium fat fish such as redfish modified from (Shewan et al., 1953; Martinsdóttir et al., 2001).

Odour	Flavour	Score	Score
Initial weak odour of boiled cod liver, fresh oil, starchy	Boiled cod liver, watery, metallic	10	10-a
Shellfish, seaweed, boiled meat, oil, cod liver	Oily, boiled cod liver, sweet, meaty, characteristic	9	9-a
Loss of odour, neutral odour	Sweet and characteristic flavours, but reduced in intensity	8	8-a
Woodshavings, woodsap, vanillin	neutral	7	7-a
Condensed milk, boiled potato	Insidid	6	6-a
Milk jug odours, reminiscent boiled clothes	Slight sourness, trace of “off”-flavours, rancid	5	5-a
Lactic acid, sour milk, TMA	Slight bitterness, sour, “off”-flavours, TMA, rancid	4	4-a
Lower fatty acids (eg acetic or butyric acids) decomposed grass, soapy, turnipy, tallowy	Strong brightness, rubber, slight sulphide, rancid	3	3-a

Table 3. Torry scoresheet for freshness evaluation of cooked fat fish such as plaice modified from (Shewan et al., 1953; Martinsdóttir et al., 2001).

Odour	Flavour	Score	Score
Butter, margarine	Meaty, shellfish flavour, slightly bitter, slight garlic flavour	10	10-b
Fatty odour, peppery	Fat reminds of herring, metallic but meaty	9	9-b
Fatty odour, “backed” odour, peppery	Spiced meat, garlic, peppery	8	8-b
Caramel, boiled potatoes, musty	Neutral, slight sweet flavour	7	7-b
Metallic, slightly sour	Insidid (towards “off”-flavour), slightly rancid, sour or bitter	6	6-b
Milk jug odours, reminiscent boiled clothes	Slight sourness, trace of “off”-flavours, rancid	5	5-b
Sour beer, TMA-ammonia, spoiled cheese	Bitter, sour, traces of TMA, rancid, “off”-flavour	4	4-b
Ammonia, very sour, drain-odour	Strong brightness, sour, spoiled fruit, rancid	3	3-b

Training panellists in sensory evaluation of cooked fish

The training of the sensory panel in sensory evaluation of cooked fillets was done using the Torry scoresheet for freshness evaluation of cooked medium fat fish such as redfish (table 2), Torry scoresheet for freshness evaluation of cooked fat fish such as plaice (table 3). Samples of different storage time were used during evaluation. Ten panellists evaluated cooked fish fillets using Torry scheme on day 1, 7, 14, and 21 of sampling, during three sessions.

Samples placed in aluminium boxes coded with three-digit random numbers were served to the panellists after cooking. Odour and flavour were rated on a scale from 10 (very fresh fillets) to 3 (spoiled fillets). Score 5,5 was used as the limit of acceptable sensory quality for human consumption, with slight sourness and trace of “off”-flavours, rancid.



Figure 6. Preparation of cooked samples. Aluminium boxes (left) and pre-warmed oven (Convothem Elektrogeräte GmbH, Eglfng, Germany) (right).

3.4 Chemical analysis

3.4.1 Determination of biogenic amines in fish with RP-HPLC

The separation of biogenic amines, including histamine, was carried out on Shimadzu HPLC instrument (Shimadzu Corporation, Kyoto, Japan), with post column derivatisation using Zorbax Eclipse C₁₈ reversed-phase chromatography column as described below:

Twenty-six Atlantic mackerel were split in five different groups, stored at different temperatures.

The first group (control) was formed by six fish kept at 1,5 °C for 24 hours and then stored at 4 °C before analysis. The second group kept at 12 °C, was formed by six fish which were then stored at 4 °C before analysis. The third group was formed by six fish kept at 21 °C and then stored at 4 °C before analysis. The fourth and fifth groups, were formed each by four fish, where two were whole and other two were filleted. The fourth group was contaminated by *Photobacterium phosphoreum* and the fifth was contaminated by *Lactobacillus casei*. After contamination, both were stored at 21 °C before analysis.

The preparation of the solution with bacteria occurred as follows: after identification and isolation, the bacteria were incubated in 10 ml of brain heart infusion (BHI), for 24 hours at 30 °C and 22 °C. From these 10 ml, 1 ml was transferred to 99 ml of salt-peptone water to make the dilution 10⁻². Further dilutions were made by transferring 10 ml to 90 ml of salt-peptone water to make the dilution 10⁻³. Finally, 20 ml were transferred to 2 L of salt-peptone water to make the dilution 10⁻⁵. That gave a solution of approximately 10³ bacteria per ml. The two solutions were dumped in two containers, where the fish and fillets were dipped for one hour (Figure 7) and then incubated at 21 °C before analysis.



Figure 7. Solution of 2 L of salt-peptone water with 10^3 *Lactobacillus casei* per ml (left) and 10^3 *Photobacterium phosphoreum* (right) where fish and fillets were dipped.

3.4.2 Sample preparation

The preparation of the sample was carried out as illustrated on Figure 8.

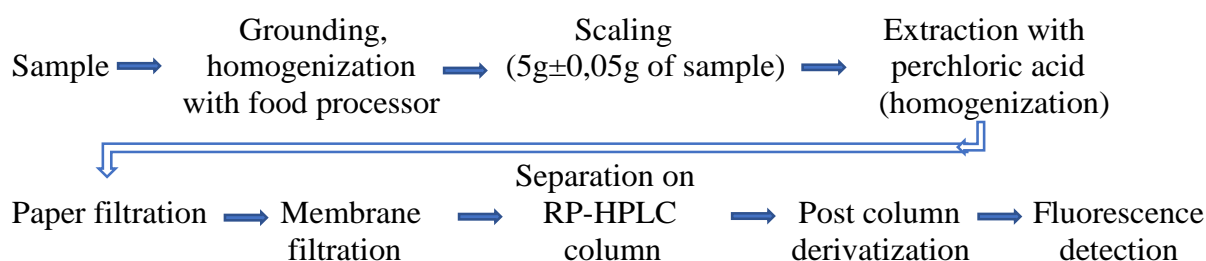


Figure 8. A schematic diagram of the sample preparation and detection.

Head, tail and guts were removed, and fish were sliced with clean, stainless steel knife. Edible parts of the fish were randomly chosen and homogenized with a food processor and transferred into plastic container. In the next step 5 g of the homogenate was weighted to the accuracy of $\pm 0,05$ g on an analytical scale in a beaker. Sample was again homogenized, now with 45ml extraction solvent (0,6 mol/l perchloric acid (HClO_4)) with a metallic staff homogenizer (T-25 digital Ultra-Turrax) for about 2 min. The extraction solvent was removed and filtered with Whatman filter papers (40, Ashless, 125 mm), followed by filtration through a membrane syringe filter (Millipore Millex-HV, hydrophilic PVDF; 0,45 μm) into an Erlenmeyer flask. The sample was then transferred into 2 ml screw cap vials (Agilent Technologies) and placed in the HPLC auto-sampler system for analysis.

3.4.3 Standards and standard stock solutions

Biogenic amines (BAs) standards were: Tyramine hydrochloride ($\text{C}_8\text{H}_{12}\text{ClNO}$), Cadaverine dihydrochloride ($\text{C}_5\text{H}_{16}\text{Cl}_2\text{N}_2$), Histamine dihydrochloride ($\text{C}_5\text{H}_{11}\text{Cl}_2\text{N}_3$), Putrescine dihydrochloride ($\text{C}_4\text{H}_{14}\text{Cl}_2\text{N}_2$) in HPLC grade. For the measurement of the target BAs

individual stock solutions were prepared with the concentration of 1 mg/ml: 182,8 mg of Putrescine dihydrochloride, 126,6 mg of Tyramine hydrochloride, 170,3 mg of Cadaverine Dihydrochloride, and 165,6 mg of Histamine dihydrochloride were measured into 100 ml volumetric flask individually and dissolved in 0,6 mol/l perchloric acid (HClO₄). 0,6 M Perchloric acid was prepared from w=60% 6 M HClO₄, also obtained from Sigma-Aldrich: 24 ml of HClO₄ was diluted in 400 ml of deionized water (> 18MΩ cm⁻¹), produced in house for chromatographic purposes with Millipore Milli-Q Academic Q-Gard[®] 1 deionizer (Millipore, Ireland). Standard mixture was prepared from the stock solutions. From each stock solution, 1 ml was measured into 10 ml volumetric flask and filled up with perchloric acid (concentration of mixture=0,1 mg/ml). Calibration curve of the standard mixture was prepared according to Table 4.

Table 4. Preparation of the calibration curve.

Dilution	Amount taken out from the mixture (ml)	Amount of perchloric acid (ml)	Concentration (mg/ml)	BA content (mg/kg) in sample
D100*	1 ml from mixture	9	0,01	100
D200	0,5 ml from mixture	9,5	0,005	50
D2000	0,5 ml from D200	4,5	0,0005	5
D4000	3 ml from D2000	3	0,0025	2,5

D100* = standard mixture of BAs is diluted 100x times

Chemical mobile phase solvents and derivatisation solution

For the gradient elution separation two solvent mixtures, eluent A and B, were prepared. In the preparation of eluent A, 8,02 g of sodium acetate (CH₃COONa) was dissolved in 800 ml of purified water. The solution was pH-adjusted with acetic acid (C₂H₄O₂, w=100%) to 4,5±0,1. Next, 2,16 g of sodium-1-octane sulfonate (CH₃(CH₂)₇SO₃Na·H₂O) was added to the solution and the solution diluted up to 1.000 ml with purified water. To prepare eluent B, 12,73 g of sodium acetate and 600 ml of deionised water was mixed. The pH was adjusted to 4,5±0,1 with acetic acid (C₂H₄O₂, w= ca. 100%) followed by an addition of 2,16 g of sodium-1-octane sulfonate and 230 ml of acetonitrile (CH₃CN). In the final step, the solution was diluted to 1.000 ml with deionised water in a volumetric flask. Both eluent A and B were vacuum filtered with 0,4 µm HTPP, Isopore Membrane filter and sonicated for 15 min prior to use as mobile phase on the HPLC.

The derivatisation solution was prepared by mixing the following substances, 3g BRIJ® L23 detergent (polyoxyethylenlaurylether) with 1 g of o-phthalaldehyde (OPA) reagent that was dissolved in 10 ml of methanol (CH₃OH), followed by addition of 1l of borate buffer as well as 3 ml of 2 mercaptoethanol. Borate buffer was prepared from 61,8 g of boric acid and 40 g of potassium hydroxide diluted to 1000 ml with purified water.

HPLC quantification of BAs using post-column derivatisation with OPA

The separation of BAs was carried out on Shimadzu HPLC instrument (see Figure 9), the details regarding the instrument used are listed in Table 5, using Zorbax Eclipse C18 reversed-phase chromatography column. The stationary phase consists of an ultra-high purity silica support (SiO₂), packed by a dense monolayer of dimethyl-n-octadecylsilane. The column is compatible with water and all organic solvent. For the protection of the column, a guard column was used with the same stationer phase to ensure a longer lifetime of the analytical column.

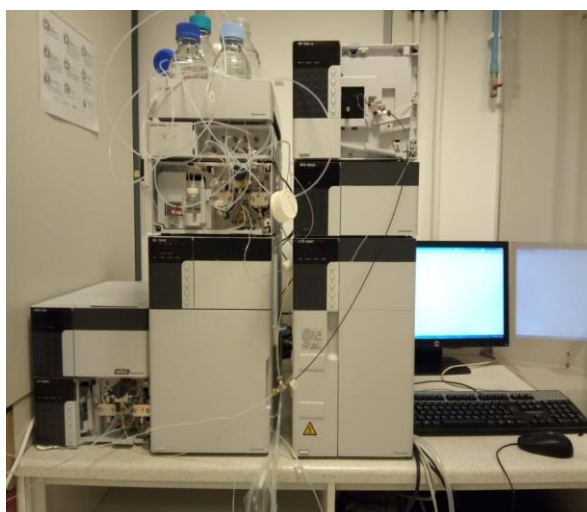


Figure 9. Shimadzu HPLC system used in this project for BAs analyses.

Table 5. Details of the HPLC instrument used for analysis of BAs.

Module	Type of module
Fluorometric detector	DGU-20A38/20A5R
Degassing unit	DGU-20A38/20A5R
Auto sampler	SIL-30AC
Column oven	CTO-20A/20AC
Solvent delivery system (pump A)	LC-30AD
Solvent delivery system (pump B) for post column derivatisation	LC-20AD
System controller	CBM-20A
Analytical column	ZORBAX Eclipse Plus C18 (4.6x250mm 5 – micron) (Agilent)

Samples were prepared according to the description section 3.4.2 and 10 μ l of the final extract was injected by the autosampler of the HPLC onto the Zorbax Eclipse column. A gradient elution separation was applied to decrease the retention of later-eluting components and to provide a better and sharper peak shape without tailing effect (common problem in isocratic elution). The gradient consisted of a binary mixture of eluent A and B prepared as explained in section “Chemicals mobile phase solvents and derivatization solution”. The mixture which contained ion-pairing reagent to control the retention strength was pumped with pump A with the flowrate of 0,926 ml/min. Gradient program in which acetate buffer and increasing proportion of acetonitrile were used is shown in Table 6.

Table 6. Time program applied for the separation of BAs.

Time (min.)	Ratio of eluent B	Event
0,01	15%	Start injecting
0,02		Zero
20,0	40%	
30,0	65%	
34,0	65%	
34,1	100%	
50,0	100%	
50,1	15%	
51,1		Rinse
60,0		End

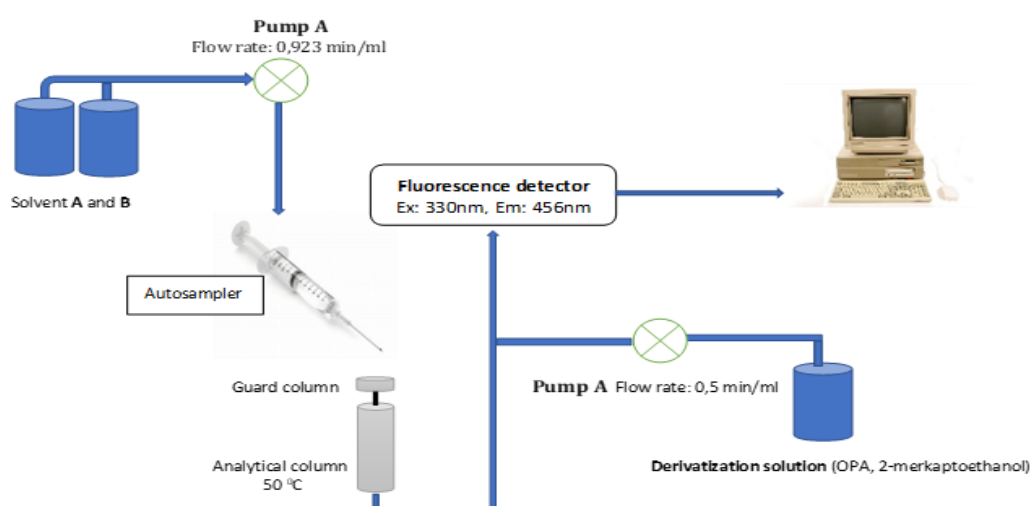


Figure 10. Schematic flow chart of post-column derivatisation used in current verification study.

Prior to fluorescent detection (excitation: 330 nm, emission: 465 nm), online post-column derivatisation took place: separated BAs were converted into fluorescent OPA-derivatives (see Figure 10 and Figure 11). The prepared derivatising solution was supplied with a flow-rate of 0,5 ml/min by pump B and pumped through a 50 cm long reaction loop, where temperature control was not necessary.



Figure 11. Derivatisation reaction of BAs with OPA.

After analysis of BAs, the analytical column was flushed with water: methanol in the ratio of 50:50% 5-10x of the column volumes to avoid any precipitation. The column volume was calculated by means of the following equation: $V = \pi r^2 L$, where V=column volume in ml; r= column radius in cm; L=column length in cm. During cleaning, the flowrate was 0,5 ml/min, approximate the 1/2-1/5 of the typical flowrate (here 0,926 ml/min).

Calculation of the results

The identification of the amines was carried out by the comparison of the retention times (RT) with those of standard substances. The quantitative determination was carried out according to the method of external standards and integrating peak area in relation to the values of the standard substances. The integration of the area was performed by LabSolution (Version 5.51; Shimadzu Corporation) which is the Shimadzu HPLC system software. The evaluation of the concentrations was done by using Microsoft Excel. By means of linear equation of the regression line: $y = ax + b$ where y= area measurement in mAbs; x= concentration of the substance in mg/kg; a = slope of regression curve and b = y-intercept. The amine contents were calculated in mg/kg on a wet weight basis in fish flesh and on a product basis in fish meal.

3.5 Data analysis

Microsoft Excel 365 was used to calculate means of the QIM, to plot graphs for all results against the storage time, to fit linear correlation equation on QIM graph and calculate the correlation coefficients (R^2) of QI. The same was used to calculate means of the concentrations of BAs and to plot graphs of concentrations of BAs.

4 RESULTS

4.1 Sensory evaluation of whole Senegalese sole

A preliminary QIM scheme for farmed Senegalese sole was developed during the pre-observation sessions by four sensory assessors. The QIM scheme for farmed Senegalese sole was then developed further and finalised during panellists training sessions.

4.1.1 *Pre-observation results*

Changes were observed in the appearance of Senegalese sole during 1, 6, 8 and 13 days of storage in ice (Figures 12, 13, 14 and 15). The changes in parameters skin, texture, eyes and gills were clear. With storage time skin spots on the ocular side faded and became unclear, appearance on the ocular side become dull, pale, and purple discoloration was evident (seen at the edges of the fins), the skin became shrunken and contained slight yellowish discoloration. The mucus on the skin became clotted, slightly yellow, thicker and drier. The appearance on the blind side became slightly duller and purple discoloration at the edges of the fins. The parameter texture became less firm and less elastic.

At the beginning of storage, the eyes were flat and the eye socket convex, with black and clear pupil, golden rim around the pupil, brown and grey bluish iris. They became sunken and/ or swollen, eye socket more shrunken with rather matte pupil, faded golden rim, pinkish/ purple colour of the iris on day 13 of the storage (Figures 12, 13, 14 and 15).

The gills were at the beginning fresh or neutral with dark purple/dark red colour. With storage time they became grassy, slightly sour/ fermented with faded colour and slightly discoloured.

Table 7. Preliminary QIM scheme developed for Farmed Senegalese Sole after pre-observation.

Quality parameter		Descriptors/demerit points	Score
Skin	Spots on ocular side	Very clear, light	0
		Less clear	1
		Faded / unclear	2
	Ocular side	Dark colour, bright shiny appearance. No discoloration	0
		Rather dull or pale, colour slightly faded or colour slightly paler, somewhat shrunken skin	1
		Dull, pale, purple discoloration (seen at the edges of the fins), shrunken skin evident. Slightly yellowish discoloration	2
		Pale, dull, yellowish shrunken skin evident	3
	Blind side	Bright white, no discoloration / dark colour, bright shiny appearance (if no white area on blind side)	0
		Some purple discoloration at the edges of the fins	1
		Dull, purple, yellow discoloration at fins and in the middle	2
	Mucus	Abundant and almost clear/ translucent, not clotted	0
		Slightly clotted and milky	1
		Clotted and slightly yellow	2
		Clotted yellowish and brownish, less mucus	3
	Odour	Neutral	0
		Grassy, metallic	1
Sour, fermented		2	
Rotten		3	
Texture (ocular side)	Very firm and stiff when lifted (In-rigor)	0	
	Firm, elastic	1	
	Less firm, less elastic	2	
	Rather soft, reduced elasticity	3	
Eyes	Form	Flat eye, eye socket convex	0
		Slightly sunken, eye socket sunken	1
		Sunken and/ or swollen, eye socket shrunken	2
	Clarity/ brightness	Black and clear pupil, golden rim around the pupil, bluish/whitish at the top of the eye	0
		Rather matte pupil, faint golden rim around the pupil	1
		Matte and milky pupil, pinkish/yellowish colour of the rim around the pupil	2
Gills	Odour	Fresh or neutral	0
		Metallic, cucumber	1
		Grassy, slightly sour / fermented	2
		Rotten, sour, sulphurous	3
	Colour	Dark purple/dark red	0
		Slightly faded colour, gill slightly yellow near the gills	1
		Faded colour, slightly discoloured	2
		Yellowish, brownish discoloration	3
	Mucus	Mucus almost clear and transparent	0
		Milky and slightly clotted	1
Yellowish, thick, clotted		2	
Quality index			(0-28)

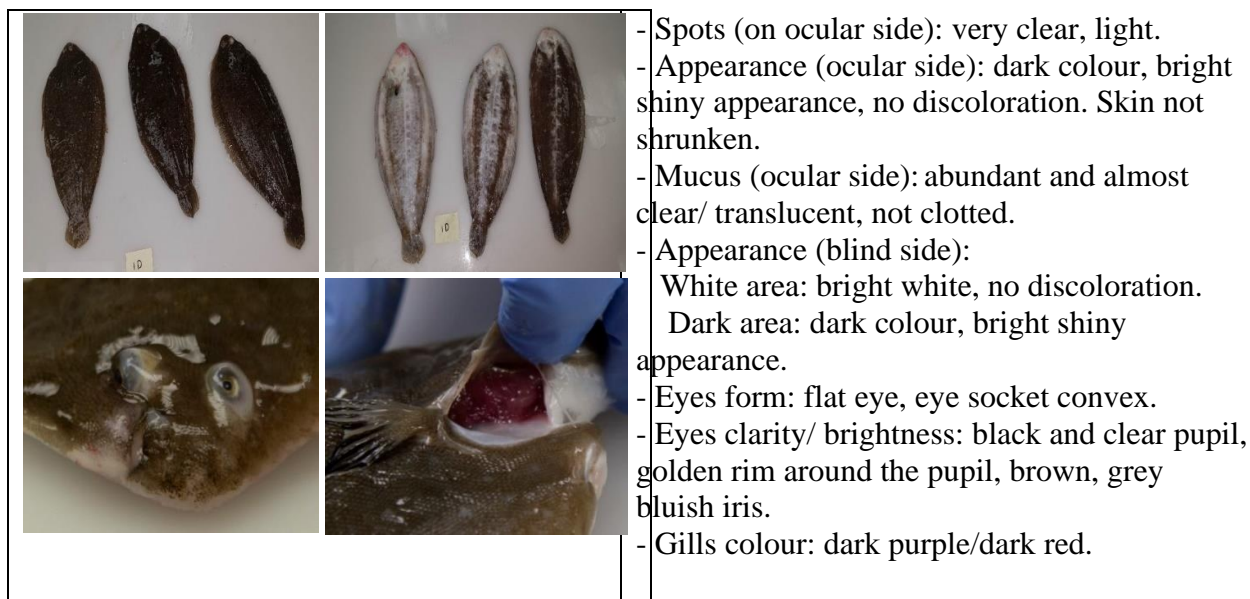


Figure 12. Senegalese sole stored 1 day in ice.

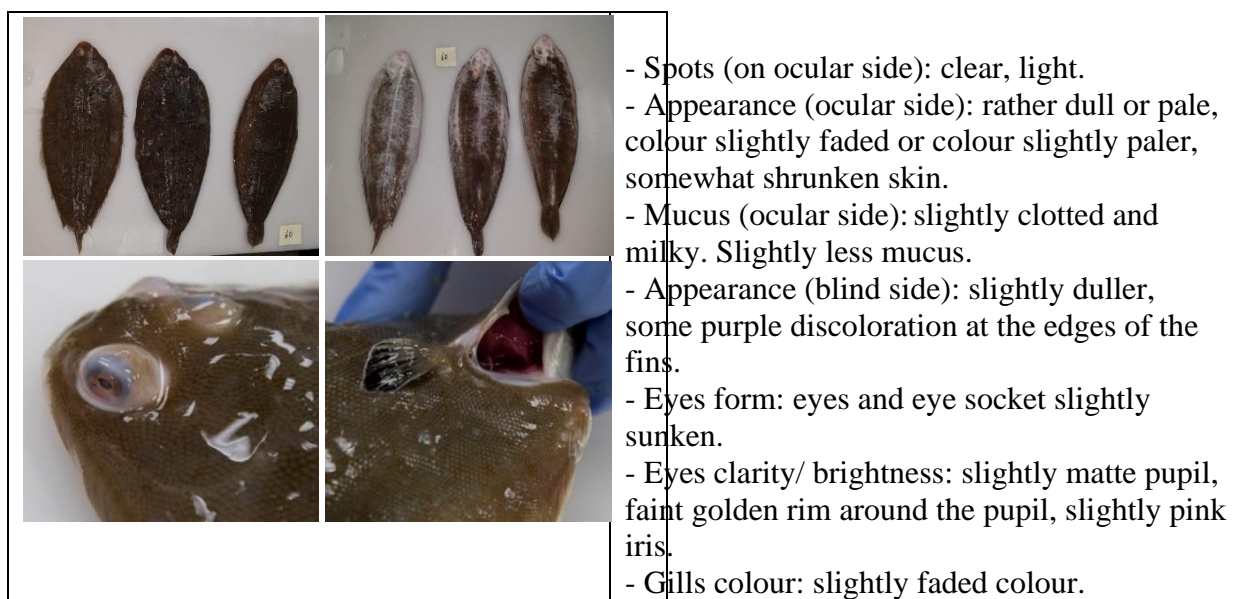


Figure 13. Senegalese sole stored 6 day in ice.

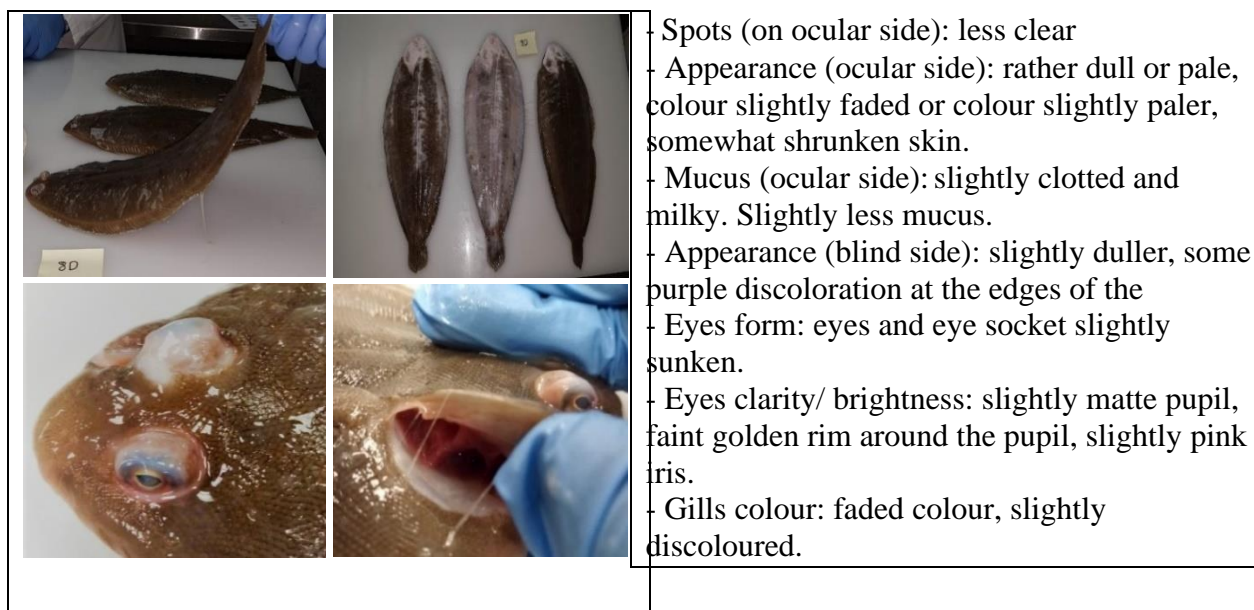


Figure 14. Senegalese sole stored 8 day in ice.

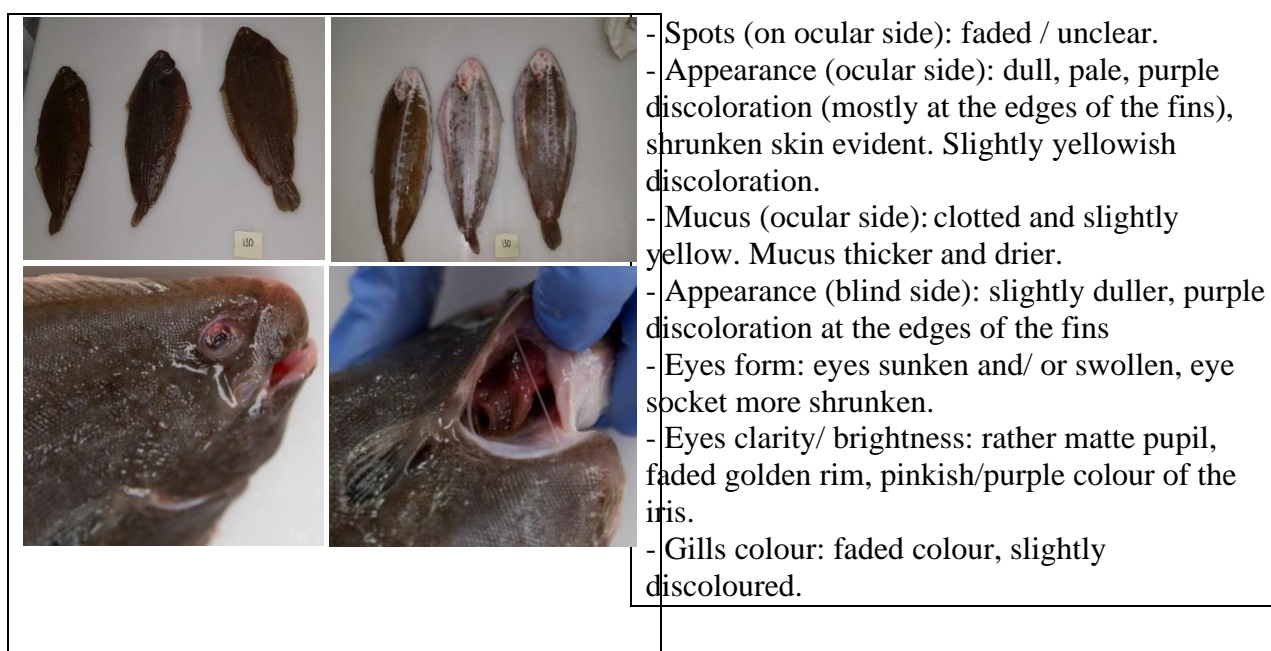


Figure 15. Senegalese sole stored 13 day in ice.

4.1.2 Panel training results

The QIM evaluation was carried out with 10 panellists. During the first session, the panellists worked with the scheme developed during pre-observation (preliminary QIM scheme). The preliminary QIM scheme was changed in the descriptions corresponding to skin (appearance on the ocular side, mucus, odour and appearance on the blinde side), eyes, gills (colour and mucus). The quality index (QI) was reduced from 0-28 to 0-26 points (table 8).

The QI based on an average from the whole panel was calculated for each storage day of the Senegalese sole and formed linear relationship with storage time (Figure 17). QI was around 16 on day 20 of storage which was the day the panel judged the Senegalese sole unfit for human consumption by Torry score. After these changes, the preliminary QIM scheme version of Senegalese sole was finalised. Table 8 shows the suggested new QIM scheme developed for Farmed Senegalese sole after training.

Table 8. QIM scheme developed for Farmed Senegalese Sole after training.

Quality parameter		Descriptors/demerit points	Score
Skin	Spots (ocular side)	Very clear, light	0
		Less clear	1
		Faded / unclear	2
	Appearance (ocular Side)	Dark colour, bright shiny appearance, no discoloration. Skin not shrunken	0
		Rather dull or pale, colour slightly faded or colour slightly paler, somewhat shrunken skin	1
		Dull, pale, purple discoloration (mostly at the edges of the fins), shrunken skin evident. Slightly yellowish discoloration	2
		Pale, dull, yellowish. Shrunken skin evident	3
	Odour (ocular side)	Neutral, weak	0
		Grassy, cucumber	1
		Slightly sour, dirty table cloth	2
		Sour, fermented, rotten	3
	Mucus (ocular Side)	Abundant and almost clear/ translucent, not clotted	0
		Slightly clotted and milky. Slightly less mucus	1
		Clotted and slightly yellow. Mucus thicker and drier	2
		Clotted yellowish and brownish. Mucus thicker and almost dry	3
	Appearance (blind Side)	White area: bright white, no discoloration. Dark area: dark colour, bright shiny appearance	0
		Slightly duller, some purple discoloration at the edges of the fins	1
		Dull, purple, yellow discoloration at fins and in the middle	2
Texture (ocular side)	In rigor, very firm and stiff when lifted	0	
	Firm, elastic	1	
	Less firm, less elastic	2	
Eyes	form	Flat eye, eye socket convex	0
		Eyes and eye socket slightly sunken	1
		Eyes sunken and/ or swollen, eye socket more shrunken	2
	clarity/ brightness	Black and clear pupil, golden rim around the pupil, brown, grey bluish iris	0
		Slightly matte pupil, faint golden rim around the pupil, slightly pink iris	1
		Rather matte pupil, faded golden rim, pinkish/purple colour of the iris	2
	Matte and milky pupil, faded golden rim, pinkish/yellowish colour of the iris	3	
Gills	Colour	Dark purple/dark red	0
		Slightly faded colour	1
		Faded colour, slightly discoloured	2
		Yellowish, brownish discoloration	3
	Odour	Fresh or neutral	0
		Metallic, cucumber	1
		Grassy, slightly sour / fermented	2
		Rotten, sour, sulphurous	3
Quality index			(0-26)

The QI was calculated for each storage day in the panel training which indicated, in general, a clear linear relationship to time in ice storage (Figure 17).

The results on each quality attribute are presented as averages of all panellists. QIM assumes that score for all quality attributes increase during the storage time of iced Senegalese sole. The rate of increase was variable for different attributes, in some of them the increase was less pronounced than the other such as spots on ocular side and eye form.

For the remaining attributes, the change was more pronounced. Farmed Senegalese sole stored in ice was evaluated. In the parameter describing presence of spots on ocular side of skin from day 1 to 7 there were very increased changes, the spots were less clear and changed to faded/ unclear. From days 7 to 13 and 15 to 20 the spots were very decreased, i.e., were less clear, while from days 13 to 15 and 20 to 21 they were increased from less clear until being faded/ unclear, as shown in figure 16. The changes on the skin appearance on ocular side and the presence of mucus were very increased between day 1, day 2 and day 7, decreased between day 7 to 8, after day 8 they increased until day 15. From day 15 to 20 the skin appearance increased, and the mucus remained constant. The odour of skin increased from 1 to 2, 7 to 13 and 15 to 21 days starting with a neutral/ weak smell which increased linearly with storage time until sour, fermented/ rotten smell.

A small difference in the appearance of the skin appearance on the blind side was observed. On day 1 the characteristics were on white area: bright white, no discoloration, and on dark area: dark colour, bright shiny appearance these characteristics maintained until day 7, which were increased until the end, being dull, purple, yellow discoloration at fins and in the middle.

The parameter texture on ocular side increased from 1 to 2 and 8 to 21 days. Only remained constant between 2 to 8 days. The texture was between firm/ elastic and less firm/ less elastic. The eye form on days 1 to 2 and 13 to 15, showed constancy in the form of eyes. From 2 to 8 and 15 to 20 an increase was observed, while from 8 to 13 and 20 to 21 days a decrease was observed. The variation was between flat eye, eye socket convex and eyes sunken and/ or swollen, eye socket more shrunken. The eyes clarity/ brightness had a large variation of characteristics, causing an oscillation so that days 1 to 2, 7 to 8 and 13 to 20 were increased.

The gill colour and gill odour were decreased between days 1 to 2. From day 2 until 15 for colour, and until 21 for odour, an increase was observed. The colour varied from dark

purple/dark until yellowish, brownish discoloration. The odour initially was fresh or neutral until rotten, sour, sulphurous.

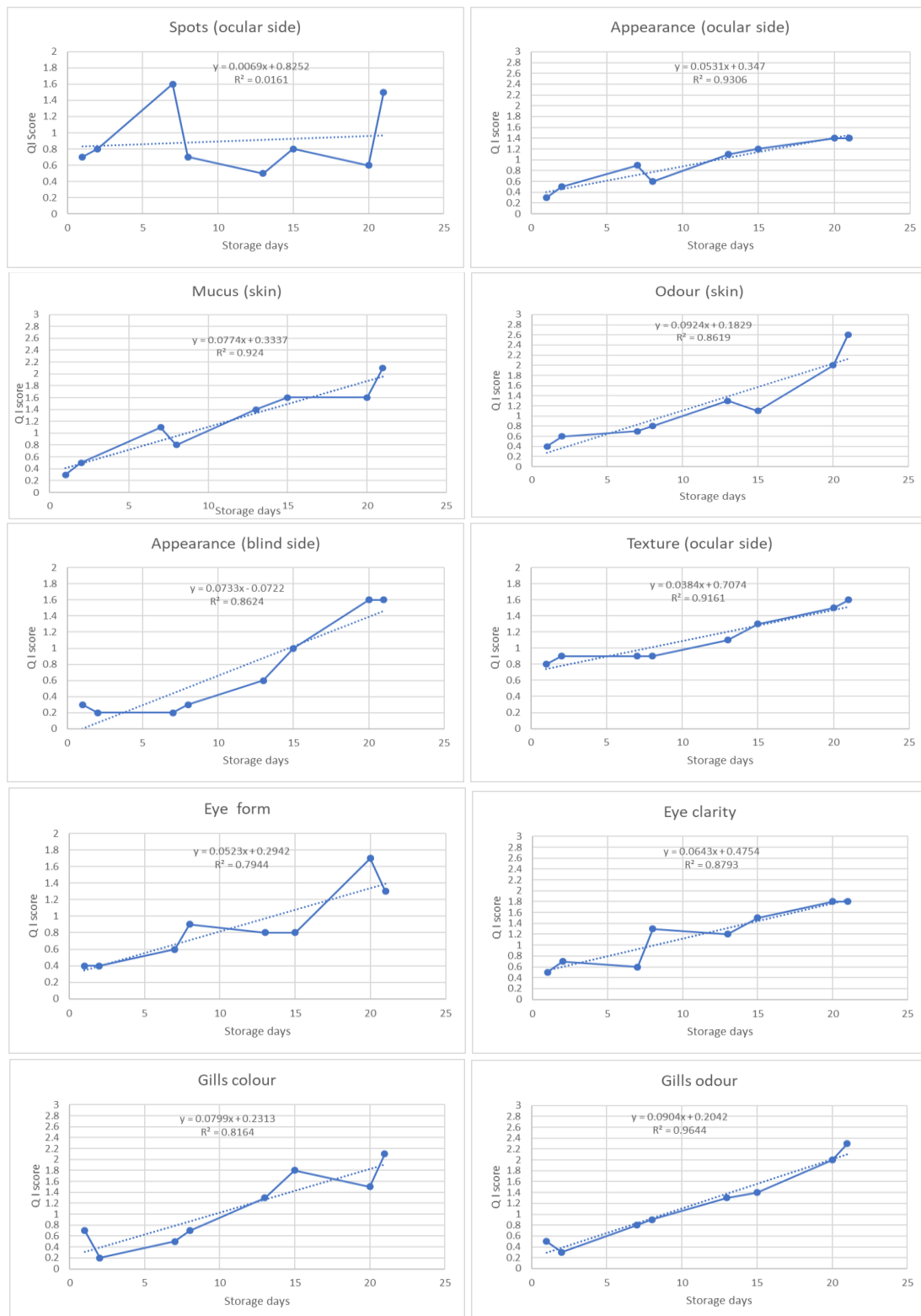


Figure 16. Average score for each parameter evaluated with QIM scheme developed for Farmed Senegalese sole stored in ice after training. UNU Fisheries Training Programme

During the last training day, the panellists were in more agreement (lower variation between panellists). This indicated that the panellists agreed more when evaluating the samples at the middle and end of storage time.

The Quality Index (QI) based on an average from the panel (10 panellists) was calculated for each trial day of storage (1, 2, 7, 8, 13, 15, 20 and 21). There was a strong linear relationship with significant correlation ($R^2 = 0.969$) between the average QI for each storage day and storage time in ice (Figure 17).

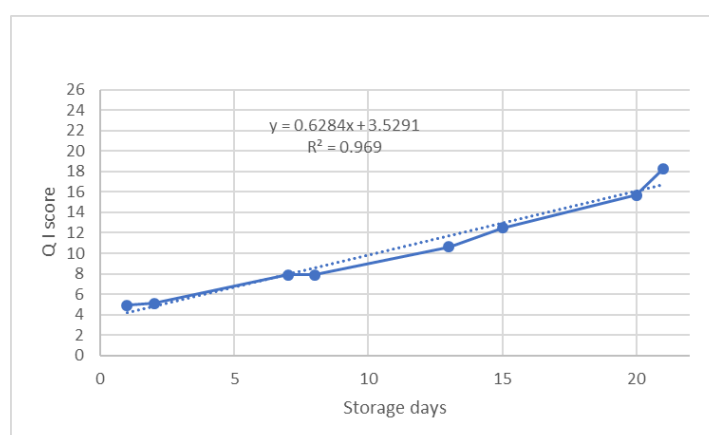


Figure 17. Quality Index of whole farmed Senegalese sole over days in ice storage.

4.1.3 Sensory evaluation of cooked fillets using Torry scheme

Sensory evaluation of cooked fillets occurred in two parts: pre-observation of cooked fillets, made by assessors, and panel training, made by panellists. Using the Torry scheme assessors and panellists evaluated odour and flavour.

Pre-observation sessions produced a list of words that described the quality parameters of odour and flavour of cooked Senegalese sole (Table 9). The Torry scores range from 10 (very fresh) to 3 (very spoiled) and score 5,5 was used as the limit for human consumption. Torry scores reduced with storage time during the evaluation of cooked Senegalese sole samples.

Fillets from 21 days of storage were observed to have high spoilage characteristics during the Torry evaluation. At the beginning of storage, freshness attributes describing the odour and flavour during the first 8 storage days were dominant and decreased towards end of storage time. Most attributes indicating odour spoilage were detected at the final day of storage, while the attributes indicating flavour spoilage were slightly detected from 8 days of storage and beginning to increase to the final day of storage. The results of pre-observation of cooked fillets,

made by assessors, indicate that neither Torry for medium fat nor fat were very good for Senegalese sole (Table 9).

Table 9. Sensory characteristics of cooked farmed Senegalese sole as evaluated with Torry score sheets for fat (-a) or medium fat (-b) fish.

Storage days	Odour	Score	Flavour	Score
Day 1	Butter, margarine	10-b	Oily, boiled cod liver, sweet, meaty, characteristic	9-a
Day 8	Boiled potatoes	6-a	Neutral, slightly sweet flavor	7-b
Day 13	Metallic, not sour	6-b	Neutral, slightly sweet flavor	7-b
Day 15	Metallic	6-b	Almost neutral	7-a
Day 21	Sour beer, TMA-ammonia, spoiled cheese	4-b	Slight bitterness, sour, "off"-flavor, TMA, rancid	4-a

10-b* = description corresponding to score 10 for Torry scoresheet for freshness evaluation of cooked medium fat fish such as redfish shown in Table 2.

6-a* = description corresponding to score 6 for Torry scoresheet for freshness evaluation of cooked fat fish such as plaice shown in Table 3.

4.2 Determination of biogenic amines in fish

The experiment was carried out with Atlantic mackerel which were split into five different groups and stored at different temperatures.

4.2.1 First group (control)

The first group (control) was kept at 1,5 °C for one and two days and then stored at 4 °C before analysis. After 26 hours, the first sample was harvested and the second and third harvest were carried out 50 and 74 hours later. No BAs were detected.

4.2.2 Second group

The second group was divided into two subgroups kept at 12 °C respectively for one (b3) and for two (b5) days long prior to storage at 4 °C before analysis. Samples were taken after 26, 50 and 74 hours. As figure 18 shows, the amount of BAs is higher in the subgroup, kept for two days at 12 °C compared to that one which was kept just for one day. Prolonged storage time at 4 °C shows no effect on the BA content. The concentration of histamine has only reached 1 ppm and no formation was detected in subgroup b3 26.

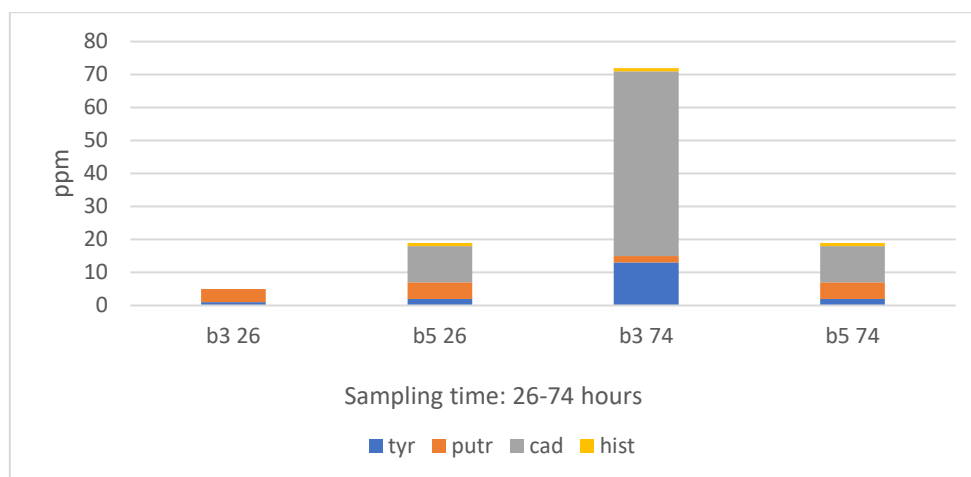


Figure 18. Concentration of BAs in uncontaminated fish kept at 12 °C and stored at 4 °C.

b3 26* = description corresponding to one sample kept at 12 °C for one day (b3), for two days (b5) and stored at 4 °C and then analysed after 26 hours (26), 74 hours (74). **BAs*** = Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

4.2.3 Third group

The third group was kept at 21 °C respectively for one (c1) and for two (c2) days and then stored at 4 °C. Samples were taken after 26 hours (26), 50 hours (50) and 74 hours (74) and then analysed (Figure 19).

In the sample, kept at 21 °C for one day (c1 26) there was no formation of BA, but in the following days the increase in the concentration was observed for all samples. In the concentration of histamine, there was an increase during these two days (Figure 19).

In the sample kept for two days the concentration of BA showed a higher amount than in the sample kept for one day. Sample abused by moderate temperature for long time showed no significant difference in BAs through the prolonged storage against the other sample.

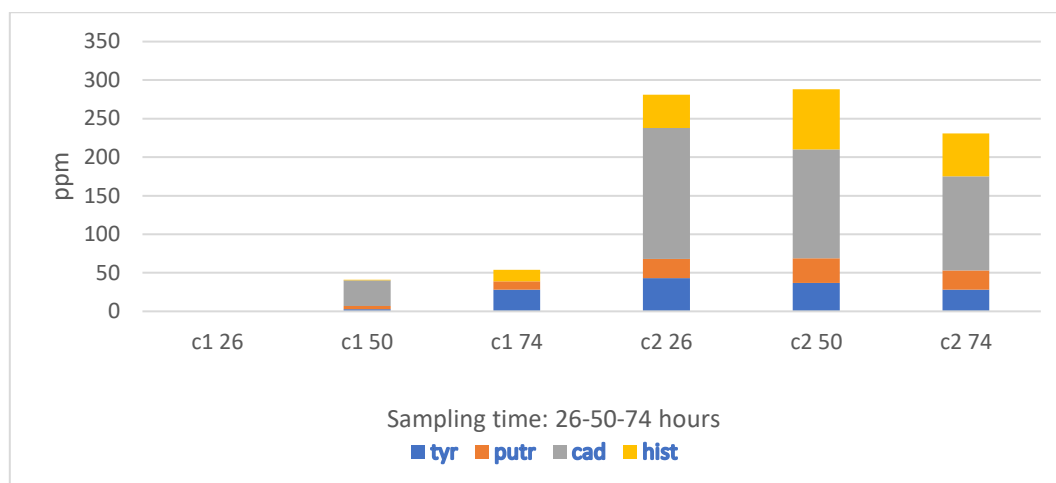


Figure 19. Concentration of BAs in uncontaminated fish kept at 21 °C and stored at 4 °C. Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

4.2.4 Fourth group and fifth group

In the fourth and fifth groups (Figures 20 and 21) both whole fish and fillets were contaminated by two different species of bacteria, such as *Photobacterium phosphoreum* (pw / pf) and *Lactobacillus casei* (lcw / lcf). After the contamination (one hour) samples were kept at 21 °C respectively for one and two days and were analysed after 26 hours (26) and 50 hours (50).

By analysing the whole fish samples, contaminated by *Photobacterium phosphoreum* (pw) and *Lactobacillus casei* (lcw), it may be noted that there was an increase of BAs formation from the first to the second day. On the first day samples contaminated by *Lactobacillus casei* (lcw) contained the BAs in a higher concentration (107 ppm) in relation to samples contaminated by *Photobacterium phosphoreum* (pw) (49 ppm) (see Figure 20). On the second day there were no significant differences in BA content between the two species (133-134 ppm).

The concentration of histamine was very low both days. On the first day no histamine was detected in the case of *Lactobacillus* and only 1ppm in the case of *Photobacterium*. On the second day again *Photobacterium* proved oneself to be better in histamine formation (7 ppm) versus *Lactobacillus* (3 ppm). In the filets contaminated by *Photobacterium phosphoreum* (pf) 10 x more BA were found than in whole fish. First day 552 ppm was formed from which 61 ppm was histamine and on the second day the BA content was 1029 ppm including 554 ppm histamine. In the case of *Lactobacillus casei* (lcf), there was also an increase in BAs formation

from the first (75 ppm) to the second day (793 ppm) (Figure 21). The concentration of histamine increased from 0 ppm up to 256 ppm during these two days (Figure 21).

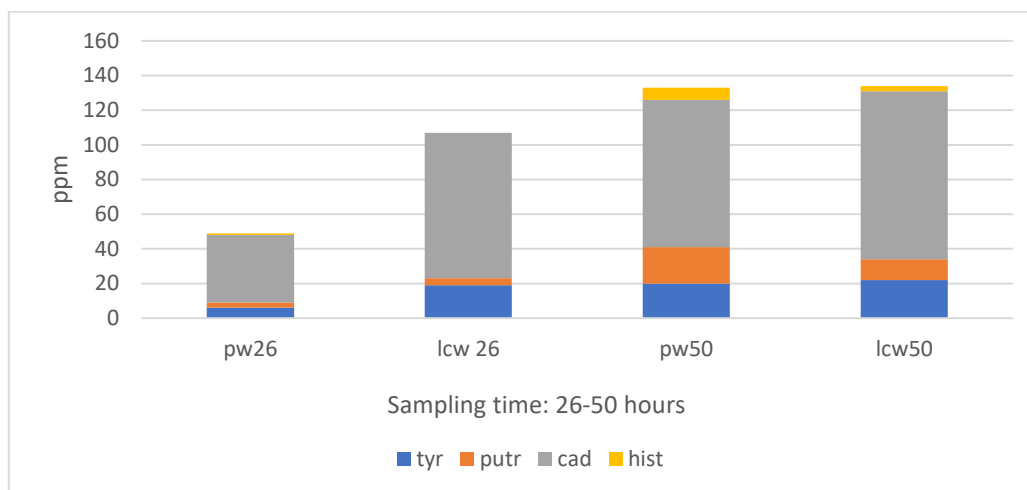


Figure 20. Concentration of BAs in whole fish contaminated by *Photobacterium phosphoreum* and by *Lactobacillus casei* and stored at 21 °C for one and two days. Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

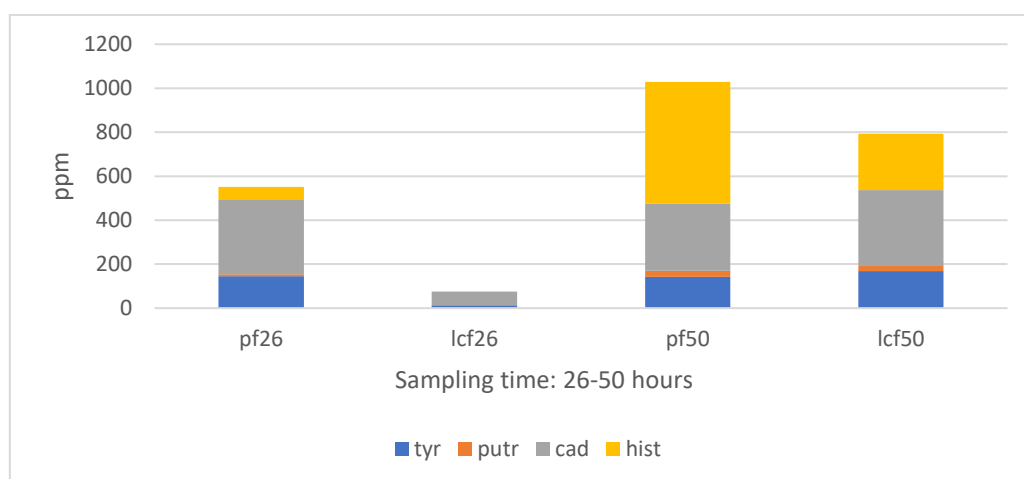


Figure 21. Concentration of BAs in fillets contaminated by *Photobacterium phosphoreum* and by *Lactobacillus casei* and stored at 21 °C for one and two days. Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

5 DISCUSSION

5.1 Sensory evaluation of Senegalese sole

During the pre-observation and panel training sessions, the spots on ocular side were difficult to evaluate. They were less clear at the beginning of storage and on seventh day of storage they were faded/ unclear. Around storage for 8-20 days, the spots were still less clear, but at the last storage day they were faded/ unclear, reaching the average score of 1,5 of maximum score of 3 for this parameter. For common sole and Senegalese sole, both farmed, the spots are normal (FAO, 2014). The observation of different shades of spots on the skin of different fishes was noted and for some other fishes it was difficult to observe these spots even on the first day of storage. Once the panellists encountered fish with less clear spots and others faded/ unclear, it is normal that there were different shades of skin spots. Kraup (2006) had reported that Senegalese sole had numerous small spots in life, tending to disappear on death.

The average score of skin odour reached 2,6 at the end of the storage time, which is close to the maximum score of 3 for this parameter. At the beginning of the storage time when the Senegalese sole was very fresh, the odour was described as neutral or weak, then a grassy, cucumber-like odour dominated the Senegalese sole skin odour. During later stages, the odour was described as slightly sour or dirty table cloth and finally as sour, fermented and rotten. Freshly caught fish generally contains low levels of volatile compounds like 2,6-nonadienal, which has a very characteristic cucumber aroma and a low odour threshold (0,001 ppb), which contribute to fresh-like odours. Short-chain acids, alcohols, amines, and sulphur compounds from microbial activity probably caused the sour and rotten odour (Sveinsdóttir et al., 2009). Sensory attributes including sour, condensed milk, off-flavour become event after extended storage time and are indicators of spoilage whereas sweet, characteristic and seaweed attributes indicate freshness (Sveinsdóttir et al., 2002).

The evident changes were found in the skin appearance (blind side), eyes clarity, mucus skin, as well as in the gills (colour and odour) (Figure 16), based on the steady increase of the scores for each attribute with storage time. This is in accordance with how the QIM scheme is constructed, where seafood evaluated shortly after catch should be scored low and subsequently increase with storage time reaching close to maximum score at the end of shelf life (Martinsdóttir et al., 2001).

The texture was found to be rather difficult to evaluate during training sessions. The loin was sometimes firm, and sometimes less firm, and appeared not to be related to storage time. The tail part was firm throughout the storage time. Therefore, it is important to always evaluate the texture at the same location on the fillet and give detailed guidelines to panellists. These guidelines include recommendations to evaluate the texture in the middle of the spine muscle at ocular side, by pressing a finger firmly on the skin. By raising the finger a little from the caudal fin and then lowering and observing if and how fast the flesh recovers, the texture firmness was evaluated. Using this method, the score around 1 (of 2 maximum score) with description firm / elastic was frequent until storage day 15, and for storage days 20 and 21 reached score around 2 (less firm / less elastic). Propagation of rigor caused the muscle to relax again, and through storage in ice, the flesh became less firm or elastic, which may be due to autolysis influenced by both fish muscle enzymes and microbial enzymes (Sveinsdóttir et al., 2002).

In the case of eyes, the scores were increased with the storage time. Some improvement was observed and a significant linear increase with storage was achieved. The subjective QIM developed for many species has demerit points if the eyes become cloudy and the initial clear black colour changes to a whitish cloudy appearance (Kyra et al., 1997).

During the panel training sessions, the attribute gills mucus was omitted from the scheme as was difficult to evaluate because of the abundant skin mucus that was penetrating into gills during the storage time. Additionally, one score was added for clarity/brightness of the eye. Changes were made in the setup of the scheme and selection of words to describe the parameters in the scheme, mainly to make each description more precise and to facilitate the QIM assessment. This is in agreement with Sveinsdóttir et al. (2003) who reported removal from the scheme of parameters whose evaluation was destructive and difficult.

The deterioration of iced Senegalese sole was observed during the pre-observation and panel training sessions with 28 and 26 points of total sum of scores. The QIM scheme that is suggested for farmed Senegalese sole, based on this study, consists of 10 parameters (Table 8), resulting in a total of 26 demerit points. There was a strong linear relationship with significant correlation ($R^2 = 0,969$) between the average QI for each storage day and storage time in ice (Figure 17). Gonçalves et al. (2007) found a significant correlation ($R^2 = 0,878$) between the average QI for each storage day and storage time in ice for whole raw farmed Senegalese sole. This shows that the attributes gradually deteriorated with time and results of quality index score

increase linearly with storage time as recommended in the manual for sensory evaluation of fish freshness (Martinsdóttir et al., 2001). Also, all individual attributes analysed indicated, in general, a clear linear relationship with storage time.

The results of pre-observation of cooked fillets, made by assessors, indicate that the sensory attribute descriptors of Torry for medium fat or fat did not describe the Senegalese sole sufficiently (Table 9). However, after 15 days, the fish was not spoiled, but was clearly spoiled after 21 days of storage, according to the sensory evaluation of the cooked fish using the Torry scheme. The results indicate a longer shelf life of Senegalese sole than reported by Gonçalves et al. (2007), who found the shelf life to be 15 days in ice. According to Martinsdóttir et al. (2001) the shelf life of fat fish such as plaice was estimated as 13 days, and for sole, the shelf life was estimated as 15 days.

Freshness parameters such as texture, odour and flavour are used in the Torry freshness scoring to determine the sensory response of cooked fish samples (Martinsdóttir et al., 2001). Martinsdóttir et al. (2009b) state that the attribute to be assessed must be clearly defined and understood and in all cases the assessors require intensive training and a detailed briefing before each session. People are very sensitive to various compounds produced in fish during storage, and especially spoilage, such as several sulphur and nitrogen compounds.

5.2 Determination of biogenic amines

As expected, BAs/histamine was not detected in the control group stored at 1,5 °C for three days. These results are in accordance to the results of Barbuzzi et al. (2009), who did not detect histamine in Atlantic mackerel stored under same condition until 10 days. The Atlantic mackerel used in the present study was stored before use at -25 °C for five months. Oucif et al. (2012), demonstrated that the accumulation of histamine in Atlantic mackerel exceeds the threshold, only after 5 days stored at 4 °C and never at -18 °C.

In the sample kept for one day at 12 °C (b3) the histamine did not appeared after 26 hours storage just after 74 hours (Figure 18). According to James et al. (2013) at 10 °C, the concentration of histamine in many species begins to increase after 2 and 3 days, respectively, and increases more significantly throughout longer storage period. The muscle tissues of fresh fish from at-risk species contain very little, if any, histamine. Histamine formation occurs in spoiled fish flesh that is high in histidine and contaminated with histamine-forming bacteria (James et al.,2013).

For the sample kept at 12 °C for two days (b5) there was no quantity variation of concentration of histamine. Grau et al. (2003) has shown that in Atlantic mackerel stored at 0 °C the histamine content is constant and low (0,3-0,6 ppm) during the first thirteen days. Gutted and ungutted mackerel were below the FDA action level of histamine for one day stored at 11°C (Lokuruka & Regenstein, 2007). The same authors noticed that the concentration of BAs (histamine, cadaverine, putrescine, spermine, spermidine) were significantly different at 11°C irrespective of whether the fish were gutted, skin contaminated with guts, or untreated. Some scombrototoxic bacteria may be endogenous to the gut. Both skin and guts are potential sources of scombrototoxic bacteria (Lokuruka & Regenstein, 2007).

In the third group, where the sample was kept at 21 °C for one day (c1 26) there was no formation of BAs after 26 sampling hours, but in the following 50 and 74 hours, the increase in the concentration was observed and histamine level also increased, but did not reach the level of 100 ppm, which, according to Antoine et al. (2002) is normally expected in wholesome fish. Grau et al. (2003) reported that an initial histamine level (20 ppm tissue) did not increase up to 10 days and rose to 100 ppm after 2 weeks of storage.

At the same time, sample kept at 21 °C for two days (c2) the level of histamine increased rapidly. After 74 hours, the levels reached 50 ppm. Antoine, et al. (2002) considers 50 ppm the defect action level (DAL), which implies that some degree of bacterial decomposition has been occurring in the fish. High variation in histamine levels were observed (Figure 19) because histamine is unevenly distributed in the muscle tissue of fish (Antoine, et al., 2002). Histamine formation can be very fast, even within two to three hours at temperatures above 20 °C (James et al., 2013). The level of histidine in fish species depends on the activity patterns of that particular species. High levels are associated with active “high-speed pelagic swimmers with outstanding sprint capability”, such as mackerel (James et al., 2013).

However, in sample kept at 21 °C for two days and stored at 4 °C up to 74 hours (c2 74) a decrease in histamine content was detected. According to Oucif et al. (2012), the histamine content depends on the histamine-producing bacteria as well as on the degrading bacteria (bacteria with histaminase activity). Rawles et al. (1996) showed that histamine in spoiled fish is extremely variable. The factors of histamine formation are length of storage, temperature, body section of fish, fish species, type and level of microorganisms present.

All contaminated samples produced histamine, except the ones contaminated by *Lactobacillus casei* analysed after 26 hours (lcw 26 and lcf 26). The main bacteria responsible for histamine formation are certain species of *Enterobacteriaceae*, *Clostridium*, *Pseudomonas* and *Lactobacillus* (James et al., 2013). Other bacteria such as *Photobacterium phosphoreum*, *Photobacterium psychrotolerans*, *Hafnia alvei*, *Morganella morganii*, *Klebsiella pneumonia* have been isolated from fish incriminated of scombroid poisoning incidents, and are considered as strongest histamine producers (EFSA, 2011).

For fillet samples contaminated by *Photobacterium phosphoreum* (pf) and *Lactobacillus casei* (lcf) and stored at 21 °C, there was an increase of histamine formation from the first to the second day (Figures 20 and 21).

A few hours after storage (26 hours), initial histamine content in mackerel is already high (61 ppm) for fillet samples contaminated by *Photobacterium phosphoreum*. It exceeds the toxic level of histamine of 100 ppm muscle rapidly after 50 hours of storage at ambient temperature (21±1 °C). In agreement with Oucif et al. (2012), this excessive production is probably due to a high amount of histidine in red muscle of fish (especially the Scombridae), a large proliferation of mesophilic bacteria capable of histidine decarboxylation and an optimum temperature (26±3 °C) and a suitable pH for the synthesis and activity of histidine decarboxylase, reached rapidly in the flesh of mackerel. Considerable increase in histamine levels was also noticed for fillet samples contaminated by *Lactobacillus casei*. However, any detection of histamine levels 150 ppm confirm the presence of decomposed fish tissue (Antoine, et al., 2002). Antoine, et al. (2002) also found that the rate and level of histamine formation varied with fish species, which makes it necessary to stipulate different defect action levels (DALs) for different species.

Indeed, whole fish and fillet samples contaminated by *Photobacterium phosphoreum*, produced higher concentration of histamine than whole fish and fillet samples contaminated by *Lactobacillus casei*. Several studies had shown that a number of psychrophilic and psychrotolerant bacteria (such as *Morganella psychrotolerans* and *Photobacterium phosphoreum*) have been identified as significant histamine formers at low temperatures. A number of the histamine-forming bacteria are facultative anaerobes, such as *P. phosphoreum*, that can grow in reduced oxygen environments (FDA, 2011). The optimal temperatures for growth for most of histamine-forming bacteria are in the range of 20 °C to 30 °C (James et al., 2013), although some histamine-forming bacteria are capable of growing below 10 °C.

Histamine formation is generally considered to be “more commonly the result of high temperature spoilage than of long term, relatively low temperature spoilage” (FDA, 2011). And it was concluded by EFSA (2011) that generally the amine production rate increases with the temperature.

However, other authors (Grau et al., 2003) have shown that histamine is synthesised at significant levels at temperatures as low as 2-5 °C. Indeed, *Photobacterium histaminum* and *Photobacterium phosphoreum* show a decarboxylase activity at 4 °C and even at –20 °C.

In this study, no sample of Atlantic mackerel, except sample contaminated by *Photobacterium phosphoreum* (pf 26), had histamine above 50 ppm for 26 hours analysis. It might be inferred that the spotted mackerel fillet had low post-catching contamination or contained low histamine originally (Yeh et al., 2004).

Whole fish samples contaminated by *Lactobacillus casei* had BAs higher than *Photobacterium phosphoreum*. According to Hosseini-Nezhad, Hussain & Britz. (2015), *Lactobacillus casei* is a lactic acid bacterium used in the production of many fermented foods and feed products with adaptive response to adverse conditions, including low pH, bile salts, high osmotic pressure, high and low temperature. In other hand, *Photobacterium phosphoreum* is psychrophile (an organism capable of growth and reproduction in cold temperatures) and a piezophile (an organism which thrives at high pressures) (EFSA, 2011).

Figure 21 also indicated that samples contaminated by *Photobacterium phosphoreum* (pf 26), stored at 21 °C and analysed after 26 hours, may contain histamine higher than 50 ppm. Yeh et al. (2004) reported that 8 out of 107 (7.5%) of the smoked fish from New Zealand markets had histamine levels above 50 ppm.

On the other hand, fillet samples (pf 50, lcf 50) contained higher amount of histamine than the limit recommended by the EU, EC regulation 2073/2005 (100 ppm). Fish and certain varieties of cheese contain the highest amounts of histamine (up to 1.000-2.000 ppm) and are the foods most frequently associated with cases of histamine poisoning (Ladero et al., 2010). Oucif et al. (2012) reported that histamine content exceeded the limit of 100 ppm, after 24 hours at T_{amb}, 5 days of Atlantic mackerel storage at 4 °C only. Also, Yeh et al. (2004) reported that 1000 ppm of the amines in food could be considered as a hazard criterion for health.

In general, the concentration of all BAs increases with the storage time. EFSA (2011) concluded that the quantitative production of BAs is usually reported to be temperature and time dependent. Generally, the amine production rate increases with the temperature. Conversely, for EFSA (2011), BAs accumulation is minimised at low temperatures through inhibition of microbial growth and the reduction of enzyme activity. The optimum temperature for the formation of BA by mesophilic bacteria is between 20 to 37 °C, while production of BA decreases below 5 °C or above 40 °C.

The toxicological level of BAs is very difficult to establish because it depends on individual characteristics and the presence of other amines, however, a maximum total BAs level of 750–900 ppm has been proposed as well as a threshold values of 100 ppm for tyramine (Ladero et al., 2010). There is some evidence that tyramine, cadaverine and putrescine, potentiates histamine toxicity by inhibiting the histamine-metabolizing enzymes diamine oxidase (DAO) and histamine-N-methyltransferase (HMT) (FAO-WHO, 2010).

6 CONCLUSION AND RECOMMENDATIONS

The QIM scheme developed for farmed Senegalese sole consisted of 10 parameters grouped in four main categories, resulting in a total of 26 demerit points. The scores for quality attributes in the QIM scheme increased differently with storage time in ice, but a strong linear relationship with significant correlation was found between QI and storage time in ice.

Based upon the sensory evaluation of raw and cooked Senegalese sole, the shelf life may be estimated to be 16 – 21 days when stored in ice.

Skin odour (sour, fermented and rotten) was the parameter that defined spoilage of fish.

The effect of the temperature on histamine formation was investigated to see how histamine/BA level changes at high temperature (21 °C) abuse versus moderate temperature abuse (12 °C); and on the other hand, whether the room temperature after the high/ moderate temperature abuse had any effect on the BAs/histamine level.

High temperature abuse (21 °C) resulted in much more formation of all BAs compared to the moderate temperature abuse (12 °C). It seems that the storage did not significantly affect the level of BA/histamine. At 12 °C histamine formation was very low (1 ppm) versus at 21 °C where it was significant.

In the case of the contaminated whole fish there was no significant differences between the two different stains respectively to all BAs and both produced very low amount of histamine, after only the second day still there were only 3-7 ppm detectable histamine.

On the other hand, when bacteria had more excess to fish muscles (fillet) 10x more BAs were produced. In both cases after two days exposed to 21 °C the concentration of all BAs exceeded 800 ppm, but *Photobacterium phosphoreum* (pf) could form 2 times more histamine (554 ppm) after two days storage versus *Lactobacillus casei* (256 ppm).

Recommendations:

- The panellists need more time for training, as the Torry scheme needs to be adapted to the sensory characteristics of Senegalese sole.
- In continuation of the development of a QIM scheme, the scheme should be tested in a full-scale shelf life study. Such a study would include sensory evaluation of both raw

and cooked samples by a trained sensory panel, and preferably microbial and/or chemical analysis.

- As the European food legislations require histamine measurements, the laboratory in Mozambique is going to set up histamine measurements on the new HPLC machine. In the future the laboratory is going to participate in interlaboratory testing and obtain the accreditation for this measurement.

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APPENDICES

Appendix 1. Concentration of BAs in whole fish kept at 12 °C and stored at 4 °C.

Table 10. Concentration of BAs in whole fish kept at 12 °C and stored at 4 °C.

Concentration of BAs in ppm (group kept at 12 °C for one day – b3 and for two days – b5)				
BAs	b3 26	b5 26	b3 74	b5 74
tyr	1	2	13	2
putr	4	5	2	5
cad	0	11	56	11
hist	0	1	1	1

b3 26* = description corresponding to one sample kept at 12 °C for one day (b3), for two days (b5) and stored at 4 °C and then analyzed after 26 hours (26), 74 hours (74).

BAs* = Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

Appendix 2. Concentration of BAs in whole fish kept at 21 °C and stored at 4 °C.

Table 11. Concentration of BAs in whole fish kept at 21 °C and stored at 4 °C.

Concentration of BAs in ppm (group kept at 21 °C for one day – c1 and for two days – c2)						
BAs	c1 26	c1 50	c1 74	c2 26	c2 50	c2 74
tyr	0	3	28	43	37	28
putr	0	4	11	25	32	25
cad	0	33		170	141	122
hist	0	1	15	43	78	56

c1 26* = description corresponding to one sample kept at 21 °C for one day (c1), for two days (c2) and stored at 4 °C and then analyzed after 26 hours (26), 50 hours (50), 74 hours (74).

BAs* = Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

Appendix 3. Concentration of BAs in whole fish contaminated by *Photobacterium phosphoreum* and by *Lactobacillus casei* and stored at 21 °C.

Table 12. Concentration of BAs in whole fish contaminated by *Photobacterium phosphoreum* and by *Lactobacillus casei* and stored at 21 °C.

Concentration of BAs in ppm (group of whole fish contaminated by <i>Photobacterium phosphoreum</i> (pw) and <i>Lactobacillus casei</i> (lcw) and stored at 21 °C)				
BAs	pw 26	lcw 26	pw 50	lcw 50
tyr	6	19	20	22
putr	3	4	21	12
cad	39	84	85	97
hist	1	0	7	3

pw 26* = description corresponding to one sample contaminated by *Photobacterium phosphoreum* (pw), *Lactobacillus casei* (lcw) and stored at 21 °C and then analyzed after 26 hours (26), 50 hours (50).

BAs* = Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

Appendix 4. Concentration of BAs in fillet fish contaminated by *Lactobacillus casei* and *Photobacterium phosphoreum* and stored at 21 °C.

Table 13. Concentration of BAs in fillet fish contaminated by *Lactobacillus casei* and *Photobacterium phosphoreum* and stored at 21 °C.

Concentration of BAs in ppm (group of fillets contaminated by <i>Photobacterium phosphoreum</i> (pf) and <i>Lactobacillus casei</i> (lcf) and stored at 21 °C)				
BAs	pf 26	lcf 26	pf 50	lcf 50
tyr	145	13	142	169
putr	9	1	28	23
cad	337	61	305	345
hist	61	0	554	256

pf 26* = description corresponding to one sample contaminated by *Photobacterium phosphoreum* (pf), *Lactobacillus casei* (lcf) and stored at 21 °C and analyzed after 26 hours (26), 50 hours (50).

BAs* = Biogenic Amines: **tyr** = Tyramine. **putr** = Putrescine. **cad** = Cadaverine. **hist** = Histamine.

Appendix 5. Chromatogram of sample contaminated by *Photobacterium phosphoreum*, stored at 21 °C and analyzed after 26 hours (pf 26)

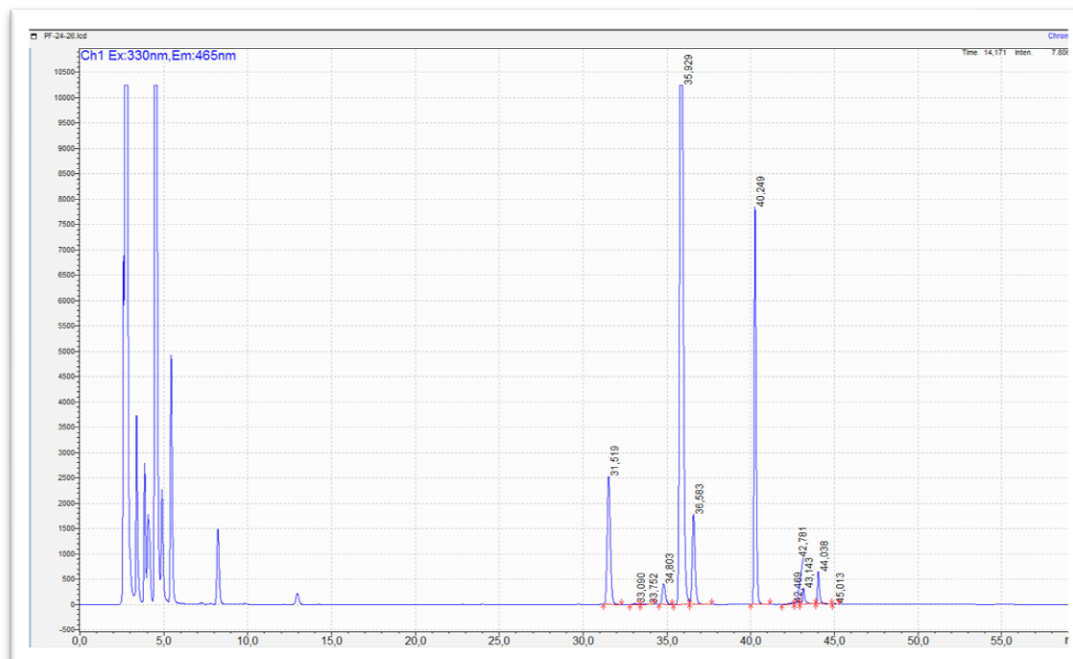


Figure 22. Identification of BAs on column for sample contaminated by *Photobacterium phosphoreum*, stored at 21 °C and analysed after 26 hours (pf 26)

Appendix 6. Chromatogram of sample contaminated by *Photobacterium phosphoreum*, stored at 21 °C and analyzed after 50 hours (pf 50)

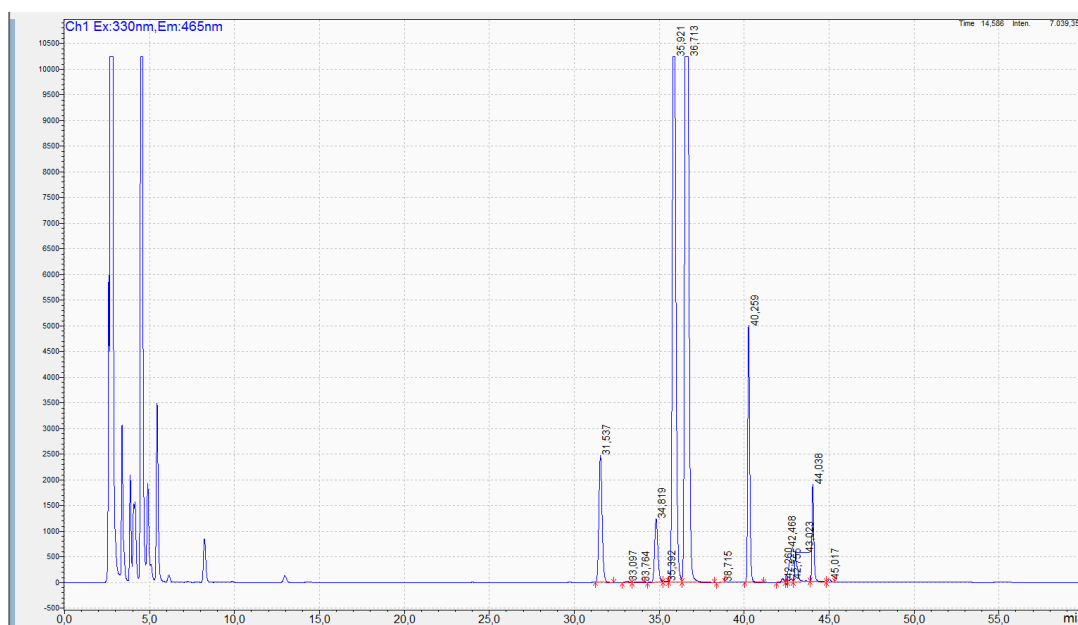


Figure 23. Identification of BAs on column for sample contaminated by *Photobacterium phosphoreum*, stored at 21 °C and analysed after 50 hours (pf 50)

Appendix 7. Chromatogram of sample contaminated by *Lactobacillus casei*, stored at 21 °C and analyzed after 26 hours (lcw 26)

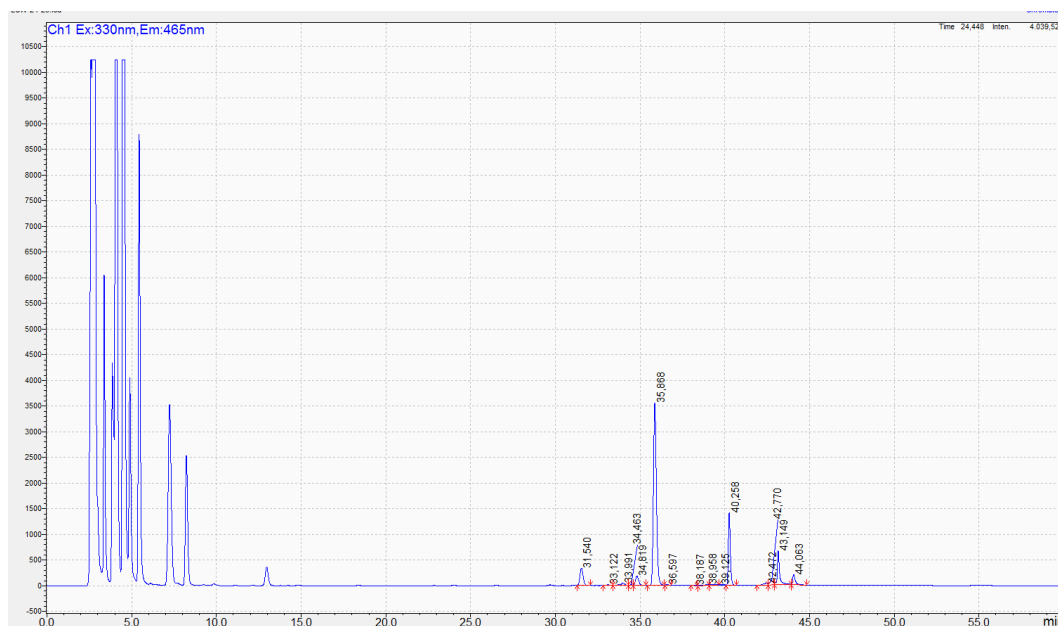


Figure 24. Identification of BAs on column for sample contaminated by *Lactobacillus casei*, stored at 21 °C and analysed after 26 hours (lcw 26)

Appendix 8. Chromatogram of sample contaminated by *Lactobacillus casei*, stored at 21 °C and analyzed after 50 hours (lcw 50)

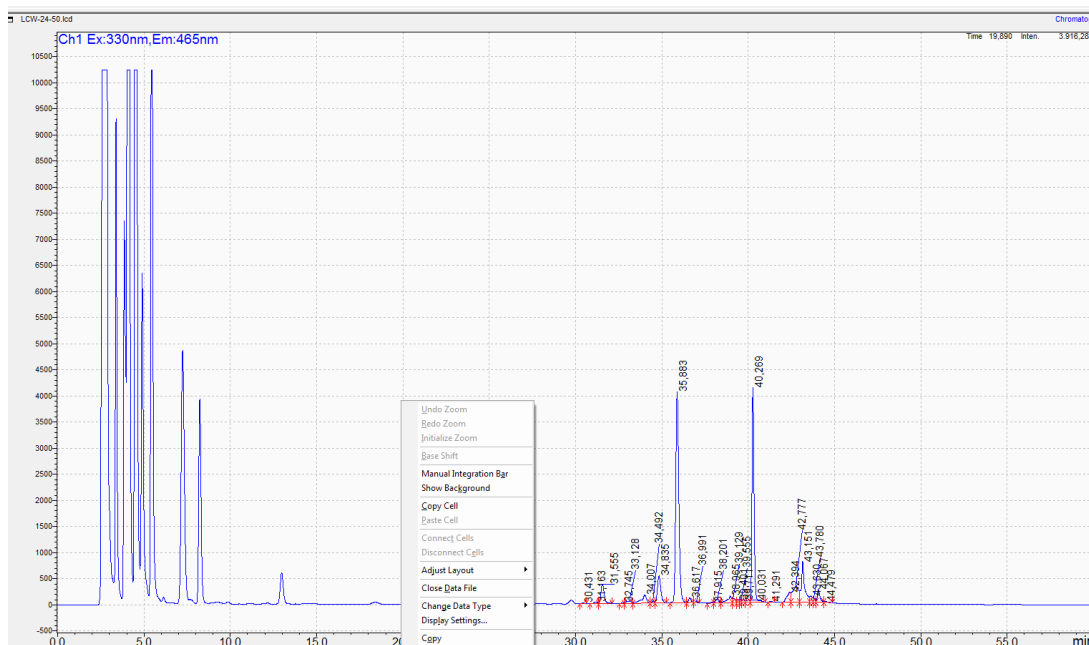


Figure 25. Identification of BAs on column for sample contaminated by *Lactobacillus casei*, stored at 21 °C and analysed after 50 hours (lcw 50)

Appendix 9. Chromatogram of fillet sample contaminated by *Lactobacillus casei*, stored at 21 °C and analyzed after 26 hours (lcf 26)

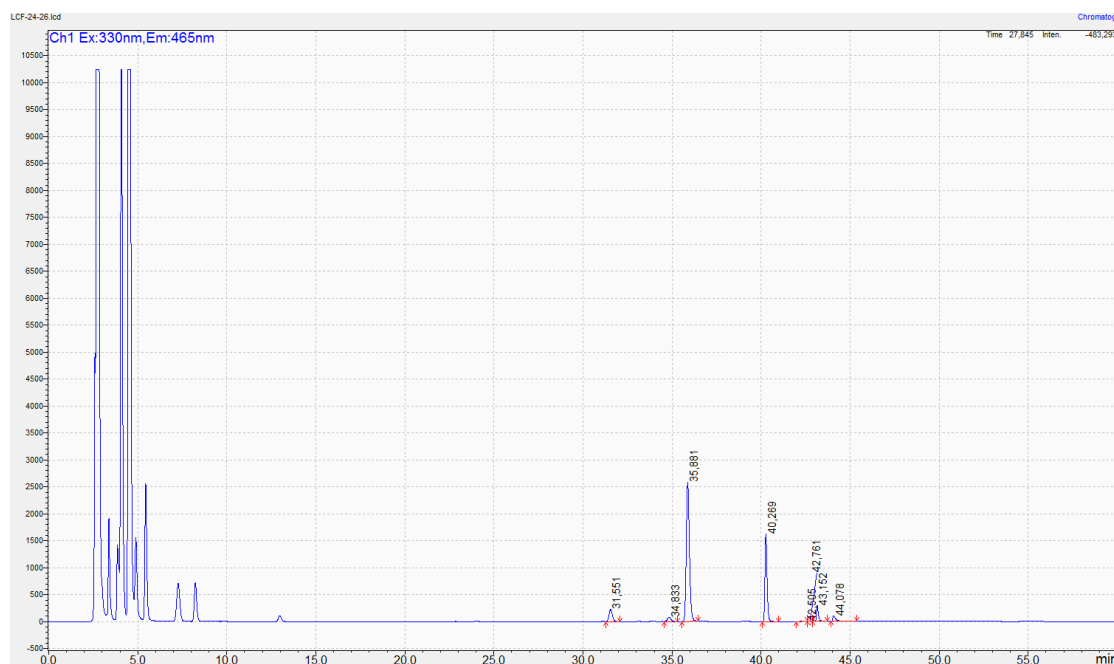


Figure 26. Identification of BAs on column for sample contaminated by *Lactobacillus casei*, stored at 21 °C and analysed after 26 hours (lcf 26)

Appendix 10. Chromatogram of fillet sample contaminated by *Lactobacillus casei*, stored at 21 °C and analysed after 50 hours (lcf 50)

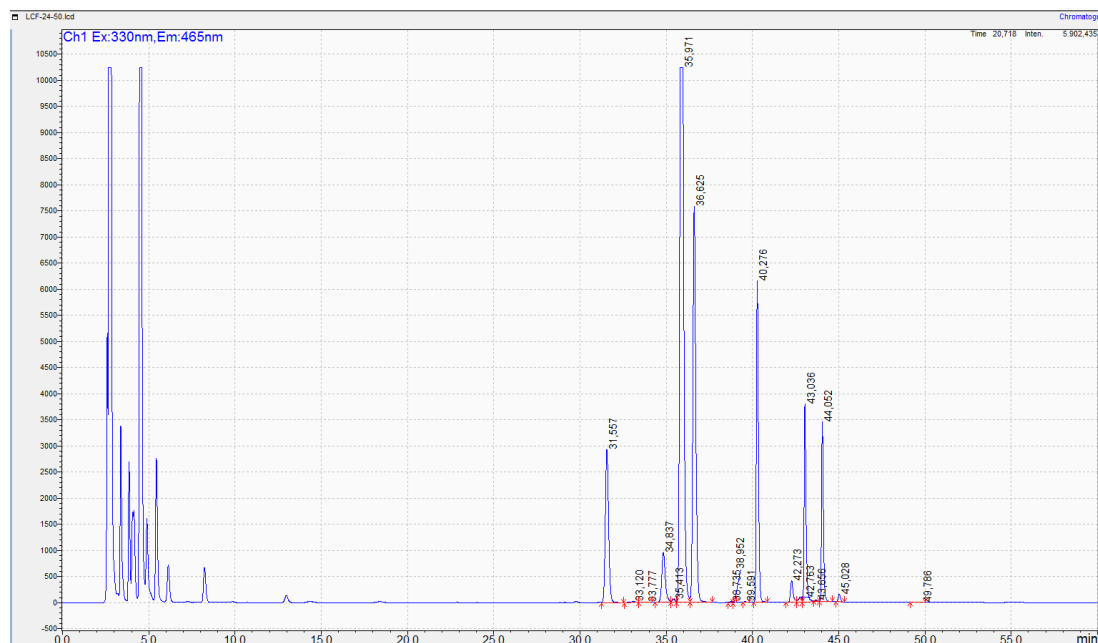


Figure 27. Identification of BAs on column for sample contaminated by *Lactobacillus casei*, stored at 21 °C and analysed after 50 hours (lcf 50)

Appendix 11. Chromatogram of sample kept at 21 °C for two days, stored at 4 °C and then analyzed after 50 hours (c2 50)

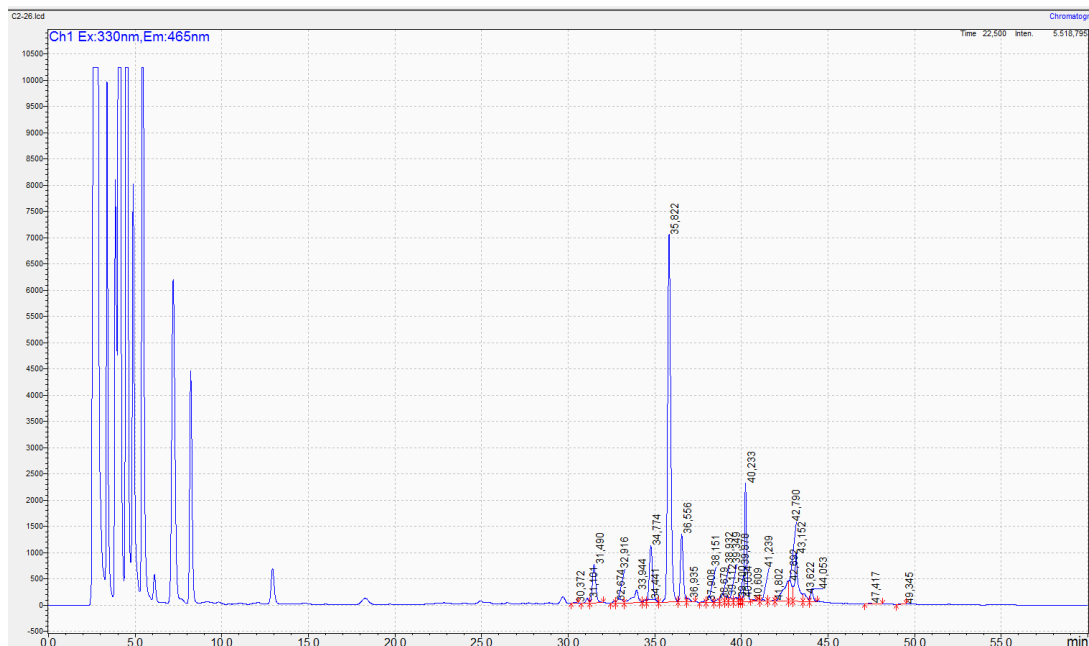


Figure 28. Identification of BAs on column for sample kept at 21 °C for two days, stored at 4 °C and then analysed after 50 hours (c2 50)

Appendix 12. Chromatogram of standard mixture of BAs diluted 100x times (Dil-100)

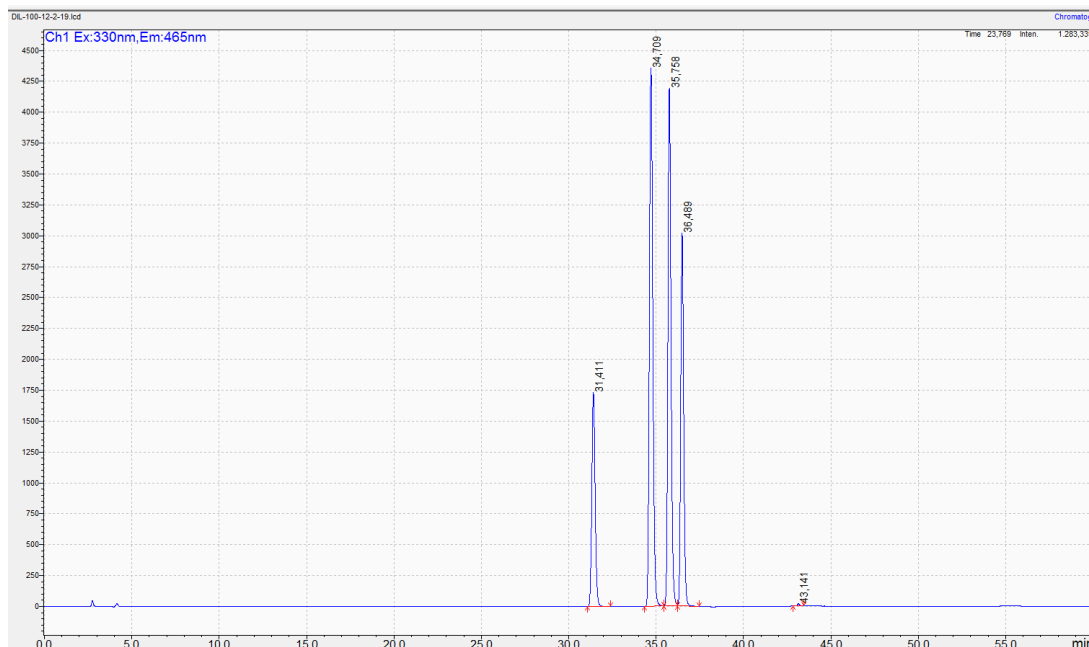


Figure 29. Identification of standard mixture of BAs diluted 100x times (Dil-100)