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CORSO DI DOTTORATO DI RICERCA IN
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SCUOLA DI DOTTORATO IN
SCIENZE VETERINARIE PER LA SALUTE ANIMALE E LA SICUREZZA
ALIMENTARE

TESI DI DOTTORATO DI RICERCA

SEAFOOD SAFETY: A MATTER OF ANY CONCERN ?

VET/04

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"The common aim of all science is the gradual removal of prejudices"
Niels Bohr

Contents

Abstract

In this thesis is reported my research activity, the investigation was focused on seafood safety. The thesis consists of a collection of articles, which reports the research works onto peer-reviewed journal. Below title, authors, abstract and references are reported.

Determination of Carbon Monoxide in Tuna by Gas Chromatography with Micro-Thermal Conductivity Detector.

C. Bernardi, L.M. Chiesa, S. Soncin, E. Passerò e P.A. Biondi.

Journal of Chromatographic Science 46 pp 392-394.

Abstract:

The suitability of a portable gas chromatograph equipped with a micro-thermal conductivity detector for the head-space determination of carbon monoxide (CO) in tuna samples is evaluated; CO is estimated after its liberation from tissue by acidic treatment at 70°C. Using the tested technique, the CO contents in untreated and suspected treated samples are analyzed. A limit of detection of approximately 13 ng/g is reached. The results demonstrate that this apparatus has performances similar to more expensive and sophisticated instruments.

Preliminary study on prevalence of larvae of Anisakidae family in European sea bass (Dicentrarchus labrax).

C. Bernardi.

Food Control 20, 433-434**.**

A total of 561 fresh European sea bass from North-East Atlantic ocean (F.A.O. zone 27) were examined for Anisakidae larvae detection. It is the first record on the prevalence of Anisakidae larvae in this fish specie. An unexpected high prevalence was found. The prevalence range was between 65 and 85%, in

relation with the body weight of the subject. The results have an important consequence on epidemiology of anisakidosis and public health risk assessment.

A case study: shelf-life of smoked herring fillets by volatile compounds analysis.

C. Bernardi, P. Cattaneo, L. Chiesa.

The open food science journal, in review

Abstract

Two different products of vacuum packed cold smoked herrings were analyzed at time intervals in order to evaluate the efficiency of the processing and product stability. Microbiological total counts, lactic acid bacteria, total coliforms, pH, water activity, water content, salt content (WPS) were determined. Differences in hygienic conditions and salt content were found. Principal components analysis (PCA) of volatile compounds determined by GC-MS analysis allowed the differentiation not only of the processing but also of the storage time.

Shelf-life of vacuum packed cold-smoked salmon from italian market.

C. Bernardi, B. Ripamonti, S. Stella, A. Campagnoli, P. Cattaneo.

Abstract

Fourteen samples of different vacuum packed cold smoked salmon products purchased in Italian retail were analysed at about half shelf-life and at the expiry date. In order to evaluate product stability, total psychrotrophic count, lactic acid bacteria, *Enterobacteriaceae, L. monocytogenes, Salmonella* spp, coagulase positive staphylococci, TVBN, TBARS, colour and texture were performed. Preservation parameters (water content, salt content-WPS, aw) and product analysis (net weight, defects) were determined. High TVBN and

TBARS values were already determined at 30 days before the expiry date. On the expiry date 12 samples were above the limits. In one sample WPS was less than 3,5%, with microbiological risk. Microbial counts at half shelf life were acceptable in most samples; TPC at expiry date was very high (> 10^6 CFU/g) in almost all samples (12 of 14), and was dominated by Lactobacilli. Levels of Enterobacteriaceae and coagulase-positive staphylococci were very low, and no potential pathogenic bacteria were detected. Significant negative differences in declared weight were determined.

Carbon monoxide in yellowfin tuna (*Thunnus albacarens*)

The freshness of tuna meat is normally judged by its bright red colour. The desirable bright red colour is due to the presence of oxygenated myoglobin (Mb), which is present in the muscle fibres; however, the amount of Mb varies as a function of the age and species as well as the way the meat is treated (Livingston & Brown, 1981). The colour of the muscle tissue in fish is the result of the oxidation state of myoglobin protein; Mb serves to bind oxygen for cellular use and contain a heme group with an Fe(II) center bound to four nitrogen molecules of the porphyrin ring. The fifth bond orbital of Fe(II) is bound to a histidine side chain of the globin protein, while the sixth site is available to molecules affecting colour of the product. In its reduced form, the oxygen molecule is bound to the heme group of myoglobin resulting in flesh that has a bright red colour. Muscle tissue of tuna (*Thunnus obesus*) is susceptible to autoxidation (Kitahara, Matsuoka, Kobayashi, Shikama 1990) upon cutting and freezing, forming metmyoglobin (MetMb), which is brown in colour (Tajima & Shikama, 1987). Treatment of tissue with CO results in the stabilization of colour (bright cherry red) due to the greater affinity (>240 times) of CO for the Fe(II) binding site of myoglobin (Sørheim, Aune, Nesbakken 1997).

In figure the challenge between a yellowfin tuna slice(*Thunnus albacares*) CO added (on the left) and a yellowfin tuna slice not CO added (on the right).

Thus, autoxidation and discolouration are prevented when CO is bound, preserving the bright red colour associated with tissue that is presumably fresh for an extended period of time. To increase sales and add values, the seafood industry is continuously attempting to improve quality and create attractive attributes in aquatic foods for consumers. One such action in last years has led to the use of CO or tasteless smoke (TS), which contains CO gas to fix the bright red colour in the muscle tissue of fish. A number of studies have demonstrated the positive effects, mainly in the improvement of colour stability, of low amounts of CO in modified atmosphere packaging (MAP) and its purported safety for packaged meat (Sørheim, Aune, Nesbakken 1997; Sørheim, Nissen, & Nesbakken, 1999). There are no direct health implications from eating CO-treated tuna. However, since CO treatment makes tuna appear fresh, CO-treated tuna can obscure the colour changes, which normally follow aging of

the fish, so the real health risk is due to the lack of freshness of the product. More recently, research at the Danish Institute for Fisheries Research demonstrated significant histamine formation by marine psychrotolerant bacteria in vacuum packaged tuna during refrigeration storage (Emborg, Laursen, Dalgaard 2005). These findings raise substantial concerns over the potential for product abuse leading to high levels of histamine in the product. In the United States, tuna treated with CO or TS must display labelling indicative of that process because the CO treatment is considered general recognized as safe (GRAS). In EU is not allowed the CO use for the possible health risk of consuming old products. Qualitative determination of CO treatment on a product can be determined by persistence of flesh colour upon thawing and holding at room temperature (FDA 1999), unnatural colour determined by quantitative sensory evaluation (Canadian Food Inspection Agency 1999), or colorimeter/image analysis measurements (Seafood Products Research Center 2003). However, these techniques do not consider penetration or total absorption of CO into the muscle tissue. Many spectrophotometric methods have been used to determine various myoglobin derivatives from fish muscles (Chow, Hsieh, Hwang 1998; Yang, Lee, Chow 2001) after myoglobin extraction and purification. These methods give quantitative information by measuring amounts of carbonilmyoglobin (MbCO) in the muscle tissue. Other researchers developed CO quantisation procedures using GC coupled with flame ionization detector (FID) detection of methane reduced from CO released from fish tissues (Chow, Hsieh, Hwang 1998). The GC/FID-nickel catalyst approach was employed by the Japanese government in its attempt to regulate imported seafood products (Hsieh, Chow, Chu, Chen 1998; Chow, Hsieh, Hwang 1998). This pilot study describes a direct measurement of CO using gas chromatography/mass spectrometry (GC/MS) following chemically induced CO liberation from fish muscle. Determination of CO using GC/MS for biological

samples has been previously reported in forensic application (Oritani, Zhu, Ishida, Shimotouge, Quan, Fujita, Maeda 2000). Recently, a simple, confirmative method for quantitative determination of carbon monoxide in commercially treated tuna and mahi–mahi tissues has been reported using gas chromatography/mass spectrometry, following chemical liberation of CO (Anderson & Wu, 2005). The official laboratories of food control need not only confirmatory methods but also rapid low cost screening methods for everyday activity. The aim of this preliminary study is obtain a simple, rapid, quantitative and low cost method.

Determination of Carbon Monoxide in Tuna by Gas Chromatography with Micro-Thermal Conductivity Detector

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Abstract

The suitability of a portable gas chromatograph equipped with a micro-thermal conductivity detector for the head-space determination of carbon monoxide (CO) in tuna samples is evaluated; CO is estimated after its liberation from tissue by acidic treatment at 70°C. Using the tested technique, the CO contents in untreated and suspected treated samples are analyzed. A limit of detection of approximately 13 ng/g is reached. The results demonstrate that this apparatus has performances similar to more expensive and sophisticated instruments.

Introduction

Fish products treated with carbon monoxide (CO), not allowed by the current rules, are actually present on the European markets (1,2,3). Therefore, an analytical method for distinguishing the physiological CO content from that resulting from CO treatment is helpful for surveillance systems. Head-space sampling of CO followed by gas chromatography (GC) analysis, which has previously been used to determine CO in blood and tissues (4,5,6), was adopted for its determination in fish samples as well (7,8). A recent work states that better-releasing CO yields were obtained using a sulphuric acid solution instead of a potassium ferrycianide solution, and by direct treating of flesh homogenate instead of preliminary extraction of CO-myoglobin (7). According to the procedures introduced for blood and tissue analysis, different detection techniques have also been proposed for quantitating the CO liberated from the tuna muscle. The lowest detection limit was reached with a nickel catalyst system placed before flame ionization detection (8). On the other hand, an unsurpassable selectivity was achieved by mass spectrometry (MS) detection (7).

The thermal conductivity detector (TCD), which is the simplest and cheapest detection technique, was used to determine CO in blood only (4), and no studies on tuna are actually reported.

The aim of this work was to test the ability of a recently introduced micro-machined TCD on a portable GC apparatus (9) to reach the sensitivity of the more expensive MS detector for quantitating the endogenous CO content in tuna. The sample preparation already introduced for CO GC–MS analysis in tuna (7) has been followed with only slight modifications with a view to reduce the specimen amounts and reagent volumes used before GC–TCD. This new method was applied to the analysis of *Thunnus albacares* fillets.

Materials and Methods

Samples and materials

All tuna samples of *Thunnus Albacares* were purchased from local trade market. Three dorsal fillet aliquots from an entire subject were withdrawn and used as untreated samples. Suspected CO treated samples were vacuum-packed frozen fillets from Indian Ocean.

Head-space vials of 20-mL were purchased from National Scientific Company (Quakertown, PA). All reagents used were of analytical grade.

Apparatus and chromatographic conditions

The chromatographic system was from Varian (Palo Alto, CA) and composed of a Micro GC CP-4900 equipped with an automatic injection system, a micro TCD, a 10 m × 0.25 mm column containing the Molecular Sieve 5 Å stationary phase (MSA), and StarWS mod. 6.2 software. The injection time and temperature were 250 ms and 70°C, respectively, corresponding to a final injection volume of nearly 10 µL. The column temperature was maintained at 80°C.

Sample preparation

The recently introduced procedure (7) was modified by reducing 20 times both the sample weight and reagent volume.

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Briefly, approximately 100 g of frozen tuna specimen were homogenized for 30 s, then a 2 g aliquot was placed in a vial, followed by 4.2 mL of water and 5 µL of octanol. 0.5 mL of 5M sulphuric acid was then added to the vil, previously capped and shaken, using a syringe to reach a final volumne of 6.7m:. The vial was shaken again, heated at 70ºC for 1 h and allowed to cool at room temperature before the head-space was analyzed by the GC apparatus.

Calibration curve

The mixtures containing known amounts of CO were prepared according to the procedure of the previously cited work (7): first, pure CO was bubbled in vials by displacing water, and then stock gaseous mixtures were prepared by transferring pure CO aliquots by gastight syringe to closed vials containing air. The standard mixtures for the calibration curve were then obtained by adding stock mixture aliquots in closed vials containing a solution volume equal to that indicated in the sample preparation section. In fact, 2 mL of additional water were used instead of tuna homogenate, as in our preliminary experiments we measured the specific weight of tuna homogenate, which resulted in 1.00 g/mL ± 0.03 g/mL (n = 5, 25°C). Using this procedure, six different standard mixtures in the range 33.9 ng–6342.5 ng were prepared and analyzed in triplicate.

Results

In the tested range, the relationship between the peak areas (µvolt × s) and the CO amounts (ng) was linear (r^2 = 0.9999) with the following regression parameters: slope (b) = 0.1091 ± 0.0001, intercept = 0.0451 ± 0.2248, and residual standard deviation ($\sigma_{y/x}$) = 1.671.

According to a simple theoretical approach (10), the limit of detection (LOD = $3\sigma_{y/x}b^{-1}$) and limit of quantitation (LOQ = $10\sigma_{y/x}b^{-1}$) resulted as 13.28 ng and 44.28, respectively.

In Figure 1, the profiles corresponding to untreated and suspected treated samples are shown. The repeatability on a CO content of 72.8 ng/g was measured by analyzing untreated sample (RSD % = 11.8, n = 5). In Table I, the contents found in the analyzed suspected tuna samples are reported.

Discussion

Due to its low sensitivity, TCD has not been not used until now for the determination of CO in food. Recently, a new portable micro-GC equipped with a miniaturized TCD was introduced for gas analysis in environmental fields (9). The main novelties of this apparatus were the micro-machined injector, with no moving parts, the chip detector, with internal volume of 200 nL, and the new designed electronics, giving a very low electronic noise. In this work, a micro-GC was applied for the first time to the determination of CO in fish. Regarding the analytical conditions with respect to the most recently reported GC method on CO determination in tuna (7), the significant difference introduced was the use of the microTCD on the portable GC instead of the MS detector. In the described conditions, the detection suitability appeared satisfactory; in fact, only approximately 44 ng of CO were the theoretical LOQ, while the lowest quantitated amount in the already cited work (7) was 1 µL of CO corresponding to 1158 ng at 22°C. Thus it is not necessary to use an expensive GC–MS apparatus to reach the high sensitivity needed for regulatory purposes. The CO content found in the analyzed untreated tuna sample resulted, as expected, lower than the 200 ng/g value, which was considered the accepted limit for the CO physiological content (8). On the other hand, this value was clearly higher than the LOQ of the micro-TCD technique. Therefore, the method presented here appears suitable for distinguishing the untreated samples from the treated. In the analyzed samples of *Thunnus Albacares* from Indian Ocean, CO contents higher than 200 ng/g were found, confirming the frequent use of CO in those countries. In conclusion, considering its lower price and portability, the apparatus used is more convenient than GC–MS instruments for CO determination in either technological studies or inspection investigations.

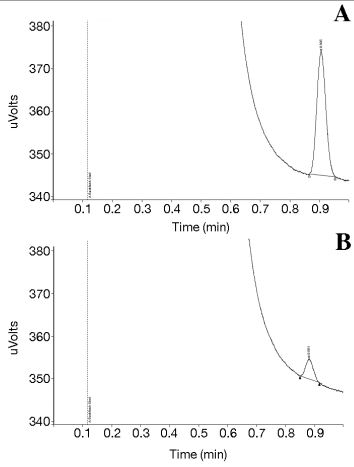

Figure 1. Chromatographic profiles corresponding to a sample from a vacuum packed frozen fillet (A) and to an untreated sample from an entire subject (B). The resulting CO contents were 559.5 ng/g and 72.8 ng/g, respectively.

Table I. CO Contents in *Thunnus Albacares* Samples*

Sample	CO content (ng/g)†
1	525.0
2	559.5
3	744.8
4	660.0
5	558.4
6	351.8

* All samples were vacuum-packed frozen fillets from the Indian Ocean.
† Mean of three determinations.

Acknowledgments

The authors wish to thank Mr Mario Voglino (Varian S.p.A., Italy) for his helpful technical suggestions.

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Discussion

The method described above is simple, fast and low cost, further experimentation would have to be conducted to determine the natural CO level in tuna and if the natural CO presence is always distinguishable from the treated tuna.

In further experiments 2 g of muscle were placed in a vial, followed by 4.7 mL of NaCl saturated solution, then the vial was capped and shaken, heated at 70°C for different times and allowed to cool at room temperature before the head-space was analysed by the micro GC-TCD apparatus. The results obtained are reported below in figure n.1. The same experiment was performed replacing NaCl saturated solution with 5M H_2SO_4 solution (figure n.1).

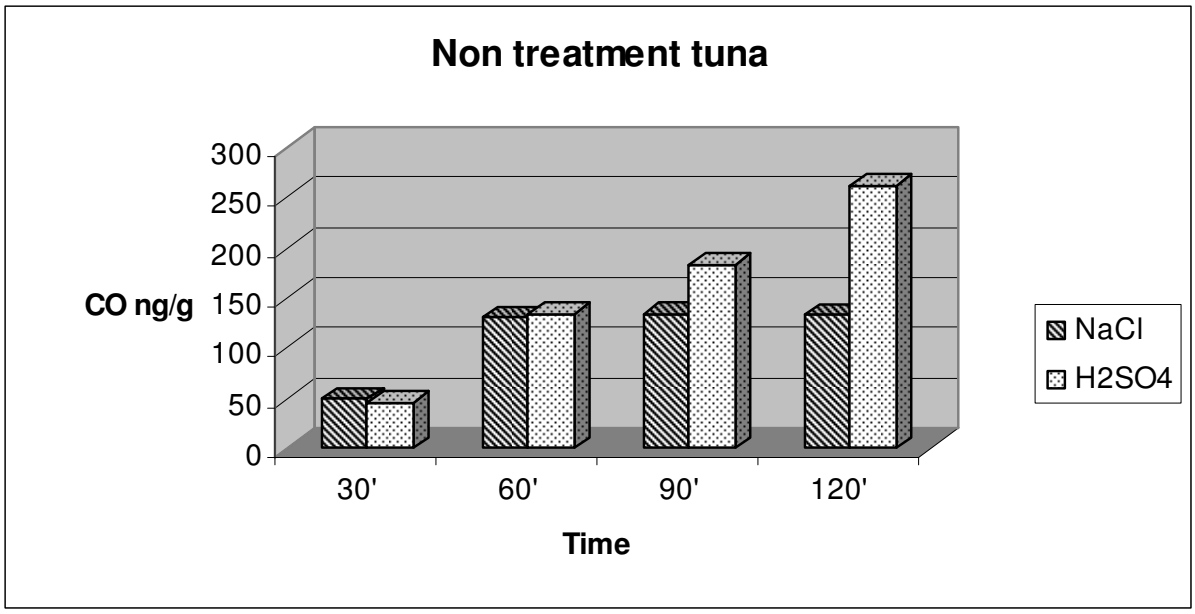

Figure n.1 -

In figure n.1 the CO quantities determinated increases with warming time when in reaction the 5M sulphuric acid solution was used. The analogous phenomenon is not observed in the case of NaCl saturated solution. In method proposed by Anderson the not CO added samples warmed for 60' liberated 134 ng/g of CO, while warmed for 120'the same samples liberated 262 ng/g of CO.

These data are cause of some concern about the Anderson's method, it is not clear the increasing of CO in not treated samples. An hipotesis is the new CO formation cause to the uncompleted combustion of muscle in the vial.

Analogous experiments with tuna CO adding was performed, the results are reported in figure n.2.

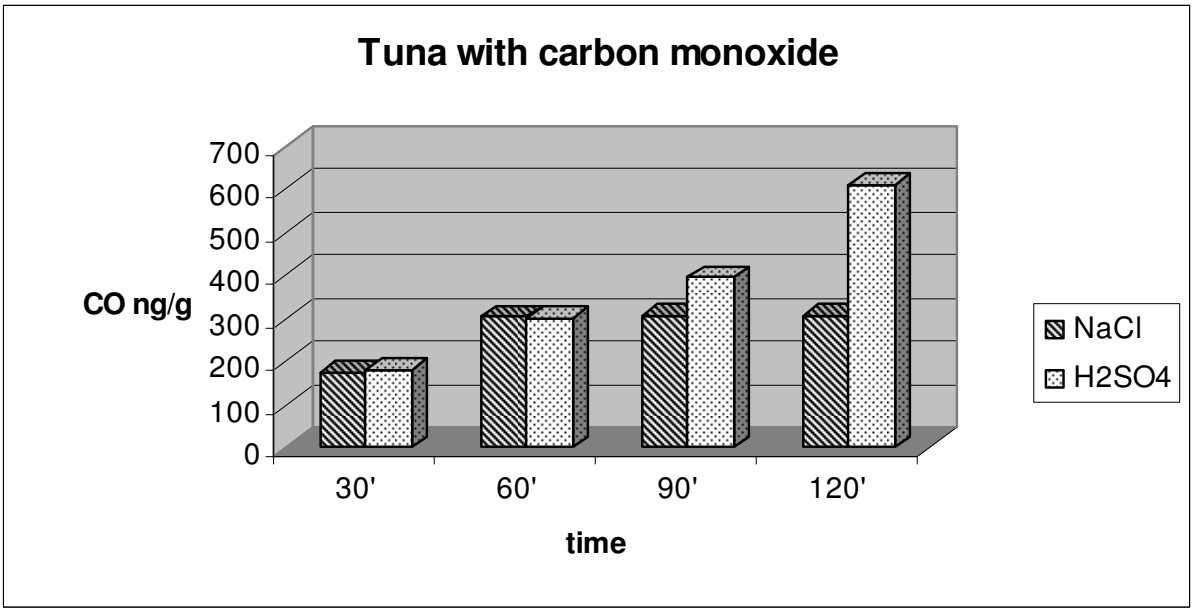

Figure n.2

In Japanese legislation the limit to distinguish between natural and treated sample is 200 ng/g, Anderson and Wu consider sample below 150 ng/g as non treated tuna; in my experience the limit is strictly bound to the extractive method. In fact the CO quantitative liberated from fish muscle results from the temperatures/time and from the solvent power.

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Anisakidae larvae in european sea bass

Introduction

The larvae of Anisakis, whose adult form lives on sea mammals such as whales, seals and dolphins, are parasitic upon many species of salt-water fish. When the final host animals eat paratenic hosts, the larvae grow to adulthood in the hosts stomach. However, the humans eat these infested fish, the larvae die instead, causing a disease called anisakiasis. In 1960, in the Netherlands, Van Thiel found a worm in the intestinal wall of a patient, who had eaten raw herring and had suffered symptoms of acute abdomen. Today as is well known from literature the pathogenic species of Anisakidae larvae are *Anisakis simplex*, *Anisakis physeteris*, *Pseudoterranova decipiens* and *Contracecum osculatum sp.*, but only *A. sinplex* larvae get into the human intestinal wall by the consumption of raw paratenic host fish and squid. Normally this larva is not found in the tissue of gastrointestinal tract, but may also invade the peritoneal or thoracic cavity, causing extra-gastrointestinal anisakiasis.

Annually 2000 cases of anisakiasis were reported in Japan, in Italy only 19 cases had reported until today. The allergic form of anisakidae larvae is underestimated for the complexity of Anisakis antigenes and the cross-reaction with other antigenes. Some researchers found that ingestion of a large number of lyophilized A. simplex larvae or a large amount of the parasite secretory and excretory allergens did not induce allergic symptoms in individuals allergic to A. simplex (Baeza, Rodriguez, Matheu, Rubio, Tornero, de Barrio, Herrero, Santaolalla, and Zubeldia 2004; Astre, Lluch-Bernal, Quirce 2000), suggesting that only the ingestion of live parasite larvae may induce allergic symptoms. However, other researchers have described clinical symptoms in sensitized patients following the ingestion of frozen or canned fish (Audicana, Ansotegui, Ferna´ndez de Corres, and Kennedy. 2002). Allergic reactions to A. simplex also have been described after consumption of meat from chickens fed with fishmeal

(Armentia, Martin-Gil, Pascual, Martı´n-Esteban, Callejo, and Martı´nez. 2006). Freezing and frozen storage of fish at temperatures lower than -20°C is recommended for killing Anisakis larvae and avoiding infestation of the consumer. Excreted and secreted products released by the migratory larvae into their environment are thought to play a role in tissue penetration, and in infested humans these products can induce an immunoglobulin (Ig) E–mediated immune response (Moneo, Caballero, Rodriguez–Perez, Rodriguez-Mahillo, and Gonzalez-Munoz 2007). Among the proteins excreted and secreted by A. simplex, an allergen called Anis s 4 has been identified (Moneo, Caballero, Gonza´lez-Mun˜oz, Rodrı´guez-Mahillo, Rodrı´guez-Perez, and Silva 2005). Ani s 4 is pepsin resistant and heat stable and, therefore, could be a clinically relevant fishborne allergen. Recently Solas, García, Rodriguez-Mahillo, Gonzalez-Munoz, de las Heras, Tejada observed the migration of the larvae from the surface of the steaks to the interior of the flesh immediately when the larvae were placed in contact with the muscle. The muscle penetration by the larvae was deeper as the storage period increased. Ani s 4 was detected in the interior and on the exterior of the larvae and in the areas immediately surrounding the larvae. Ani s 4 and other antigens detected by the anti–crude extract antiserum also were observed in areas not surrounding the larvae, where the structure of the sarcomeres was preserved. These findings indicate that allergens released by the larvae were able to disperse into the muscle. The presence of Ani s 4 or other allergens excreted or secreted by A. simplex in the muscle could cause further allergic reactions in sensitized patients, even if the parasite is dead when ingested. The prolonged stability of these allergens may explain the disagreement found in the literature about whether allergic reactions can occur when only dead larvae or larvae included in fish muscle are ingested. Anisakidae larvae have been recorded from numerous species of marine fish. Japanese authors (Ishikura, Kikuchi 1990)

listed 123-164 fish species as hosts of this kind of parasite. Many studies have been made in commercially important teleosts as: herrings, atlantic salmon and pacific salmon species, gadoids, scombers, clupeids, achovy and squid. Although from 1960 until today more was known about anisakidae, there are futher aspects to be deeply know as host range of anisakidae larvae and the prevalence rates in definitive intermediate or paratenic hosts. The aim of this preliminary study is to calculate the prevalance of anisakidae larvae in a unkonwn host specie as european sea bass.

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Short communication

Preliminary study on prevalence of larvae of Anisakidae family in European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

A total of 561 fresh European sea bass from North-East Atlantic Ocean (F.A.O. zone 27) were examined for Anisakidae larvae detection. It is the first record on the prevalence of Anisakidae larvae in this fish specie. An unexpected high prevalence was found. The prevalence range was between 65% and 85% in relation with the body weight of the subject. The results have an important consequence on epidemiology of anisakidosis and public health risk assessment.

1. Introduction

Many zoonoses are caused by parasitic helminths of fish, one of these is anisakiasis. Anisakiasis is the human infection with third larval stage of nematodes belonging to the families Anisakidae or Raphidascaridae.

Anisakis and Pseudoterranova are the two genera most associated with anisakiasis. Marine mammals and piscivorous birds are definitive hosts, while aquatic invertebrates and fish are intermediate hosts. In intermediate host, the larvae penetrate the intestine and invade the celomatic cavity or the muscle, where they moult in third stage and become encapsulated. Humans are accidental host for larvae, as the parasite does not develop further in the human gastrointestinal tract. Nevertheless, Anisakidae larvae cause a number of clinical forms, some of which can be very serious. People can incur in this pathology when consuming raw or lightly cooked seafood products. About 20,000 cases of anisakiasis are reported in the literature, over 90% from Japan and the rest from European Union, United States of America, Canada, New Zealand, Chile and Egypt (Chai, Murell, & Lymbery, 2005). The high prevalence of anisakiasis in Japan is clearly correlated to the tradition of consuming raw fish. In Italy, 23 identified cases of human anisakiasis are reported over the period 1996–2007; in all the cases the patient referred the consumption of raw or marinated fish. Anisakiasis is misdiagnosed and underestimated, in fact the diagnosis is often obtained after surgery (Maggi et al., 2000; Montalto et al., 2005; Pampaglione et al., 2002; Pellegrini et al., 2005; Testini, Gentile, Lissidini, Di Venere, & Pampiglione, 2003; Ugenti et al., 2007). Nowadays, the increase in consumption of raw fish in Italy asks for new epidemiological studies of anisakis in fish species. For this reason, a preliminary epidemiological survey of Anisakidae larvae was carried out on 561 specimens of European sea bass (*Dicentrarchus labrax*) captured off the F.A.O. zone 27. Wild European sea bass is one of the most important species commercialized in Europe, and particularly in Italy, for commercial value and acceptance.

2. Materials and methods

Owing to the absence of prevalence studies in European sea bass, it was adopted a conservative approach. Sample size was estimated using the formula (Daniel, 1999):

$$n = [ZP(1-P)]/d^2 \quad (1)$$

where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence (in proportion of one), d = precision (in proportion of one).

In this study, Z value is 2.58 at the level of confidence of 99%. It was assumed a P value of 0.50, because, as suggested by Daniel (1999), if there was doubt about the value of P, it is best to err towards 0.50 that it would lead to a large sample size. It was chosen a precision of 10% (d value 0.10) as a good compromise between an appropriate precision and the resource due to a preliminary study. With these remarks, the resulting n was 166. Stratified random samples of European sea bass from F.A.O. zone 27, with strata defined by body weight, were collected from commercial boxes between March and June 2007. Three weight categories were defined

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Table 1
European sea bass from F.A.O. zone 27 divided in weight category. Sample (*N*) number of subject examinated, positive (*N*) number of infected subject

Weight categories	1–2 kg	2–3 kg	>3 kg
Sample (*N*)	334	180	47
Positive (*N*)	218	153	42
Prevalence (%)	65.27	85.00	89.36

(1–2 kg, 2–3 kg and >3 kg) because it was easier to use commercial categories and the specimen weight is correlated to age. The specimens were so divided: 334 of European sea bass for weight category 1–2 kg, 180 for weight category 2–3 kg and only 47 for weight category >3 kg. The fresh fish was dissected immediately after collection. The fish was dissected by making an incision along the ventral line from the anus to the oral aperture, then the whole body cavity and the abdominal organs were carefully examined to detect the Anisakidae larvae. A subject with one or more larvae was considered positive and potentially harmful for human consumption. Prevalence was defined as the percentage of infected hosts. Yates'chi-square test was used to analyse the association between fish weight and prevalence. Because of its small sample size, it was not calculate the correlation between prevalence and weight for the category >3 kg.

3. Results and discussion

The prevalence in each weight category is reported in Table 1. An unexpected high prevalence was found: the prevalence of Anisakidae larvae in the 1–2 kg European sea bass was 65.27% against 85% in 2–3 kg European sea bass and there was a statistically very significant relationship between fish weight and prevalence of parasites ($P < 0.01$). Owing to the difficulty to collect subjects of more than 3 kg body weight, this sample size group was not enough to determine prevalence with the same precision of the other weight categories, but the obtained result suggested an important infection. The present survey is preliminary, but it provides a number of points of interest, especially because it is the first report of the presence and prevalence of Anisakidae larvae in European sea bass. As the weight increase is correlated with fish age, the observed prevalence increase can be related not only to accumulation of nematodes in the host during its life, but also to the change of diet, in fact large fish preys small parasitized fish, the larvae are able to re-establishing in predator without a moult. Large European sea bass can thus accumulate a very high number of larvae. Italy is the largest European Union market for wild and cultured European sea bass. Considering that European sea bass is marketed whole without evisceration, it is strategic to study in depth the epidemiology of anisakidosis from European sea bass for assuring public health. In fact, the success of control and legislation measures against any parasitic disease is dependent on knowledge of its aetiology and epidemiology (Cox, 1993). From this preliminary work, many important issues are to be raised: the possibility of larvae migration in muscles intra vitam or post mortem; the specie identification of nematodes and their possible survival in European sea bass; the possible differences in prevalence according to fishing zone. While studying these issues, to prevent anisakidosis it is important to adopt good health practices, such as immediate evisceration of European sea bass after capture, without throwing viscera overboard and the precaution of not consuming raw wild European sea bass. After further studies, when the prevalence data in this specie will be confirmed, it will be possible to determine a sample plan for detecting the lots of parasitized fish. The following formula, suggested by Canon and Roe (1982) to determine the sample size to verify parasite free population, could be used:

$$n = [1-(1-a)^{1/D}][N-(D-1)/2] \quad (2)$$

where n = sample size, a = level of confidence, D = presumed minimum number of infected animal.

Anisakiasis is an important public health problem, this requires to deep its knowledge of each step of the food chain, from fisher to consumer.

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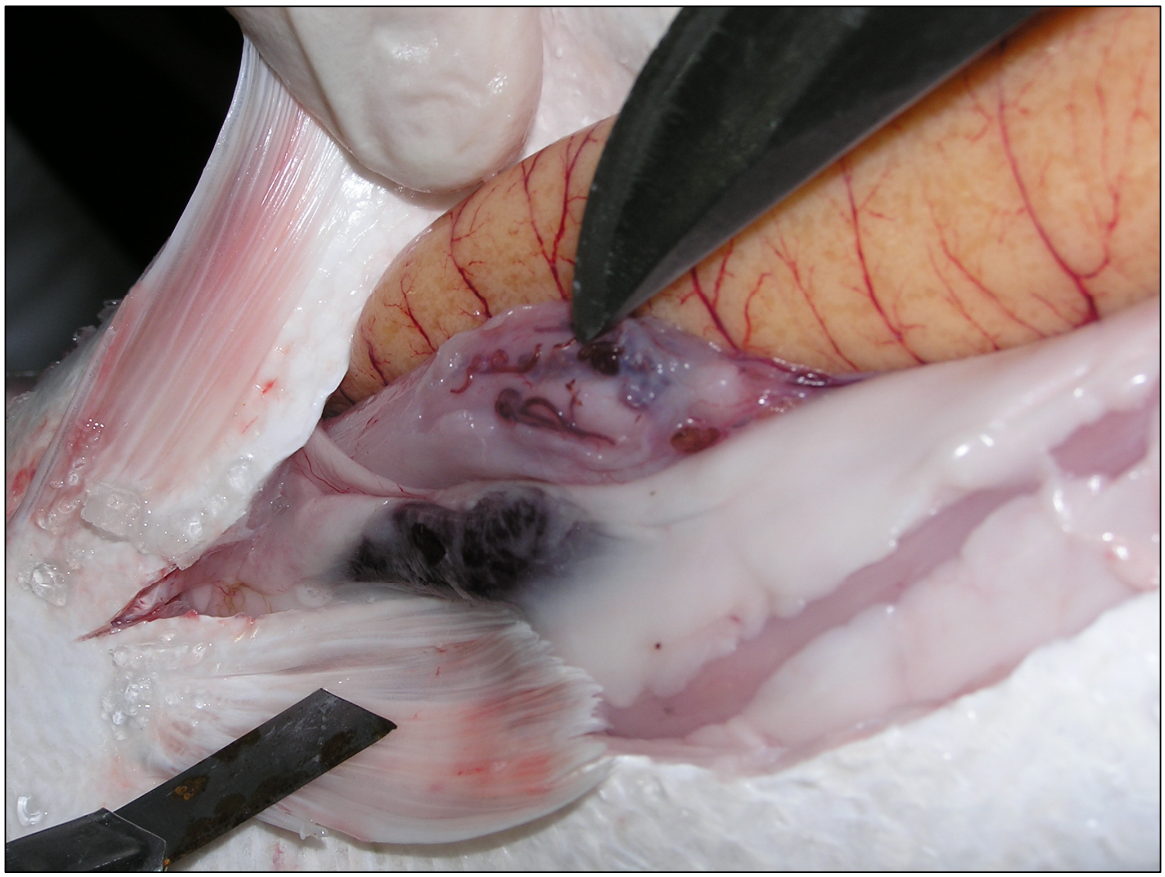
Discussion

This preliminary study imposes further research in order to obtain informations for an adequate risk assessment. The high prevalence in the abdominal cavity costitute a potential public health problem as well as an aesthetic problem.

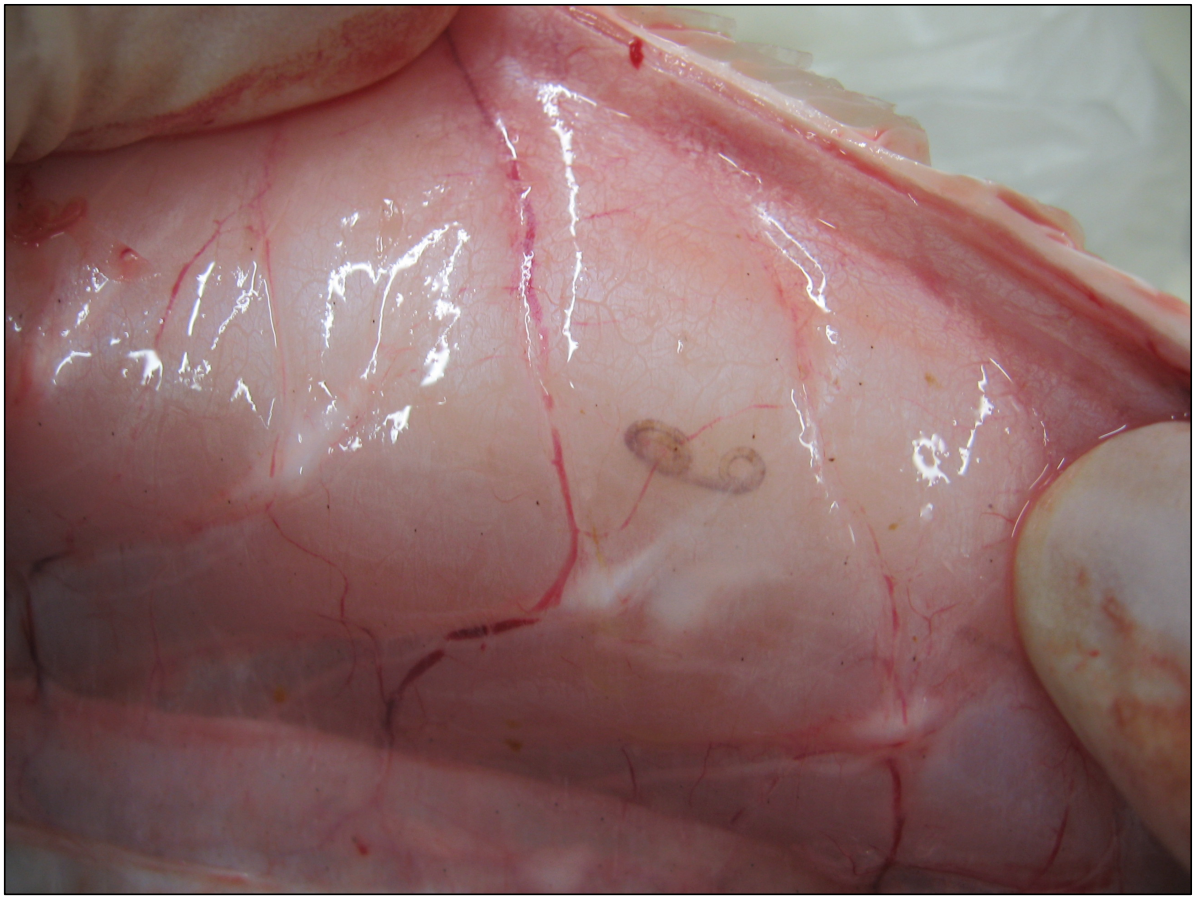


Picture n.1 Anisakidae larvae in abdominal viscera of european sea bass.

The larvae may then encoil on the surface of the internal organs, or they may migrate into the flesh. A similar migration from viscera into the flesh has been descrbed after the fish have been caught (post mortem migration) for mackerel (*Scombrus scombrus*) (Smith 1984), herring (*Clupea harengus*) (Smith, Woottten 1975), while not for fish species, such as whiting (*Merlangius merlangus*), blue whiting (*Micromessistius poutassou*) and walleye pollock (*Theragra chalcogramma*). However, some controversy exists, as other studies (Roepstorff, Karl, Bloemsma and Huss 1993) have not been able to demostrate any post mortem migration in

herring. The UE rules suggest that the fish, when possible, should be gutted on board immediately after capture to reduce the number of nematode in the fillets. This indication causes the problem of correct manipulation and safe elimination of parasitized viscera by the fisherman.

In the next studies the prevalence of anisakidae larvae will be performed, actually the anisakidae larvae presence in the flesh was recorded (picture n.2)



picture n.2 Anisakidae larva in belly wall of european sea bass.

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Seafood products ready to eat

In the last years, the consumer attitude toward seafood consumption resulted positively influenced by the development of new technologies in processing and storage. Besides, the market seems to favour products characterized first by a high nutritional value, quality and freshness, in the second place by novelty and practicality, elements which, together, more and more determine the market outlet.Some types of ready to eat fish products well agree with these requirements: they are obtained from minimally processed meat from different species, treated with salt, a little bit smoke or lightly pickled in order to increase aroma and shelf-life, then, they are usually vacuum or MAP packaged and stored at 4°C, with an expiry date of about two months. They are an example of a production that enhance the economic value of the raw matter and can locally allow the persistence of small and medium enterprises (SMEs), today more and more afflicted by the competitiveness of the global market. SMEs seem to play an important role in the increasing trend of seafood, as recorded in all European Countries, even if their actual economic weight is not full understood, because of the difficulties met in the national and international statistics processing. European Union is always paying the highest attention to SMEs, according to their central role in the European economy. It is enough to say that there are 25 Millions of SMEs which operate in Europe; they represent 86% of all the enterprises and employ about 95 Millions workers, assuring 55% of the total work places in the private sector. In Italy, SMEs of the seafood compartment are of primary economic importance in the agro-food market; the wide variety of their proposal, often deriving from culinary traditions, yet, is sometimes known only at local level. The main production is represented by smoked and marinated seafood. In Central Italy seafood respond mainly to local availability qualifications, in other Regions, among these Friuli Venezia Giulia and Emilia-Romagna, besides local catch, farmed fish or national catch or imported

wild/farmed fish is transformed, applying, with modifications even innovative, not only old technologies but also indigenous technologies of the origin countries, as for cold smoked salmon. In Lombardia, the processing of sea species has joined to those of farmed salmon, trout and sturgeon. The diffusion of new seafood and preparations brings out either hygienic-sanitary or technological questions. In fact, the contamination and recontamination possibilities, which can occur during the processing, are various. The productive situation itself is composite for culture degree, product quality, awareness of peculiar risks of these foods and the applied procedures are sometimes empiric, if not slapdash. Fish microbiological condition after catch or harvest is strictly bound to environmental condition and microbiological characteristic of water: temperature, salt content, distance from polluted areas (animal and human waste), natural presence of bacteria in water, feeds, fishing methods and chilling conditions. Seafood-borne pathogens are often divided into three groups: I) naturally present in the aquatic environment, or indigenous bacteria (Vibrio spp., Aeromonas spp., Plesiomonas shigelloides, non-proteolytic C. botulinum), II) bacteria associated with aquatic environment pollution (Salmonella spp., pathogenic E. coli, Shigella spp., Campylobacter spp., Yersinia enterocolitica), III) handling and processing contaminants (L. monocytogenes, S. aureus e Clostridium perfringens) (FDA 2001). Although only a small fraction of food-borne diseases is reported in statistics, in many Countries fish products cause 10% of total food-borne disease outbreaks (Huss, Reilly, Embarek 2000). In Italy, epidemiological surveillance data (WHO 2000) show that 5.2% of identified food-borne outbreaks, were due to seafood consumption. The increasing interest of consumer in ready to eat seafood has requested the processing industry to adopt new manufacturing and packaging technologies, with the purpose to extend products' shelf-life, maintaining meanwhile almost unchanged freshness characteristics expected by consumer,

applying light preservation methods (low salt levels, cold smoking, and so on). Such demands yet put important hygienic-sanitary questions: extending shelf-life can promote hazards of public health concern bound to the growth of some psychrotrophic pathogens. The fish species more processed are salmon, sword fish, tuna, octopus, mackerel, sea-bass and other ones. Generally, the hygienic-sanitary conditions of these seafood depend on various factors, related to the presence in the respective raw materials of pathogenic, spoiling microorganisms and their metabolites. Listeria monocytogenes (L.m.) is frequently isolated in seafood, either fresh or transformed (Ben Embarek 1994; Nilsson, Gram, & Huss 1999) and in cold smoked seafood represents the main health hazard. In cold smoked fish was found the presence of L.m. in the range 34-60% (Ben Embarek 1994). At present, researchers are facing the problem Listeria in a processing optics (Johansson, Rantala, Palmu, Honkanen-Buzalski 1999), particularly with predictive microbiology studies (Dalgaard, Jorgensen 1998).Processing fit to extend seafood shelf-life, such as smoking and salting, with vacuum or protective atmosphere packaging, can represent potential risk of botulism. Besides, a vast range of studies has shown that hot-smoked and cold-smoked fish are good substrates for C. botulinum and that the organisms may grow and produce toxin, depending on salt and temperature levels. A recent case owing to cold smoked salmon (Dressler 2005) occurred in Germany. Many researches evaluated either the incidence of C.b. in different seafood or its growth possibility depending on time and temperature storage (Heinitz, Johnson 1998; Hyytia, Hielm, Mokkila, Kinnunen, Korkeala 1999). Volatile compounds are chemical substances responsible of olfactory sensations (aroma); they change according to many factors, among these: specie, product transformation and conservation. Volatile compounds are therefore studied in cold smoked fish in order to assess their contribution to the spoilage off-flavour (Jorgensen, Huss, Dalgaard 2001) and to study the effect of storage (Aro,

Tahvonen, Koskinen, Kallio 2003). GC-MS with solid phase microextraction (SPME) was successful applied to study ripening and spoilage of meat products (Chiesa, Soncin, Biondi, Cattaneo 2006). Now, in spite of a wide variety of lightly preserved seafood, there are nor aimed studies and nor sufficient data in order to evaluate, in a risk assessment approach, their safety.

1 **A case study: shelf-life of smoked herring fillets by volatile compounds**
2 **analysis.**

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14

Abstract

Two different products of vacuum packed cold smoked herrings were analyzed at time intervals in order to evaluate the efficiency of the processing and product stability. Microbiological total counts, lactic acid bacteria, total coliforms, pH, water activity, water content, salt content (WPS) were determined. Differences in hygienic conditions and salt content were found. Principal components analysis (PCA) of volatile compounds determined by GC-MS analysis allowed the differentiation not only of the processing but also of the storage time.

Key words: principal components analysis, shelf-life, smoked herring, volatile compounds, microbiological analysis.

Introduction

The herring is one of the most plentiful fish specie of the planet and the commercial fishery of Atlantic Herring is one of the most important of the world in terms of biomass and value. Atlantic herring is from 12^{th} century one of the main exchange goods between Scandinavian and European Countries. The herring fished on the West Atlantic coast (USA and Canada) is mainly converted in fish meal or in oil, while in Europe the herring is more commonly

used as seafood, consumed fresh or after processing, being part of the traditional cuisine of many European Countries. The herring is a tender and fragile fish, with a very large gills area: it is highly perishable and proper handling and quick chilling are essential for fish intended for human consumption. In Italy the fresh herring is marketed as butterfly fillets.
The main food outlets are for smoked, salted, marinated and canned products. Quick freezing and cold storage are also applied on some finished products.
The herring preservation is a very ancient process, described since Middle Age. Salted and dried herring or salted, dried and smoked herring were steady foods available all year round and in the inland areas.

The herring can be hot smoked, such as buckling, or cold smoked with differences due to preparation, type, combination and duration of the processes: whole ungutted lightly smoked (bloater and corresponding Norwegian silver herring), or harder dried salted smoked (red herring, hard cured herring) to reach a drier texture and a deep red colour, more appreciated in Mediterranean countries, and gutted, splitted in two, lightly salted and smoked (kipper, golden herring) or in fillets (kipper fillets).

In Italy the consumption of smoked herring is now very reduced compared with the past, when it was added to simple foods, such as “polenta”, potatoes or salads, to give together with a strong flavour a protein contribution. Some of these dishes are part of tradition, such as “"aenghi e patatte", a Genoese dish,

56 “poenta e renga”, in Venetian cuisine, and now offered again as examples of
57 poor but tasty foods.

58 Salted and smoked herrings, in wooden box or barrel, came mainly from
59 Norway, major producer of preserved seafood, such as salted cod, dried cod
60 and other salted and/or smoked minor fish species.

61 At present, the smoked herrings more widespread in Italy are beheaded with
62 skin, vacuum packaged, silver and golden types; less frequent are skinned
63 fillets, softer and with a milder taste, vacuum packaged, which may meet more
64 with the liking of the modern consumer, while the whole smoked herrings
65 without packaging are always less widespread and available only in specialized
66 shops or in local markets.

67 The vacuum packaged cold smoked herring is at the limit of the group of the
68 lightly preserved seafood (Huss, 1994). This group comprises products with
69 low salt level (<6% NaCl-Water Phase Salt WPS), preservatives or smoke, pH
70 value > 5, mainly vacuum packaged, requiring refrigeration temperatures and
71 typically consumed as ready to eat with no heat treatment.

72 The process must allow the survival of an adequate number of spoilage
73 organisms, which have the important role to compete with the growth and
74 toxin formation of *C.botulinum* type E and non –proteolytic types B and F (FDA
75 Center for Food Safety & Applied Nutrition, 2001a), considering that the

packaging conditions, such as vacuum sealed pack, reducing the quantity of oxygen in the environment, extend the product shelf-life inhibiting the growth of aerobic spoilage organisms but increase the possibility of toxins formation of *C. botulinum*, before spoilage makes the product unacceptable for consumption.

Besides *C. botulinum*, whose spores are very widespread in nature and occur in gills and gut, being a natural, even if not frequent, presence in seafood, another possible hazard is *Listeria monocytogenes*, halo- and psychrotolerant pathogen, indigenous to the general environment. In fact, cold smoked seafood processing has no Listericidal step; salt content and water activity (Huss, Ababouch, Gram, 2003) are not always enough to inhibit its growth and, given to the ability of the organism to survive in the processing environment and re-contaminate products, it is realistically difficult to produce RTE foods consistently free of *L. monocytogenes* (FDA Center for Food Safety & Applied Nutrition, 2001a).

The control measures of potential hazards are based mainly on salt control (nitrite addition is not allowed by EC regulation in these products), on control of exposition to temperatures that favour C. botulinum throughout processing steps, on maintenance to refrigeration temperatures of the final products. With regard to this, US HACCP regulations suggest a critical limit for vacuum packaged cold smoked products of at least 3.5 % NaCl –WPS at ⌾ 4.4°, allowing for short time periods temperatures up to 10°C (FDA, Center for Food Safety and Applied Nutrition. 2001b).

97 The purpose of this investigation was to answer an importer's request, that was
98 to verify the established shelf-life of smoked vacuum packed herring fillets
99 produced in two European countries. With this aim traditional analytical
100 techniques and SPME headspace-gas chromatography-mass spectrometry were
101 applied.

102

103 **Materials and methods**

104 Samples of smoked herring fillets, peeled and vacuum packed (product A) were
105 furnished by the importer on their 27th day from production. The following
106 information was on the packaging: ingredients (herring, salt); fishing area
107 (North-eastern Atlantic Ocean); storage temperature (+ 2 - + 5°C); "best before"
108 date (45 days from production). The product was to be compared to another
109 one (product B), which was apparently the same, but with a "best before" date
110 of 60 days and a different producer. The processing technologies of both were
111 unknown. The products were furnished with the guarantee of a proper storage.
112 The packages were stored at 0-2°C for the whole period of the trial.

113 Experimental design: Chemical, physical and microbiological analyses were
114 performed on product A on the arrival day (27th day from production), on the
115 "best before" date (45th day) and on the 49th , 55th, 60th day from production.

Product B, available only in smaller quantity: on the 9^{th} , 26^{th}, 30^{th}, 41^{st} day from production.

SPME GC-MS: Product A: on 27^{th} day (L1 and L2), 45^{th} (L3 and L4), 60^{th} (L5 and L6) day from production. Product B: on the 30^{th} (D1 and D2) and 41^{st} day (D3 and D4).

Analyses were performed separately on two packages for each time and in duplicate.

Chemical-physical analyses: pH, water activity, water content (Official Methods of Analysis of the A.O.A.C., 1990); salt – WPS content (Pearson, 1973).

Microbiological analyses: Total Psychrotrophic Count (Count Agar Sugar Free, Merck); Lactic Acid Bacteria (Man Rogosa Sharpe, MRS, Oxoid, Italy), total coliforms (Chromocult® , Merck)

SPME GC-MS analysis

Sample preparation: ten grams of homogenate of herring muscle were transferred to a 20 mL headspace vial, sealed with polytetrafluoroethylene (PTFE)-coated silicone rubber septum (20 mm diameter) and allowed to equilibrate for 1 hour at room temperature. After this time the headspace was sampled with SPME fibre 75 μm CAR-PDMS (Superchrom-Italia) for 180 min at

134 room temperature (Aro, Tahvonen, Koskinen, Kallio, 2003), (Chiesa, Soncin,
135 Biondi, Cattaneo, 2006), (Triqui, Reineccius, 1995).

136 Gas chromatographic analysis : all analyses were performed on an Finnigan
137 Trace GC Ultra gas chromatograph coupled with an Finnigan Trace DSQ mass
138 selective detector. The compounds were separated on a Rtx-WAX capillary
139 column (30m × 0.25mm i.d.; film thickness 0.25 μm). The SPME fibre was
140 desorbed in the PTV injector port held at 220 °C. The GC oven temperature was
141 held at 35 °C for 8 min, then increased to 60°C at 4°C/ min, then to 160°C at 6°C/
142 min and to 200°C at 20°C/ min and was finally held at 210°C for 15 min. The
143 interface and ion source temperatures were maintained at 230°C and 250°C,
144 respectively.. Helium was used as a carrier gas with a flow rate of 1 mL/min.
145 Mass spectra were obtained under Electron Impact condition at 70 eV in the 35–
146 350 amu range. Ion source was held at 250 °C. Volatile compounds were
147 identified by matching their retention times and mass spectrometry data with
148 those of authentic compounds as standards.

149 After sampling for microbiological and SPME GC-MS analyses, pH and a_W
150 were determined; afterwards the whole package was blended and destined to
151 salt and water determinations.

152 **Statistical analysis**

153 Principal component analysis (PCA) was performed on the areas of the
154 chromatographic peaks detected in samples, in order to visualize data trends
155 and to detect possible clusters within samples, thus providing a first evaluation
156 of the discriminating efficiency of the considered variables.

157 Statistical analysis was performed by using the SPSS package version 9.0 (SPSS
158 Italia, Bologna, Italy).

159

160 **Results and discussion**

161 In table n.1, values belonging to a_W, salt - WPS and water contents are
162 displayed. Product A had water content significantly higher than B, but for its
163 higher salt value, WPS of product A was higher than B's; WPS rate was amply
164 above the 3.5 % NaCl-WPS which allows food safety of refrigerated cold
165 smoked seafood. In product B, salt content was more variable. Water Activity
166 was similar in both, but more variable in product A.

167 Product A had on the first sampling day (27^{th}) a very high TPC, afterwards
168 TPC decreased and LAB counts overcame 7 log units (figure n.1). Product B had
169 always lower TPC than A and so LAB counts (figure n. 2). Total coliforms were
170 absent both in A and B.

Forty four peaks were determined and identified using GC-MS in table n. 2 the main volatile compounds from smoked herrings A and B are reported. In figure n. 3 the analysed samples and the identified variables (volatile compounds) are represented in the space described by the two principal components, in this case the first component explains 78.3% variability among data. Samples are clearly distinguished in two groups, coinciding with A and B productions. The second component allows to distinguish product A on 27^{th} day from the subsequent samples.

Particularly, benzaldehyde and furfural were present only in product B, so small quantities of propionaldehyde and hexanal, absent in product A. Phenol was present in both, without significant differences. Acetic acid was found more in product B. Dimethyl sulfoxide and dimethyl sulphide were more represented in A than in B. As a whole, the herring B showed an aromatic profile more complex and rich in qualitatively superior compounds, while the herring A showed a very little articulate profile.

It was possible to distinguish the different storage times analyzing only the volatile compounds found in herring A, as presented in figure n. 4.

On the "best before" date, after 45 days, the herring A had an initial softening with presence of fluid material in the pack, without other sensorial changes; the herring B maintained a better firmness, also for a lesser water content. The

191 bacterial count at the first sample of A was high, indicating bad hygienic
192 condition of raw material, while the product B was in better hygienic conditions
193 on the same storage time.

194 The product A had a higher salt content; this was a negative aspect, not only
195 from a nutritional point of view, but also respect to the consumer's present
196 preferences. The WPS ratio of the product A was enough to give the same
197 stability of the product B in presence of a very high water content. WPS more
198 than 6% puts both products at the limit between lightly preserved and semi-
199 preserved seafood (Huss, 1994).

200 The salt level of product A was not enough to control the growth of alotolerant
201 bacteria, such as lactic acid bacteria, that reached on the "best before" date
202 numbers of $2.4 * 10^7$ cfu/g, levels very high that, nevertheless, do not cause
203 detectable deterioration of flavour.

204 The obtained results allowed to suppose a different processing technology: a
205 brine salting process in A, either for the high water level or for the uniform salt
206 content of the fillets, while in B, because of a lower uniform salt concentration
207 (Coefficient of variation 13.8% in WPS ratio) and a considerably lower water
208 content, a dry salting process with purging was probable.

209 From the chemical and microbiological analyses, it can be argued that the good
210 manufacturing practices were improperly applied to the product A processing.

In the verified conditions, the shelf life of product A is to be stated up to 4 weeks. Microbiological parameters of B were stable for the whole period of the trail (41 days), but in the impossibility to analyze the product B to the end of its shelf life and after, it was not possible to give a conclusive opinion. Water activity and WPS allowed to evaluate positively both products from a food safety point of view.

The exclusive presence in the product B of benzaldehyde and furfural, aldehydes known for giving a strong taste of smoke, confirmed the perception of a more typical aroma of the herring B.

The presence of propionaldehyde and hexanal in B, as small and constant in time, was to be attributed more probably to smoking than to lipid oxidation. The clear finding of dimethyl sulfoxide and dimethyl sulphide only in the product A, well match with the microbial condition of A; these sulphur compounds may be the expression of bacterial activity besides of ripening (Chiesa et al. 2006).

Conclusions

Granted that the available material was very short, a very probable answer could be given to the importer. The obtained results confirm that the principal components analysis applied to the identified volatile compounds determined

by SPME GC-MS is a promising analytical tool, either to define the quality or the processing characteristics of seafood. The results related to the study of the volatile fraction confirmed what deduced by the more traditional analyses: in spite of the close outward likeness between the two products, their processing technologies were very different, resulting in distinct products as regards shelf life and quality. The reported case is an example of the result of the delocalization, due to economical reasons, of food processing in countries applying less advanced or less consolidated technologies or procedures with inferior process standard.

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Table n. 1: Preservation parameters

sample	A					B				
	m	M	Mean	SD	CV	m	M	Mean	SD	*CV*
Water activity a_W	0,923	0,966	0,9433	0,014	0,36	0,940	0,949	0,9450	0,0034	*1.48*
Water %	72,25	75,18	74,12	1,1182	1,51	60,39	66,73	63,26	2,6170	*4,1284*
Salt %	5,25	5,59	5,43	0,1435	2,6432	3,76	4,85	4,38	0,5308	*12,133*
Salt-WPS	*6,57*	*6,92*	*6,83*	*1,464*	*2,14*	*5,62*	*7,43*	*6,48*	*0,897*	*13,838*

Legend:
m = minimum value
M = maximum value
SD = Standard Deviation
CV = Coefficient of Variation

279 Table n.2 Volatile compounds identified in smoked herring (*Clupea harengus*) by
280 GC-MS.

Peak number	Compound
1	2-pentene
2	heptane
3	dimehyil sulphide
4	Methyl-cyclo-hexan
5	propanal
6	furan
7	Propanone
8	methyl acetate
9	2-methyl-furan
10	ethyl acetate
11	2-butanone
12	3-methyl-butanale
13	ethanol
14	benzene
15	2-ethyl-furan
16	Branched hydrocarbon
17	2-pentanone
19	chloroform
20	hexanal
21	p-xylene
22	cyclopentanone
23	2-methyl-cyclo-pentanone
24	3-methyl-cyclo-pentanone
25	pyrazine
26	cyclohexanone
27	acetoin
28	2-methyl-2cyclo-pentenone
29	3-furaldehyde
30	acetic acid
31	2-furaldehyde
32	acetyl-furan
33	benzenaldehyde
34	propanoic acid
35	Dimethylsulfoxide
36	5-metil 2-furaldehide
38	gamma-butyrolactone

39	butanoic acid
40	3,4-dimethyl-3-penten-2-one
41	5-metil-2(5H)-furanone
44	2- pyranone
45	2-methoxy-phenol
46	guaiacol
47	phenol
50	2-furanone

281
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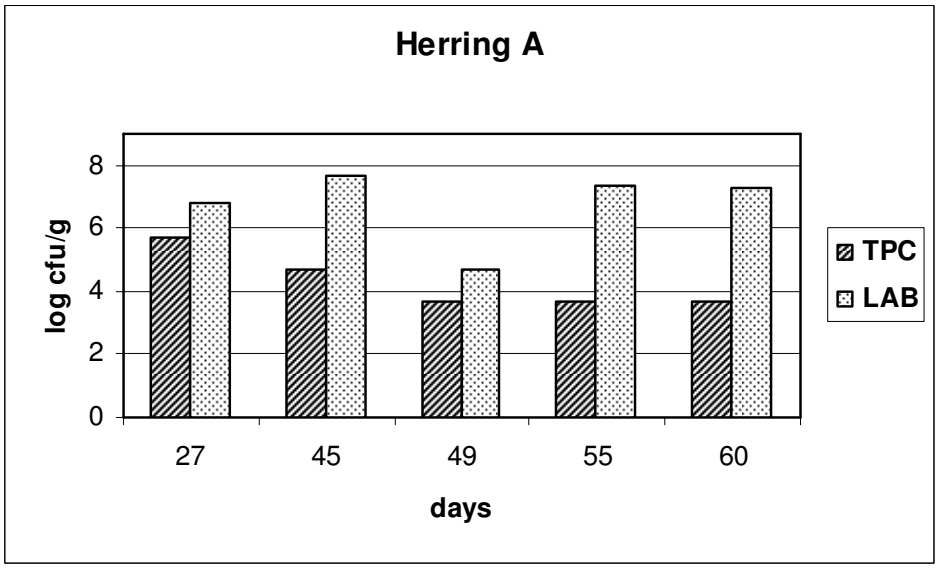
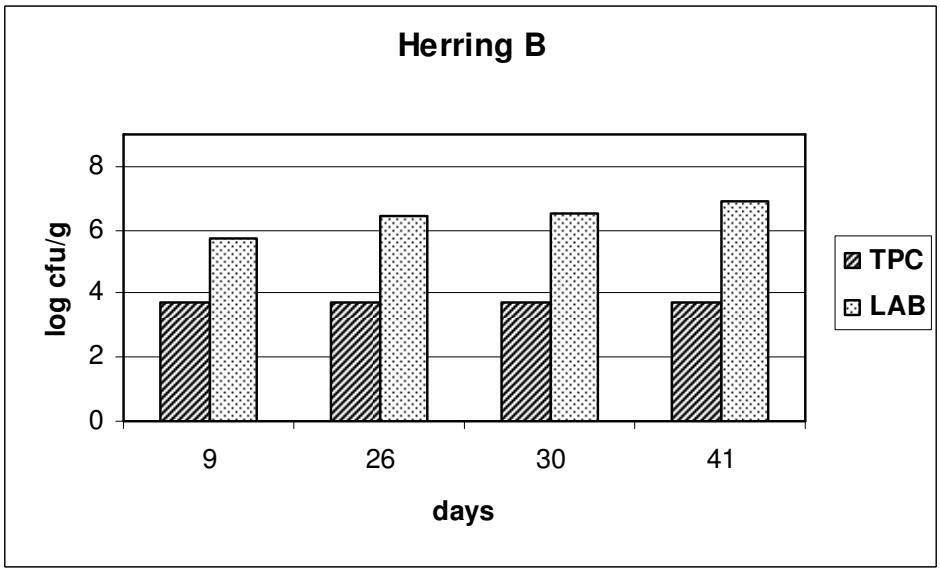
285 Figure n.1: TPC and Lactic acid bacteria in sample A.



286
287

288 Figure n.2: TPC and Lactic acid bacteria in sample B.



289
290

Figure n.3. Biplot of principal component scores and factor loadings from principal component analysis applied to volatile compounds of product A (scores L) and product B (scores D).

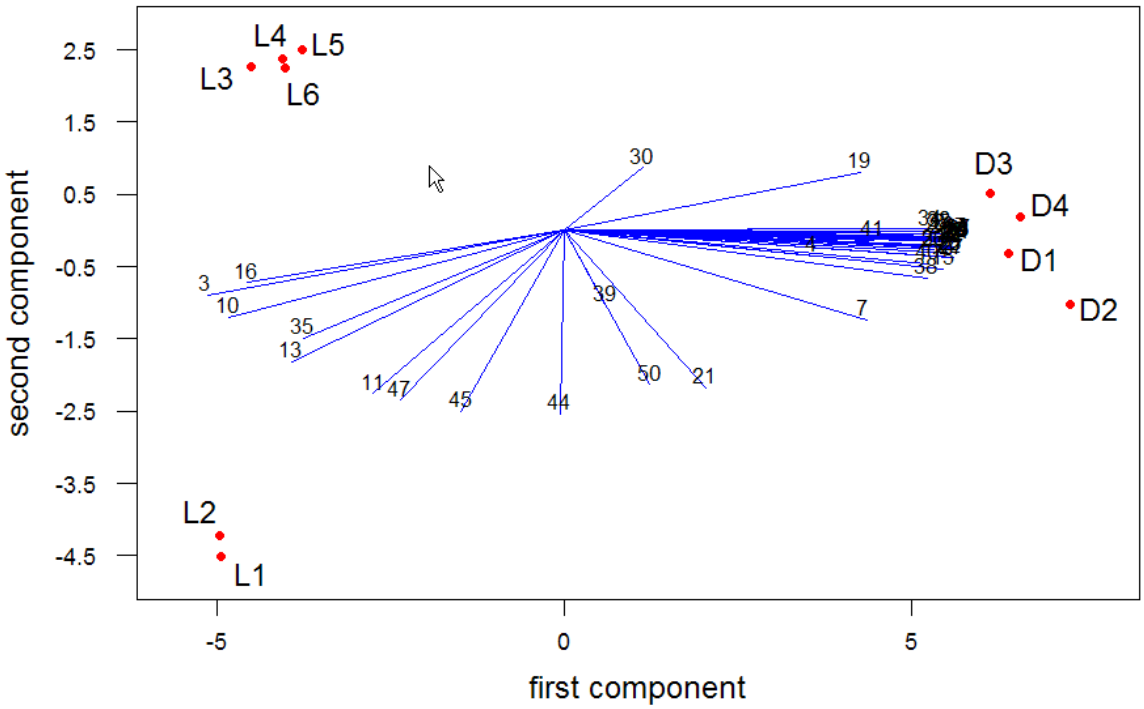
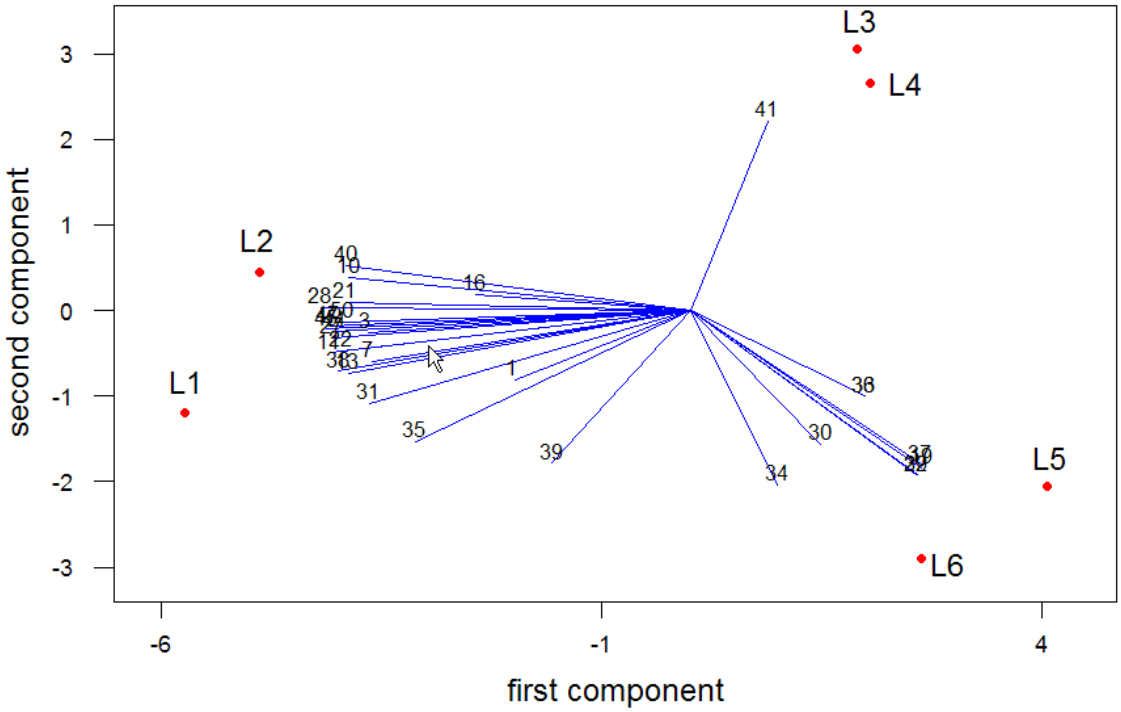


Figure n.4 Biplot of principal component scores and factor loadings from principal component analysis applied to volatile compounds of product A at different time of shelf-life.



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Quality and shel-life of cold smoked salmon -

Cold smoked salmon is a highly valued product in Italy. Most often the smoked salmon are sold sliced and vacuum packed in 100 and 200 g portions. Most of the fissh that are sold as smoked salmon within Italy are bought from different countries and are salted and smoked in further countries, finally are packed by Italian suppliers. Generally salmon from Scotland and Ireland are more expensive and have a, somewhat, higher reputation than the Norwegian salmon. In general sensory analysis employs several criteria, including the appearance of the skin, eyes, mucus and gills, the firmness of the flesh and odour. This determination of freshness is in part subjective. For transformed products, such as fillets, sensory analysis is less reliable because the number of assessment criteria diminishes. Adopting chemical, microbiological, physical criteria is possible to classify objectively different smoked salmon products. Smoked salmon is a highly nutritious food containing highly unsaturated fatty acids (HUFA), fat soluble vitamins, essential minerals as well as proteins containing the amino acids essentials for human beings (Espe, Nortvedt, Lie, Hafsteinsson 2001). The high amounts of HUFA in smoked salmon are susceptible to lipid oxidation during smoking and storage. Although oxidation analysed as thiobarbituric acid reactive substances (TBARS) has been reported to occur in Atlantic salmon during the smoking process, values has not been found to be so high that the fissh should be regarded as oxidized (Espe, Nortvedt, Lie, Hafsteinsson, 2001 and 2002). The prevalence of Listeria sp. in seafood is reported to vary products, one of the products showing the highest prevalences of L. monocytogenes has been reported to be vacuum-packed cold smoked fissh (Jemmi, 1993; Jorgensen, Huss, 1998). It has been shown that the external surfaces of raw fissh represent one of several sources for L. monocytogenes contamination of production lines in processing plants, and that plant specific strains of L. monocytogenes is frequently found to

contaminate smoked fissh products (Eklund, Poysky, Paranjpye, Lashbrook, Peterson, Pelroy 1995). L. monocytogenes will not be inactivated during the processes involved in cold smoking (Civera, Parisi, Amerio, Giaccone, 1995). L. monocytogenes is also shown to survive the commonly encountered concentrations of salt during salting and brining (Farber, Coates, Daley, 1992). The presence of L. monocytogenes in smoked and lightly processed ready to eat fissh products is therefore of special concern, because these products are often kept for a substantial period of time under refrigerated temperatures and under anaerobic conditions and are commonly eaten without further heating. Moreover in Italy the smoked salmon shelf-life generally is long sixty days, this is a matter of some concern in relationship to shelf-life of thirty days in other European countries as France, U.K., Norway.

The first aim of the present study was to analyse the quality and food safety indicators in cold smoked salmon with different origin on the Italian marked. To study this, cold smoked salmon was collected in the most important GDOs.

Commonly used approaches for monitoring the quality of fresh fish include sensory, microbiological, texture, and chemical analyses. Of these approaches, the most comprehensive measure of quality (in relation to acceptability by the consumer) is sensory analysis, which requires the use of a trained sensory panel for quality assessment of fish appearance, odour, taste, and texture. This is not only time-consuming but also costly to set up. The other analytical methods measure specific attributes that impact on fish quality, for example microbiological testing will give an indication of microbial spoilage and various chemical tests will target the products of specific chemical or enzymatic reactions (for example oxidation) or microbial metabolism. A need therefore exists for an instrumental method that can be used to monitor the quality of salmon and is related to the sensory changes and the analytical results obtained by robust and universally accepted methods. Solid-phase microextraction

(SPME) is a proven tool in volatile analysis and has previously been used for the analysis of flavour volatiles in seafood (Li, Zeng, Zhou, Gong, Wang, Chen 2004; Mansur, Bhadra, Takamura, Matoba 2003; Serot, Lafficher 2003; Triqui, Bouchriti 2003). In general, SPME is both simple and cost-effective to use and can be used to analyse the levels of a wide range of volatile compounds. Odour is the primary parameter determining the sensory quality of products and consequently it is of interest to study if key volatile compounds contributing to the characteristic odours can be measured as indicators of quality. Many factors influence the quality of smoked salmon including the properties of the farmed salmon feed, size, starvation and storage and for wild salmon maturity, age, seasonal variations and for both categories the factors involved in the smoking procedure such as type of wood, composition of the smoke, temperature, humidity, velocity and density of the smoke.

Specific volatile compounds in particular phenolic compounds have been related to the different smoking techniques which directly influence the sensory characteristics of smoked salmon (*Salmo salar*). The aim of the volatile compounds study presented herein was to identify the key characteristic volatile compounds in cold smoked salmon by gas chromatography mass spectrometry (GC–MS) and study their suitability as quality indicators to predict sensory quality. The objective was to verify if the variation in the volatile compounds could explain the differences observed by sensory, chemical and microbial measurements.

SHELF-LIFE OF VACUUM PACKED COLD-SMOKED SALMON FROM ITALIAN MARKET

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ABSTRACT

Fourteen samples of different vacuum packed cold smoked salmon products purchased in Italian retail were analysed at about half shelf-life and at the expiry date. In order to evaluate product stability, total psychrotrophic count, lactic acid bacteria, *Enterobacteriaceae, L. monocytogenes, Salmonella* spp, coagulase positive staphylococci, TVBN, TBARS, colour and texture were performed. Preservation parameters (water content, salt content-WPS, aw) and product analyses (defects, colour, texture and net weight) were determined.

High TVBN and TBARS values were already determined at 30 days before the expiry date. On the expiry date 8 samples were above the limits. In one sample WPS was less than 3,5%, with microbiological risk. Microbial counts at half shelf life were acceptable in most samples; TPC at expiry date was very high (> 10^6 CFU/g) in almost all samples, and was dominated by Lactobacilli. Levels of Enterobacteriaceae and coagulase-positive staphylococci were very low, and no potential pathogenic bacteria were detected. Significant negative differences in declared weight were determined.

Key words: shelf-life of salmon, quality of cold smoked salmon, volatile compounds, quality indicators.

INTRODUCTION

Cold smoked salmon, vacuum packed, is a product whose consumption has an increasing trend in Italy. Smoked salmon is consumed all over the year as "ready to eat" food, while in the past it was purchased only on the occasion of the December festivities. In the last year it was confirmed the increase of request of smoked salmon (+8,2%) against the decrease of the purchase of other semi preserved seafood, such as salted and dried cod (Ismea, Consumi 7.1.07-5.1.2008). The availability of smoked salmon is now very high, being the raw material obtained from aquaculture in many European Countries, first of all Norway. Salmon farmed in Ireland and Scotland is considered of best quality. Smoking processing is made mainly in France, followed by Denmark, United Kingdom but also in Norway, Italy and Poland. A lesser quote is represented by wild salmon, either *Salmon salar* either *Onchorhyncus* species.

The quality of the finished product depends on numerous factors, first of all the quality of the raw material, depending from the aquaculture techniques and freshness, and from the processes used (salting methods, drying and smoking techniques, premises). Very important is the hygienic level of the process, from which depends the shelf-life of the product. The expiry date is attributed by the producer; in fact this is a precise responsibility of the producer who, by means of shelf-life studies, can establish an expiry date that assure the hygienic quality of the product. In France the use by date (Date Limite de Consommation DLC)

of smoked salmon vacuum packed is about 3-6 weeks at 4°C (Leroy, Ifremer, 2005), in Italy most producers fix a use by date of 2 months, in the same storage conditions.

The main problems observed from a French market survey are quality and texture (Cardinal n. 21). Bad hygienic conditions were already observed at the expiry date in smoked salmon marketed in Italy (Vergara et al., 2001).

The aim of this work was to verify the quality and the durability of sliced smoked salmon vacuum packed available on the Italian market, by means of product, physical, chemical, microbiological analyses.

MATERIALS AND METHODS

Fourteen samples of cold smoked salmon, sliced vacuum packed, from eight different processors and of different origin (Norwegian, Scottish, Wild from Alaska) were purchased in supermarkets in Italy in summer-autumn 2007 (table n.1). All the samples were in retail packs of 100 g each, except samples n. 101 (50 g) and n. 114 (250 g). The samples (18 retail packs each of the same lot) were stored at 2-4°C and analysed about on the 30th day before the use by date (about Half Shelf Life HSL) and on the day of the use-by date (Expiry Date ED) for microbiological, chemical, physical and product analyses.

On the opening, all packs were submitted to product analysis: red muscle size, slices aspect, presence of blood spot, of rifts or gaps, of scales and bones and net weight.

The next step was to **create** a **value scale** for each of the parameters, assigning arbitrary scores depending on the importance of the defect for the consumer.

Red muscle: the consumer prefers smoked salmon without red muscle, for this reason the producers normally trim the salmon fillet after smoking. In this work the red muscle area was determined by image analysis and calculated as percent of total slice area (RM). Colour images were collected using an Epson Perfection 1200 PHOTO colour scanner (Seiko Epson Japan) with an optical resolution of 1200 dots per inch (dpi) for main scanning and set to 24 bit colour. Scanner software was the Epson software package provided with the scanner. Images were stored in the personal computer hard disk as a RGB (Red-Green-Blue) tagged image file format (tiff) files. Each salmon slice was considered as a single object and was scanned individually. The slices and a paper scale bar as a dimensional reference were placed on a black plastic base on the scanner surface. The subsequent image analysis of recorded images was performed with the Image-Pro Plus 4.5.1 software package (Media Cybernetics, Silver Spring, MD. USA). The scale bar was used for software spatial calibration. Firstly, a discrimination of each slice from the dark background was performed. In the case of salmon, all pixels of a slice (light object) had a R-colour value higher

97 than G and B-colour while black plastic base (dark object) had similar high
98 values for R, G and B-colour. On this base, an automatic option of the image
99 analysis software allowed to separate dark objects from the light ones. The
100 second step was to discriminate between red muscle and the rest of slice
101 surface. G and B-colour values of the two parts of slice were similar, but the
102 discrimination was possible on the basis of R-colour value (lower in the red
103 muscle than in muscle). Due to the considerable colour differences among the
104 salmon samples, the segmentation of images was possible by setting a specific
105 range of R-colour level for each sample. Finally, surfaces areas from the entire
106 slice and dark red muscle were automatically measured and recorded as an MS
107 excel file by the Image-Pro software. A six points scale was adopted: 0= no RM;
108 1 = RM < 2%, 2 = 2% ≤ RM <5%, 3 = 5% ≤RM<8%, 4 = 8% ≤ RM< 10% and 5= RM
109 >10%.
110 Slices aspect: (easy slice separation and differencies in size) easy slice separation
111 of salmon slices was graded in an unstructured scale, where 0 was possible to
112 obtain a entire slice and 1 was not possible. The assessment of difference in size
113 among the slices of each packs was calculated as %RSD of slice area. Every
114 packs was graded in an unstructured scale, where 0 was a RSD ≤ 30% and 1 was
115 a RSD > 30%.
116 Blood spots: In accord with Robb, Phillips and Kestin (2003) the blood spots
117 observed on the slices surface were classified in three categories: types 1, 2 and

3. Type 1 blood spots were circular with 1 -2- mm in diameter and were associated with vessels on the midline fish fillet. Type 2 blood spots appeared as small flecks, 1-4 mm long and up to 1 mm in width within the abdominal wall. Type 3 blood appeared as occasional engorged belly vessels up to 20 mm long and 2 mm in width. Elsewhere in the fillet blood spots normally tend to be type 1 or type 2 and are rare. In commercial assessment the type 3 and 2 blood spots can be trimmed off without important weight loss, whereas the trimming of type 1 blood spots causes considerable loss. In this point of view, a four point scale was set: 0= no blood spots, 1= type 1 blood spots, 2= type 2 blood spots and 3 = type 3 blood spots

Rifts and gaps in the sliced fillets were visually inspectedand graded in an unstructured scale from 0 to 5 where 0 was no visible rifts and 5 was massive tearing.

Presence of scales and bones: due to their very rare presence and very low importance, score 0 indicates the absence and score 1 the presence.

Net weight determination

Net weight was measured as mean of six packages of each sample by the method of Canadian Food Inspection Agency; in brief, for each package, the net weight was determined subtracting the individual tare weight (after careful dry cleaning the empty pack of the oily residue with laboratory paper) from the gross weight. The difference of so calculated net weight and the declared net

weight was reported as percent of the declared weight and named Net Weight Loss. (NWL). From NWL an arbitrary five point scale was create (0= 0% ,1= NWL< 1%, 2= NWL < 5%, 3= NWL< 8% and 4 = NWL> 8%).

Colour measurement

Colour was measured from three packages of each samples, using a Minolta Chromameter CR-200/CR231 (Minolta, Osaka, Japan) working at CIELab system. The chromameter instrument was calibrated using a standard white plate and was positioned perpendicular to the slice surface, after removing the superficial fat. The L*, a* and b* values, which describe the intensity of whiteness/brightness, red colour (a* > 0) and yellowness (b* > 0), respectively, were taken at ten locations on the upper layer of each pack immediately after opening. The respective mean of ten such measurements was expressed as the final value. Colour intensity (c) was calculated as $c = (a^{*2} + b^{*2})^{1/2}$. The hue angle (h) was calculated as h = arctan (b*/a*), where h = 0° for red hue and h = 90° for yellowish hue. Total colour differences (ΔE) from the respective samples at time HSL and ED were calculated as shown below:,

$\Delta E = \sqrt{(L_1-L_2)^2 + (a_1-a_2)^2 + (b_1-b_2)^2}$.

A ΔE more than 2.3 means a variation hardly perceptible to the human eye, while ΔE more than 3.0 a variation well perceptible to the human eye.

Determination of shear force

The shear force was determined using an 5542 Instron Instrument, equipped with a Warner Bratzler blade Texture of salmon slices was characterised for hardness by measuring maximum force for a complete cut. Measurements were performed at 4°C. on strips of standard size (20 mm width × 50 mm lenght × 2 mm thickness), which were carefully prepared from the slice and analyzed immediately after the preparation. Analysis was performed by cutting the strip at 90-degree angle to the longitudinal muscle fibres. Six measurements for each sample were performed.

Physical-Chemical analyses: Water (A.O.A.C., 1990) and salt (Pearson, 1973) were fourfold determined. WPS content was then calculated. TVBN (Reg. (CE) n. 2074/2005) and TBARS (Ke *et al*. 1984) were determined at HSL and at ED in duplicate.

Water activity and pH were determined on each pack after sampling for microbiological analyses.

Solid – Phase Microextraction (SPME) Headspace Analysis

Sample preparation. Headspace vials were baked at 250°C for 6 hours, and then cooled at room temperature before use. Frozen smoked salmon was homogenized and 5 g per vial were put in two headspace vials (20 ml) which were sealed with a polytetrafluoroethylene (PTFE)-coated silicone rubber septum (20 mm diameter).

Gas Chromatography – Mass spectrometry Analysis

The headspace was sampled with SPME fibre 75 μm CAR-PDMS (Superchrom-Italia) for 180 min at room temperature (Aro, Tahvonen, Koskinen, Kallio, 2003), (Chiesa, Soncin, Biondi, Cattaneo, 2006), (Triqui, Reineccius, 1995). All analyses were performed on a Finnigan Trace GC Ultra gas chromatograph coupled with a Finnigan Trace DSQ mass selective detector. The compounds were separated on a Rtx-WAX capillary column (30m × 0.25mm i.d.; film thickness 0.25 μm). The SPME fibre was desorbed in the PTV injector port held at 220 °C. The GC oven temperature was held at 35 °C for 8 min, then increased to 60°C at 4°C/ min, then to 160°C at 6°C/ min and to 200°C at 20°C/ min and was finally held at 210°C for 15 min. The interface and ion source temperatures were maintained at 230°C and 250°C, respectively. Helium was used as the carrier gas with a flow rate of 1 mL/min. Mass spectra were obtained under Electron Impact condition at 70 eV in the 35–350 amu range. Ion source was held at 250 °C. Volatile compounds were tentatively identified by matching mass spectral data with the Wiley and NIST reference libraries of standard compounds. The identification was confirmed by comparison of the retention times and mass spectra with authentic standards for the following compounds: carbon disulfide, acetone, 2-butanone, ethanol, 2,3-butanone, 2 butanol, 1-propanol, 2,3-pentanedione, hexanal, 1-propanol-2-methyl, 1-penten-3-ol, 2-butanone-3-hydroxy, acetic acid, butyrolactone, butanoic acid and phenol.

200 Physical-Chemical analyses:

201 Water (A.O.A.C., 1990) and salt (Pearson, 1973) were fourfold determined. WPS
202 content was then calculated.

203 TVBN (Reg. (CE) n. 2074/2005) and TBARS (Ke *et al*. 1984) were determined at
204 HSL and at ED in duplicate..

205 Water activity and pH were determined on each pack after sampling for
206 microbiological analyses.

207 **Microbiological analyses**

208 Two samples from each lot of salmon were submitted to microbiological
209 analysis. 10 g of each sample were homogenized in 90 mL of diluent solution
210 (0.85% NaCl and 0.1% peptone), and then serial 10-fold dilutions were made in
211 sterile saline. Total Psychrotrophic Count – TPC was determined in duplicate
212 using a spread plate technique on Plate Count Agar (Oxoid, Basingstoke, UK);
213 plates were incubated at 15°C for 5 days. This medium was chosen since it is a
214 well known and frequently applied tool for enumeration of bacteria in food
215 quality control programs of food industries. Lactobacilli were enumerated on
216 de Man-Rogosa-Sharpe agar (Oxoid), at pH 5.5. Plates were incubated at 20°C
217 for 3 days under anaerobic conditions (AnaeroGen, Oxoid); *Salmonella* spp.
218 detection followed the method provided by the International Organization for
219 Standardization (ISO 6579: 2002 Cor. 1:2004). Detection and enumeration of
220 *Listeria monocytogenes* were performed according to AFNOR methods (AFNOR

221 BRD 07/4-09/98 and AFNOR BRD 07/0-09/01). The number of *Enterobacteriaceae*
222 was determined on Violet Red Bile Glucose agar (*ISO 21528-2:2004*), and
223 coagulase positive staphylococci were determined by Petrifilm™ Staph Express
224 (3M, St. Paul, USA), following the AFNOR 3M 01/9-04/03 method.

225 **Statistical analyses**

226 Any differences in chemical composition in smoked salmon of different
227 provenance, and the different smoke houses were analysed by ANOVA. The
228 significant level was set at 5%. Principal component analysis (PCA) was
229 performed on the areas of the chromatographic peaks, in order to visualize data
230 trends and to detect possible clusters within samples, thus providing a first
231 evaluation of the discriminating efficiency of the considered variables.
232 Statistical analyses were performed by using the SAS/STAT package version 8.0
233 (SAS Inst. Inc.,NC USA).

RESULTS and DISCUSSION

Defects

The assessment of defects is reported in table n.1, in the last column there are the sum of defects relevated. From a commercial perspective, the samples which had a low score (≤ 4) resulted better than samples with a high score (> 4 and ≤ 8); the worse sample reached a score higher of 8. In this study pointed out any difference in quality from the three commercial categories: Norwegian smoked salmon, Scottish smoked salmon and wild smoked salmon; in fact the best samples were 112 (wild smoked salmon), 105 (Norwegian smoked salmon) and 107, 111 (Scottish smoked salmon). The samples 101, 103 (Norwegian smoked salmon), 109, 110 (Scottish smoked salmon) were the worse with an excessive defects presence.

Colour

In farmed salmon, the colour reflects the pigment received through the diet. In this work we did not want to discuss of the different colours but to put in evidence possible changes due to storage. As expected, the wild smoked salmon had a lower hue value than Scottish and Norwegian salmon, (h_{ab} = 0° for red hue), The Scottish salmon had hue mean values higher than Norwegian one, (h_{ab} = 90° for yellowish). During storage the hue value had an increasing trend in all samples with the only exception of salmon number 112 (wild), which turned to yellowish. On the contrary, the colour intensity c did not differ

255 significantly during the storage time ($P < 0.1$). Only three samples did not show
256 significant total colour differences between HSL and ED samplings (expressed
257 as Delta E), four samples got Delta E acceptable, but seven samples showed
258 very strong differences in colour, indicating that the products underwent an
259 evident change in one of the most important sensorial characteristics.(table n.2)

260 **Textural characteristics**

261 The factors contributing to texture vary with processing, storage and
262 particularly with cooking, which reduces the contribution of the connective
263 tissue compartment. (Johnston et al. 2006) The texture data were reported as
264 mean and standard deviation. (table n. 2). It was observed a decrease of the
265 texture during the storage, although shear force was not significantly ($P > 0.1$)
266 higher at HSL than at ED.

267 **Microbiological analyses**

268 Total Psychrotrophic Counts (TPC) were very different among the samples at
269 half shelf-life (HSL), ranging from < 1000 CFU/g to over 10^6 CFU/g; three
270 samples exceeded counts of 10^6 CFU/g (figure n.1). At expiry date (ED) 12
271 samples had high contamination levels (TPC >10^6 CFU/g), while two of these
272 samples (n. 101 and n. 114) showed counts higher than 10^8 CFU/g (figure n.2).
273 Lactobacilli were the dominant flora at HSL in most samples; counts obtained
274 from n. 114, 112 and 101 exceeded 10^6 CFU/g; at ED Lactobacilli were the
275 dominant flora in all samples; in 11 samples counts were higher than 10^6 CFU/g.

Only in samples n. 103, 113, 104 Lactobacilli counts ranged from 10^5 to 10^6 CFU/g (figure n.2).

Enterobacteriaceae contamination levels were under the detection value (100 CFU/g), both at HSL and ED, in all excepted one sample (114 at ED), which had a count of 1800 CFU/g. Coagulase-positive staphylococci weren't detected (< 10 CFU/g) in any of the samples.

No potential pathogenic microorganism (*Listeria monocytogenes*, *Salmonella* spp.) was detected in the samples**.**

Chemical analyses

At about their HSL 5 samples had values TVN-B higher than the limit of 40 mg N/100 g (Cantoni & Coll., 1993); at the expiry date other 3 samples exceeded 40 mgN/100g, in total, 8 on 14 samples had concentration of TVN-B indicating spoilage (table n. 3).

A Multiple Compound Quality Index MCQI proposed by Leroi, Joffraud, Chevalier, Cardinal (2001) was applied on samples on the first sampling step. The index, developed by multiple linear regression, related the remaining shelf-life of cold smoked salmon at 5°C with the level of Lactobacillus and the content of TVN-B. Three samples 101, 105 and 112 and had already no remaining shelf-life, and generally the established shelf-life does not match with the analytical results.

296 Lipid rancidity: at the half of their shelf-life, in six samples TBARS were under 8
297 nmoli/g, seven were slightly rancid but still acceptable (< 20 nmoli/g), and one
298 was already rancid and unacceptable, as reported in Che Man & Ramadas, 1998
299 (24) and Ke, Cervantes &Robles-Martinez (1984). At the use-by date five
300 samples were not rancid, seven samples were still acceptable, two unacceptable
301 (table n.3). Values above 8 nmoli/g are not infrequent depending from salting
302 and smoking methods Espe, Kiessling, Lunestad, Torrissen, Bencze Røra (2004)
303 consider low levels such as 7,8 - 8,7 nmoli/g wet weight. Bugueno et al. (2003)
304 did not find changes in TBARS in salmon under vacuum until 25 days.

305 **Physicochemical characteristics**

306 Results were compared with those of Leroi, Joffraud, Chevalier, Cardinal
307 (2001), on 13 French commercial products, supposed to be representative of the
308 French traditional production and Espe, Kiessling, Lunestad, Torrissen, Bencze
309 Røra (2004), on 48 French commercial products, produced by four commercial
310 smoking-houses. Water and salt contents were very similar with those observed
311 by Leroi, Joffraud, Chevalier, Cardinal (2001). Only sample 106 had Water
312 Phase Salt value less than 3,5%; the sample had the higher TVN-B and was
313 among those with very high TPC; the other samples were well over the value
314 recommended by the Center for Food Safety and Applied Nutrition, US
315 FDA.2001, that in combination with a storage chill temperature (< 4,4°C) allows
316 control of psychrotrophic *C. botulinum.*

Water Activity

The values ranged from 0,916 to 0,956; a correlation, but not significant, was found between aw and remaining weeks, as calculated from lactobacilli counts and TVNB (r 0,5147, against a level at 5% of 0,5324)

pH determination

The pH mean value was 6.11 at HSL and 6.12 at ED, these pH values were very similar with those observed by Cornu M., Beaufort A., Rudelle S., Laloux L., Bergis H., Miconnet N., Serot T., Delignette-Muller M.L. (2006). No significant differences were found between the two trials**.**

Volatile compounds

The aim of volatile compounds analysis was to verify which of them can be quality and spoilage indicators for smoked salmon products and to verify the SPME-GC/MS method use in quality control. The analysis of the smoked salmon samples using SPME method allowed for the identification of sixty-eight compounds (table 4). Principal component analysis was carried out to establish a correlation among the chemical, physical and microbiological parameters and the volatile compounds. For this purpose all data available were included in the statistical analysis. Two principal components were extracted from data for half-shelf life and expiry date storage time. The PCA analysis of volatile compounds divided the HSL samples in two groups: one composed by 101, 106, 112 and another one by the remnants. The first group

was marked out by spoilage related compounds as: carbon disulphide, acetone, 2-butanone, 2-butanolo, acetic acid, propionic acid, butanoic acid. The short chain alcohols, aldehydes, ketones and acids are probably microbial products, in fact these volatile compounds are highly correlated with highest microbial counts. 3-hydroxy-2-butanone (acetoin) was detected in low quantities only in sample 113 at ED. This molecule is considered a marker of spoilage in both fresh and smoked salmon, It is not clear why this molecule is absent in most samples. From PCA analysis of samples on ED expiry date a high correlation results between spoilage (high TVN, TPC and Lactobacilli counts) and some volatile compounds, ethanol, 1-methoxy-propanol, 1-pentanol, 2-butanone, 1-hydroxy-2-butanone, 2-butanol, acetic acid, propionic acid, 2-methyl-propanoic acid, 3-methyl-butanoic acid. These alcohols, ketones and acids correlate with TPC and Lactobacilli, the samples 101, 102, 105, 106, 107, 111, 112 had a higher microbical counts than other samples. This is in agreement with recent studies demonstrating that short chain alcohols, aldehydes and ketones were microbial spoilage indicators (Jonsdottrin, olafsdottrin, chanie, Haugen 2008).

CONCLUSIONS

Among the 14 different smoked salmon, only four (105, 107, 111 and 112) are products good for the defects presences. The colour of the salmon is the main quality aspect of interest to consumers. Marked differences were found between the two trials, these data confirmed very important modifications during the storage. This study shows that cold smoked salmon available on the Italian market has a too long shelf- life; in spite of no pathogen microrganisms isolated from the samples, the quality of smoked salmon at the expiry date was very low. This low quality was pointed out by TVN-B value, TPC and LAB counts, On the half shelf life the 50% of samples showed already spoilage signs (TVN-B > 40 mg/100g).

The low quality of smoked salmon samples complicates the interpretation of volatile compound data, in fact, only the worst samples are discriminated, the other ones have the same volatile compound pattern. In our experiences SPME-GC-MS method for quality control had two principal limits, the first is the impossibility to use an internal standard because the effects on adsorption depend on the chemical nature of the different compounds are different for different compounds, the latter is the expensive cost of the analysis. A possible solution could be the use of a reference sample. This work confirms the high correlation between the microbial count and TVN, these rapid and cheap analyses are ideal for quality control in any step of smoked salmon shelf life.

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Table 1 – Sample description and defects.

Sample	Description	Suppliers	Origin	Blood spots	Red muscle	Rifts or gaps	Bones	Slices aspect	Net Weight loss	Total
101	N	b	PL	3	1	2	0	2	5	13
102	N	d	I	1	2	1	0	0	1	5
103	N	e	F	0	4	2	0	1	4	11
104	N	g	F	1	2	1	1	0	4	9
105	N	h	N	0	0	0	0	0	3	3
106	S	a	I	0	2	1	0	0	4	7
107	S	c	UK	0	0	0	0	0	4	4
108	S	d	I	0	1	1	0	0	4	6
109	S	f	F	2	3	0	0	2	4	11
110	S	g	F	1	2	3	0	1	4	11
111	S	h	UK	0	0	0	0	0	4	4
112	W (O.kisutch)	d	I	0	1	0	0	0	0	1
113	W (O.nerka)	g	F	1	2	1	0	1	3	8
114	W (O. nerka)	h	NL	0	4	0	0	1	0	5

N= norvegian smoked salmon, S = Scottish smoked salmon, W = wild smoked salmon. Samples with same corsive letter have same supplier.

Sample	Trial	L*	a*	b*	Hue angle	delta E	Shear force
101	1st	52.57 ± 0.57	13.09 ± 0.44	13.63 ± 1.11	46.15		0.47 ± 0.13
	2nd	60.23 ± 0.87	15.71 ± 0.47	23.11 ± 1.59	55.80	12.5	0.21 ± 0.10
102	1st	54.91 ± 0.36	14.62 ± 0.63	18.13 ± 0.99	51.10		0.78 ± 0.04
	2nd	55.03 ± 0.49	14.29 ± 0.54	19.98 ± 1.01	54.41	1.9	0.52 ± 0.38
103	1st	55.73 ± 0.51	14.35 ± 0.39	17.18 ± 0.75	50.13		1.25 ± 0.39
	2nd	54.86 ± 0.50	12.95 ± 0.44	16.26 ±0.65	51.47	1.9	0.45 ± 0.27
104	1st	49.72 ± 0.91	14.71 ± 0.42	18.11 ± 1.41	50.91		0.72 ± 0.28
	2nd	49.91 ± 0.81	12.72 ± 0.62	18.55 ± 1.25	55.56	2.1	0.38 ± 0.12
105	1st	48.97 ± 0.92	16.75 ± 0.75	19.39 ± 1.33	49.17		0.87 ± 0.40
	2nd	50.77 ± 0.50	16.65 ± 0.79	23.06 ± 1.31	54.17	4.1	0.31 ± 0.20
106	1st	53.08 ± 0.57	12.88 ±0.58	16.96 ±1.02	52.79		0.35 ± 0.14
	2nd	53.70 ± 0.50	11.84 ± 0.20	18.13 ± 0.51	56.86	1.7	0.22 ± 0.09
107	1st	50.31 ± 0.96	17.53 ±0.60	20.21 ±1.37	49.06		0.66 ± 0.15
	2nd	50.48 ± 1.62	17.86 ± 0.39	25.37 ±2.19	54.85	5.2	0.38 ± 0.08
108	1st	48.91 ± 0.57	15.86 ±0.87	19.64 ±0.94	51.07		0.48 ± 0.04
	2nd	48.25 ± 0.38	14.07 ±0.63	18.31 ±0.75	52.45	2.3	0.38 ± 0.18
109	1st	52.37 ± 1.19	16.62 ± 0.87	20.13 ± 2.28	50.46		0.59 ± 0.25
	2nd	53.97 ± 0.54	16.79 ± 0.64	20.30 ± 1.07	50.40	1.6	0.55 ± 0.28
110	1st	50.22 ± 0.55	12.93 ± 0.48	18.89 ± 0.66	55.61		0.82 ± 0.32
	2nd	49.99 ± 0.44	13.62 ± 0.60	19.84 ± 0.89	55.52	1.2	0.33 ± 0.06
111	1st	50.23 ± 1.09	15.91 ± 0.67	22.60 ± 1.78	54.86		0.92 ± 0.21
	2nd	53.52 ± 1.31	15.14 ± 0.54	23.95 ± 1.96	57.71	3.6	0.59 ± 0.20
112	1st	51.20 ± 0.30	14.61 ± 0.24	17.07 ± 0.26	49.43		0.91 ± 0.42
	2nd	51.32 ± 0.55	18.13 ± 0.22	19.94 ± 0.37	47.72	4.5	0.55 ± 0.33
113	1st	48.42 ± 0.28	26.50 ± 0.49	26.63 ± 0.52	45.13		0.45 ± 0.14
	2nd	47.45 ± 0.45	25.49 ± 0.69	25.78 ± 0.82	45.33	1.6	0.35 ± 0.07
114	1st	46.81 ± 0.29	28.68 ± 0.38	25.87 ± 0.41	42.05		0.66 ± 0.08
	2nd	48.90 ± 0.35	29.68 ± 0.39	27.12 ± 0.39	42.42	2.6	0.44 ± 0.10

Table n.2 Color analysis of smoked salmon collected from the Italian market. Means ± SE for L*, a*, b* values, Hue angle, delta E and means ± SD for shear force. The differences in hue value and shear force between 1st and 2nd trial are significant ($P < 0.01$). 1st trial: sample measured 30 days before expiry date; 2nd trial: sample measured at the expiry date.

431 Table n. 3

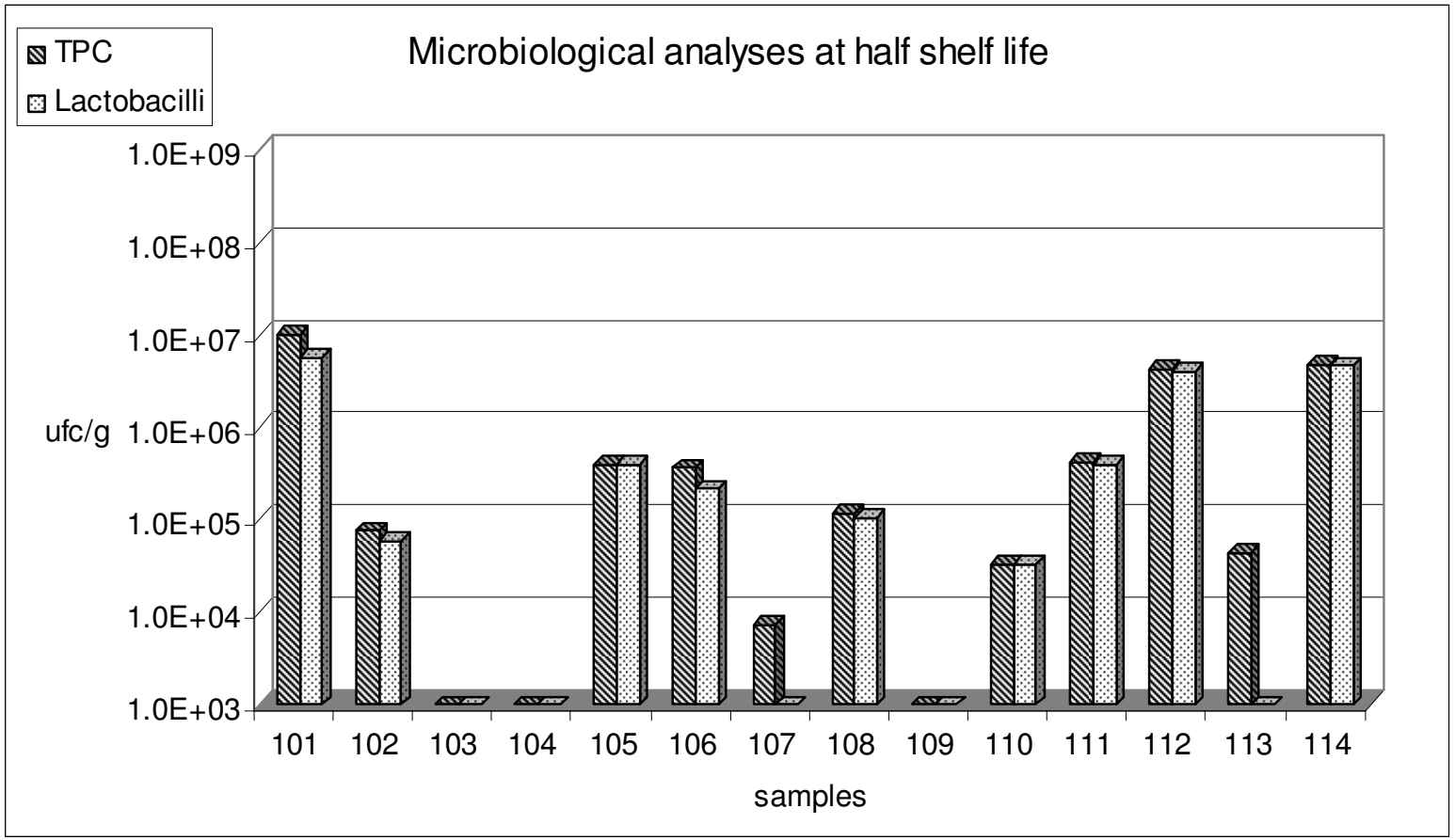
Samples	TVN-B at HSL	TVN-B on expiry date	TBARS at HSL	TBARS at expiri date	% NaCl	Water	WPS
101	63.57	64.37	3.19	7.63	2.72	67.55	3.87
102	36.36	62.83	22.15	16.81	3.22	66.27	4.63
103	30.74	28.03	10.62	12.00	3.11	67.26	4.42
104	29.21	34.71	6.87	6.97	4.17	64.15	6.10
105	47.22	54.45	12.14	3.67	3.83	63.23	5.71
106	53.93	71.33	7.63	13.21	2.11	67.87	3.02
107	35.88	50.84	7.25	13.04	3.31	66.17	4.76
108	29.40	36.82	14.36	22.04	3.95	64.10	5.80
109	34.86	34.93	10.38	18.02	3.66	65.61	5.28
110	32.75	33.40	18.39	10.13	3.46	65.23	5.04
111	45.41	69.30	14.43	8.23	3.40	65.50	4.93
112	47.46	58.80	4.30	9.08	3.45	68.55	4.79
113	31.31	37.04	14.15	6.94	4.24	67.47	5.91
114	16.50	60.47	1.54	21.37	3.41	68.82	4.72

Table n.4 Volatile compounds identified in smoked salmon by GC-MS.

Peak number	Compound	Structure
1	Carbon disulphide	sulfur compound
2	Acetone	ketone
3	2-butanone	ketone
4	2 methyl butanal	aldehyde
5	3 methyl butanal	aldehyde
6	2,4 octadiene	alkene
7	3,5 octadiene	alkene
8	1,4 heptadiene, 3 methyl	alkene
9	2 propanol	alcool
10	ethanol	alcool
11	2-ethyl furan	furan derivate
12	alkane	alkane
13	3 pentanone	ketone
14	octane, 2,5,6-trimethyl	alkane
15	alkane	alkane
16	alkane	alkane
17	2-butanolo	alcool
18	benzene methyl	aromatic
19	alkane	alkane
20	2,3-pentanedione	ketone
21	hesanal	aldehyde
22	dimethyl disulfide	sulfur compound
23	etilbenzene	aromatic
24	xilene	aromatic
25	m-xilene	aromatic
26	o-xilene	aromatic
27	2 propanol 1 methoxy	alcool
28	heptanal	aldehyde
29	1 penten3ol	alcool
30	ciclopentanone	ketone
31	limonene	terpene
32	cyclopentanone 2 methyl	ketone
33	1 butanol 3 methyl	alcool
34	1-pentanol	alcool
35	2 butanone 3hydroxy	ketone
36	2 propanone 1 hydroxy	ketone
37	2-cyclopenten-1-one	ketone

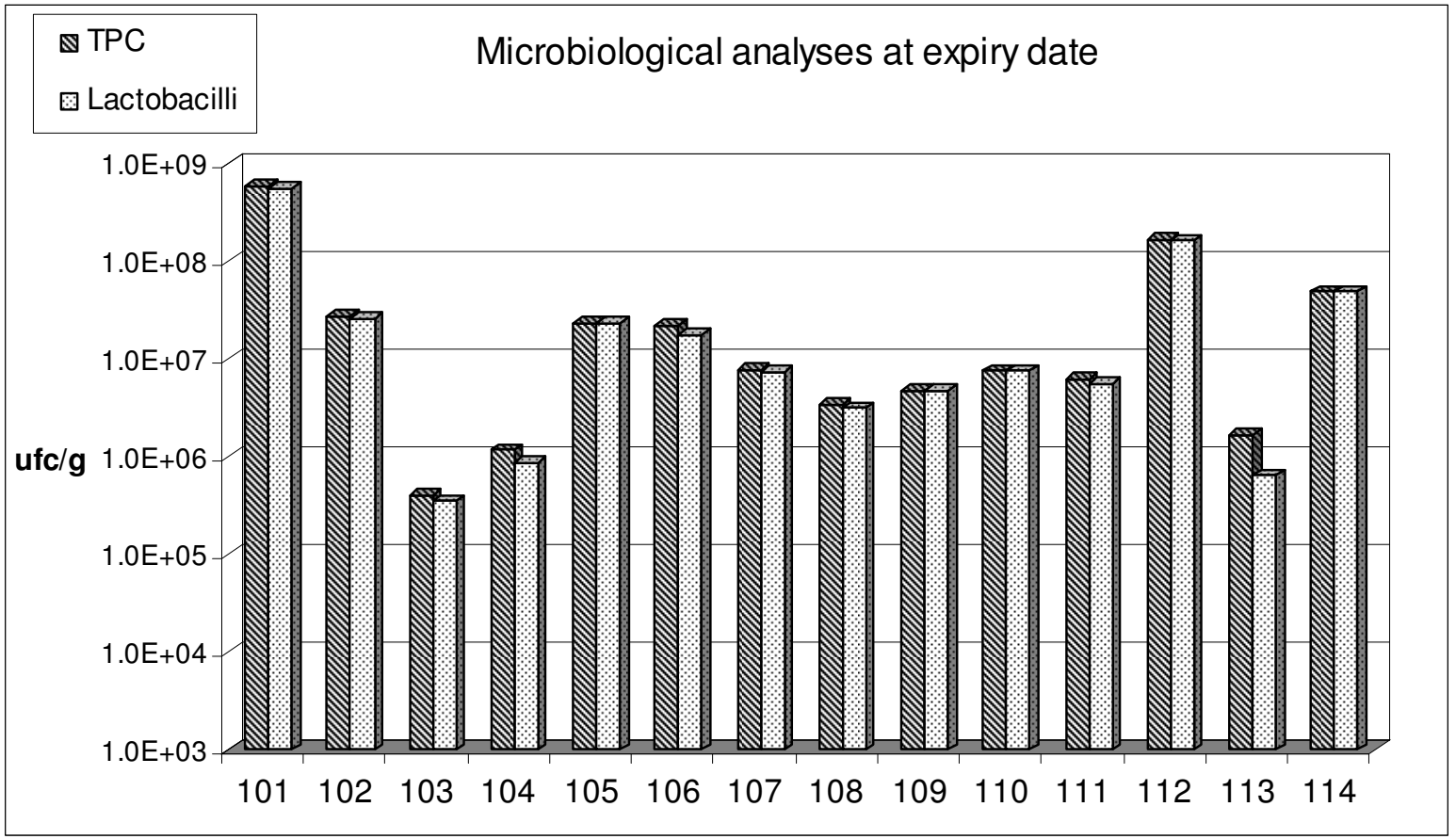
38	2-Cyclopenten-1-one, 2-methyl-	ketone
39	1-Hydroxy-2-butanone	ketone
40	trisulfide dimethyl	sulfur compound
41	nonanal	aldehyde
42	acetic acid	acid
43	furfural	aldehyde
44	4 heptanol 2, 6 dimethyl	alcool
45	2 cyclopenten 1 one 3, 4 dimethyl	ketone
46	ethanone, 1-(2-furanyl)	ketone
47	2 cyclopenten 1 one 3 methyl	ketone
48	2-furil,metilchetone	ketone
49	2-Cyclopenten-1-one,2 3-methyl	ketone
50	benzaldeide	aldehyde
51	acidopropionico	acid
52	propanoic acid 2 methyl	acid
53	2-furancarboxaldehyde,5-methyl	aldehyde
54	gamma-butirrolattone	lattone
55	Butanoic acid	acid
56	2-Furanone, 2,5-dihydro-3,5-dimethyl	ketone
57	Butanoic acid, 3-methyl	acid
58	nonadecano	alkane
59	2 furanone 3 methyl	ketone
60	2H-Pyran-2-one	ketone
61	2-cyclopenten-1-one, 2hydroxy-3-methyl	ketone
62	guaiacol	phenol
63	4-Methyl-5H-furan-2-one	ketone
64	phenol,2,6-bis(1,1-dimethylethyl)	phenol
65	phenol, 2-methoxy-4-methyl-	phenol
66	phenol	phenol
67	ethilguaiacol	phenol
68	m-cresolo	aromatic

434 Figure 1. TPCs and lactoballi counts after thridy days of storage.



435
436
437

438 Figure 2. TPCs and lactoballi counts after sixty days of storage.



439

Figure n.3. Biplot of principal component scores and factor loadings from principal component analysis applied to volatile compounds, TPC, Lactobacilli and TVN-B of smoked salmon products at half shelf life.

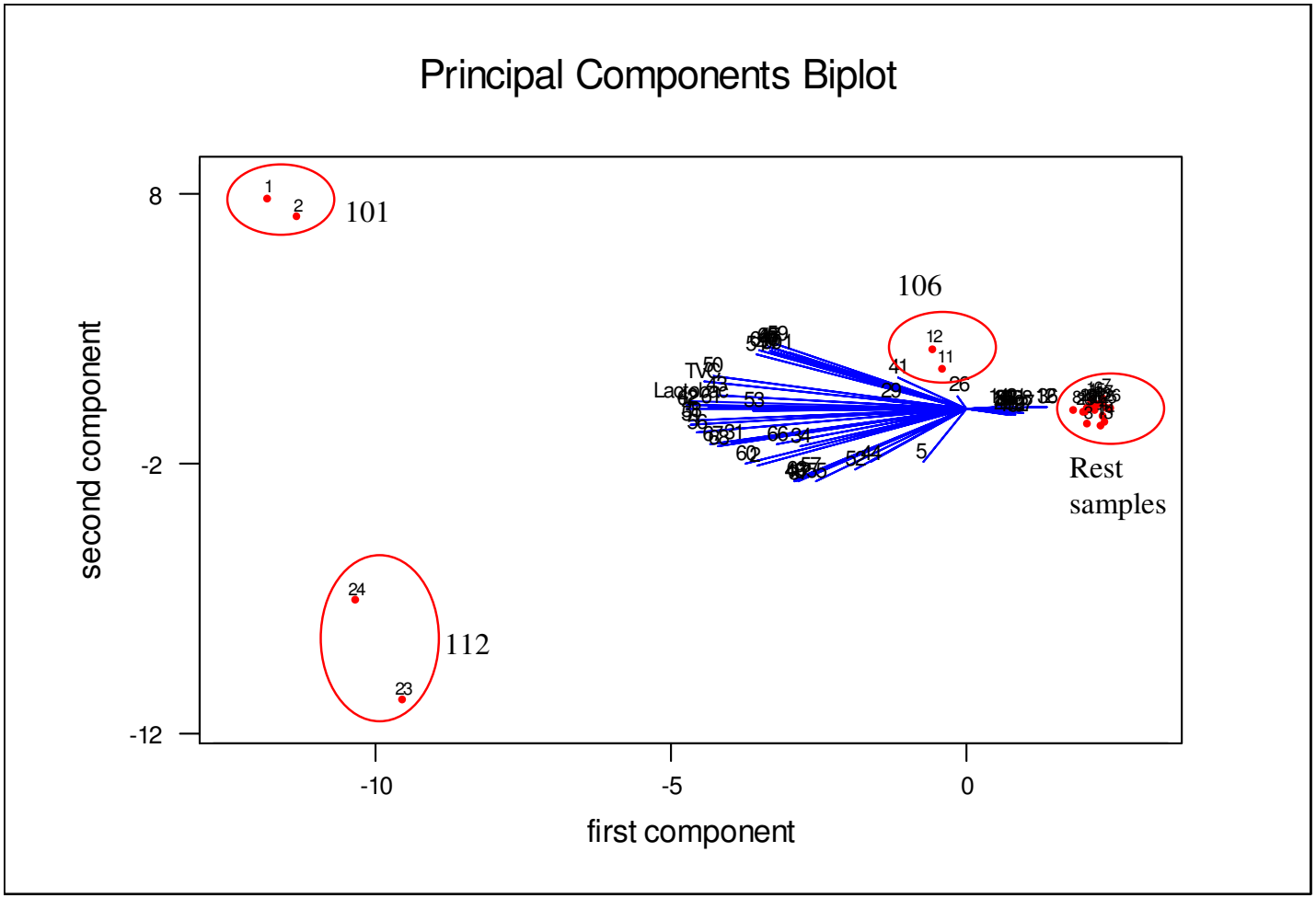
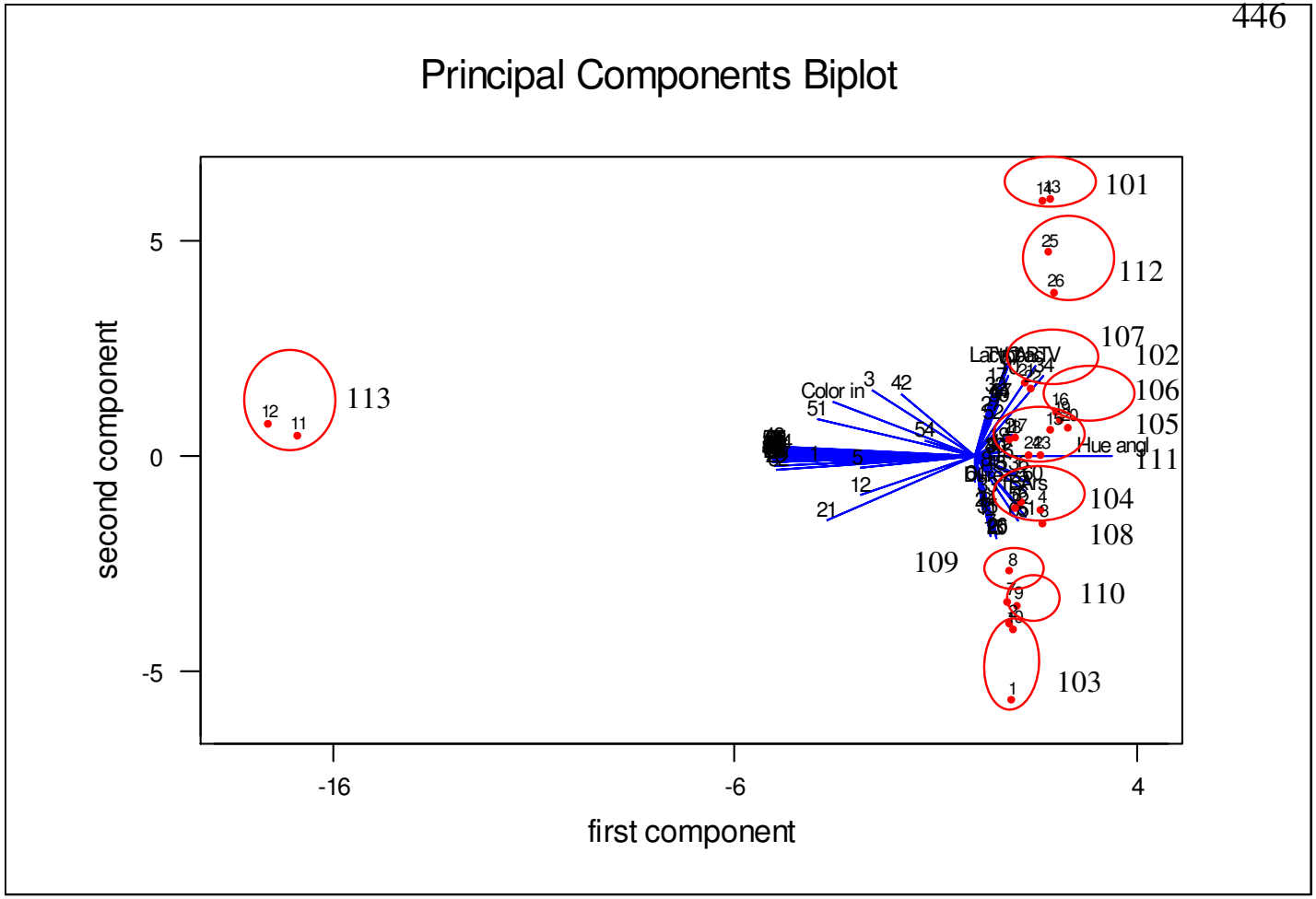


Figure n.4. Biplot of principal component scores and factor loadings from principal component analysis applied to volatile compounds, TPC, Lactobacilli and TVN-B of smoked salmon products at expiry date.



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