Fostering Mediterranean fish ensuring traceability and authenticity

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History of Changes

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0.1	CNTA	15.12.2020	Preliminary Version
0.2	UNINA	30.12.2020	Minor changes
0.3	CNTA	4.01.2021	Final Version
1.0	ENCO	07.01.2021	Quality Assessment & Formatting

Table 1: History of changes

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ABBREVIATION / ACRONYM	DESCRIPTION
AFLP	Amplified Fragment Length Polymorphism
AOAC	Association of Official Agricultural Chemists
BAP	Best Aquaculture Practices
BLAST	Basic Local Alignment Search Tool
COIBAR-RFLP	Cytochrome Oxidase I Barcode-Restriction Fragment Length Polymorphism
EEA	European Environment Agency
EFTA	European Free Trade Association
ELISA	Enzyme-Linked Immuno Sorbent Assay
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FINS	Forensically Informative Nucleotide Sequencing
FISH-BOL	The Fish Barcode of Life Initiative
FT-IR	Fourier Transform Infrared
FT-NIR	Fourier Transform Near Infrared
HSI	Hyperspectral Imaging
IEF	Isoelectric Focusing method
IEF	Isoelectric Focusing
IRMS	Isotope Ratio Mass Spectrometry
LDA	Linear Discriminant Analysis
MEDISYS	Medical Information System
NGS	Next Generation Sequencing
NIR	Near Infrared
NIRS	Near Infrared Spectroscopy
PCA	Principal Component Analysis
PCR-DGGE	Polymerase Chain Reaction Denaturing Gradient Gel Electrophoresis
PCR-RFLP	Polymerase Chain Reaction coupled with Restriction Fragment Length Polymorphism
PLS-DA	Partial Least Squares Discriminant Analysis
RAPD	Random Amplified Polymorphic DNA
RASFF	Rapid Alert System for Food and Feed
RFID	Radio Frequency Identification
RT-PCR	Real-time polymerase chain reaction
SDS	Sodium Dodecil sulfate
SIMCA	Soft Independent Modelling of Class Analogies
SSCP	Single-Stranded Conformational Polymorphism
TTI	Time Temperature Indicators
VIS	Visible

Table 2: Abbreviation and Acronyms

Executive Summary

The main goal of the SUREFISH project is to valorise traditional Mediterranean fish by fostering the supply-chain innovation and consumer confidence on Mediterranean fish products through deploying innovative solutions to achieve unequivocal traceability and confirming their authenticity, thus preventing frauds.

Accordingly, the SUREFISH project has been implementing and demonstrating a global solution to assure fish authentication and reduce fraud based on RFID, Blockchain, TTI and tamper-proof technologies as well as harmonize and validate related analytical methods and create a trans-national laboratories network in a 36-month project lifetime.

To reach the project targets and expected impact, a deep review of analytical methods (both traditional and rapid methods) for fish analysis has been conducted. The analytical methods have been also classified by its suitability to detect the most common fraud causes in fish industry.

Introduction

The purpose of this deliverable is to describe the selection of the most promising analytical methods based on suitability, simplicity, robustness, cost-effectiveness, time-effectiveness, assuring that this selection covers all relevant fish fraud causes affecting Mediterranean fish involved in the pilot cases.

First, the most common causes of fraud during the last years are compiled.

Secondly, the way of reviewing the information to complete this deliverable is described.

Finally, the most appropriate analytical techniques for each type of fraud in fish are described, completing this information with the advantages and disadvantages of each analytical technique and some of the more important references supporting this selection.

1. Main causes of fraud in fish products

The main fish fraud causes in fish industry are the following:

- **Species substitution:** where a low-value species replaces a more expensive one for economic gain, or where a high-value species is presented as a lower-value species for tax evasion purposes.
- **Origin mislabelling:** to conceal the geographical origin of illegally harvested species.
- **Addition of glaze water to frozen products:** to increase weight of fish to be sold.
- **Undeclared use of food additives:** such as water-binding agents to deceptively increase the weight of products.
- **Previously frozen fish sold as fresh:** where fresh fish is usually of higher sensorial quality and more expensive and appreciated.
- **Farmed fish sold as wild fish:** wild fish is more expensive and valued by consumers.
- **Illegal use of food additives such as carbon monoxide**: to enhance the visual quality of fish products.
- **Marketing of counterfeit products:** where brand names are fraudulently used.
- **Mislabelling of ingredients:** as batter or breadcrumbs, to bulk up the weight of processed products.

RASFF annual report reports the following types of incidents in 2018-2019:

Table 3: Incidents in fish products in 2018-2019 (Source: Rasff annual report)

Type of incident	Number of incidents		Number of notifications	
	2019	2018	2019	2018
accidental or environmental contamination	21	17	58	49
faulty labelling, processing or storage conditions	2	2	4	5
foodborne outbreak	2	6	12	30
foreign body contamination / physical danger	1	7	2	16
fraud investigation	2	1	4	5
hazardous or unauthorised composition	21	11	53	31
intentional contamination / tampering	0	0	0	0

In the case of “fraud investigation”, these are incidents that could also fall under the other incident types but are given this type to emphasise the (potential) fraud element of the investigation that spans several notifications.

According to the **Knowledge Center for Food Fraud**, the main reported causes of fish fraud in the last four years are the following[1]:

[1] SOURCES: Medical Information System (**MedISys**): an internet monitoring and analysis system. Food adulteration relevant keywords are used for queries (reviewed systematically on a weekly basis). The information processed by MedISys is derived from the Europe Media Monitor. Rapid Alert System for Food and Feed (**RASFF**): an electronic tool to exchange information about serious risks detected in relation to food or feed among EU Member States. **Food types**: The list of commodities often subject to fraud as defined by the EU Parliament in its resolution of 14 January 2014 on

Table 4: Main reports causes of fish fraud in the last four years (Source: Knowledge Center for Food Fraud))

FRAUD CAUSE	2017	2018	2019	2020	TOTAL	%
ORIGIN MISLABELLING	17	12	2	8	39	49
CONTRABAND		1			1	1
FARMED BY FRESH		1			1	1
USE OF ILLEGAL ADDITIVES	1				1	1
CONTAMINATED		6	1		7	9
NOT SUITABLE FOR CONSUMPTION		4		2	6	8
FROZEN BY FRESH		2			2	3
SPECIES SUBSTITUTION	4	8	7	1	20	25
UNDECLARED USE OF ADDITIVES	1				1	1
GLAZING				1	1	1

The data in Table 4 have been elaborated, and the single share of the fraud distribution can be observed in the Figure below:

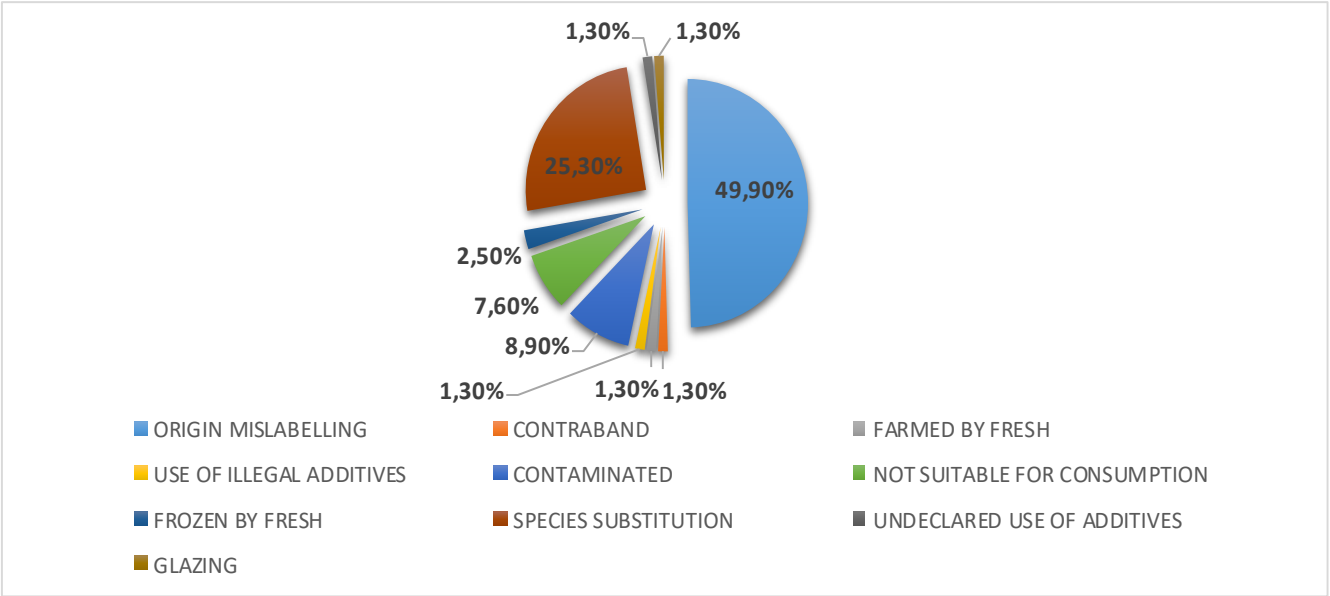

Figure 1: Percentage distribution of fraud causes, 2017-2020

The chart shows how almost half of the total causes connected to origin mislabelling (49,9 %) followed by species substitution.

The fish chain is particularly vulnerable to fraud, primarily to species substitution and mislabelling.

In 2015, the European Commission organised a control plan coordinated at European Union level to assess the prevalence on the market of white fish mislabelled with regard to its declared species. The plan was part of the Commission follow-up of the horse meat crisis in 2013, where systematic checks to assess the extent of possible fraudulent activity in a certain sector, was one of the actions. Fishery and aquaculture products

the food crisis, fraud in the food chain and the control thereof forms the basis for selecting cases: Olive oil, fish, organic products, grains, honey, coffee, tea, spices, wine, certain fruit juices, milk and meat. **Decernis Food Fraud Database** and **Food Protection and Defense Institute**.

were identified by the Commission and the EU countries' experts as a possible high risk commodity for species substitution.

In this case the objective was to assess the prevalence on the market of white fish mislabelled with regard to its declared species in unprocessed and processed products. In the context of this coordinated control plan "white fish" was defined as demersal species living in both marine and freshwater environments, including round fish which are benthopelagic species and flat fish which are benthic species.

During official controls, 27 Member States and 2 EFTA Member States collected 3906 samples of fish of predominantly white fish species. The samples were taken at different stages of the food chain and represented a broad range of products and over 150 white fish species. All the samples were collected from 1 June until 15 July 2015.

The samples were submitted to one or more of the following tests:

- IEF (Isoelectric focusing method, e.g. AOAC Official Method 980.16 using the relevant IEF database)
- PCR-RFLP (Polymerase chain reaction coupled with restriction fragment length polymorphism)
- DNA-barcoding (DNA-sequencing using a validated protocol)
- RT-PCR (Real-time polymerase chain reaction, uniplex or multiplex)

The declared species was confirmed in 94% of the samples taken. The overall non-compliance is lower than the levels of non-compliance in white fish in many of the other more limited testing programmes in the EU countries, although for certain species the levels are still quite high.

The aggregated results can only give us an idea of the situation concerning mislabelled white fish on the EU market. Based on the information gathered in this plan it is not possible to give any estimate of how many cases of intentional violations perpetrated with the purpose of financial gain this represents, as opposed to just bad or ill-informed practices.

In the case of SUREFISH project, the pilot cases participating in the project are:

- **Didon Marée (Tunisia):** Fresh and marinated anchovies. Important to certify the origin and authenticity.
- **Fish Basket (Egypt):** Farmed fish Tilapia fillets. The Best Aquaculture Practices Certification (BAP) will be implemented to achieve products authenticity.
- **Sofia (Lebanon):** Fresh Groupers. They will be analysed to confirm authenticity.
- **Balfegó (Spain).** High value fish species, that will allow to test in a real environment the use of a validates protocol.

The main fish frauds affecting the fish species involved in SUREFISH pilot cases are:

1.- Species substitution
2.- Mislabelling of fish to conceal the geographical origin
3.- Farmed fish – vs- wild fish

4.- Previously frozen fish sold as fresh

5.- Undeclared use of food additives.

2. Review of analytical fish fraud causes

From March to August 2020 a review of the different analytical methods to detect fraud in fish was performed based on the following premises and compiling the following information:

- Type of fraud to identify
- Analytical technique used
- Analytical determination
- Equipment needed
- Fish species tested
- Extra samples tested (water, feed, other…)
- Type of sample analysed (fillet, bones, mucosae, whole fish…)
- Results obtained and units
- Amount of samples required
- Time of analysis
- Cost of analysis (€)
- Robustness of method
- Simplicity
- Standardized method (yes/no)
- Protocol available
- Source/reference

All this information was compiled in an excel database in order to better analyse them and to generate the summarised information for the next section. All the analytical partners have been involved to the drafting of this work.

3. Selection of the most adequate analytical techniques for each type of fraud.

In this section, the compilation of the most appropriate analytical techniques to detect each type of fraud are described. A list of advantages and drawbacks are also included in each of them.

3.1 Species substitution

Species substitution is difficult to detect when fish morphological features such as heads, tails and fins are removed and fish are processed into fillets, ready-to-eat breaded or battered products, or highly processed in pre-prepared fish meals. With the advent of molecular identification methods, such as DNA barcoding and next-generation sequencing, the possibility exists for far greater transparency in the fish marketing chain.

Traditionally, the identification of animal species, also for fish and seafood, was performed through protein analysis, with electrophoresis, chromatography, or immunological methods [2]. The Regulatory Fish Encyclopaedia hosted by the U.S. FDA was a repository of information on protein analyses for fish

[2] Ortea I., Pascoal A., Cañas B., Gallardo J.M., Barros-Velázquez J. & Calo-Mata P. (2012). – Food authentication of commercially-relevant shrimp and prawn species: From classical methods to Foodomics: General. *ELECTROPHORESIS*, **33** (15), 2201–2211. doi:10.1002/elps.201100576.

identification, mostly IEF patterns [3]. A possible advantage of protein analytical methods is to address the presence of some specific allergens, which is relevant also for food safety purposes [4].

The most appropriate methods to detect this type of fraud are:

DNA based methods:

Genomic or mitochondrial DNA can be used for amplification. However, mitochondrial DNA is more often used due to the following advantages:

1. A higher DNA amount in the extract and a higher number of copies compared to genomic DNA.
2. Its resistance is higher in comparison with heat treatment disintegration due to its spherical arrangement [5].
3. Mitochondrial genome is maternally inherited, and then sequence ambiguities from heterozygous genotypes are theoretically avoided[6].

DNA-based techniques make use of different markers, amplified fragments or restriction profiling the most common methods used in laboratories for identification of species are:

- sequencing,
- AFLP (amplified fragment length polymorphism),
- FINS (forensically informative nucleotide sequencing),
- RAPD (random amplified polymorphic DNA),
- RFLP (restriction length polymorphism),
- SSCP (single-stranded conformational polymorphism),
- multiplex PCR and
- real time PCR for diagnostic fragments.

An important resource is the Reference Standard Sequence Library for Seafood Identification including over 1000 sequences from seafood vertebrates and invertebrates [7]. The D-loop region in mitochondrial DNA can be a good target for species differentiation because of high polymorphism and mutation rate [8].

A different approach in DNA-based analyses, the **DNA barcoding technique**, is a well-known standard to detect species of seafood in food samples, also after extreme processing. The initiative Barcode of Life Data System [9] with the FISH-BOL, fish barcode[10], is the main source of data for species identification. The marker of choice is cytochrome b (cyt-b) or cytochrome *c* oxidase I gene (COI) located on the mitochondrial DNA; other markers are 16S or 18S ribosomal DNA (16S-rDNA, 18S-rDNA), the internal transcribed spacer type I-

[3] U.S. Food and Drug Administration – Regulatory Fish Encyclopedia (RFE). Available at: https://www.fda.gov/food/foodscienceresearch/rfe/default.htm.

[4] Food Integrity Handbook a Guide to Food Authenticity Issues and Analytical Solutions. https://doi.org/10.32741/fihb.

[5] Bossier P. (1999): Authentication of seafood products by DNA patterns. Journal of Food Science, 64, 189–193.

[6] Aranishi F., Okimoto T., Izumi S. (2005): Identification of gadoid species (*Pisces, Gadidae*) by PCR-RFLP analysis. Journal of Applied Genetics, 46, 69–73.

[7] U.S. Food and Drug Administration – DNA-based Seafood Identification - Reference Standard Sequence Library for Seafood Identification (RSSL). Available at: https://www.fda.gov/food/foodscienceresearch/dnaseafoodidentification/ucm238880.htm.

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[9] Bold Systems – Barcode of life data system v4. Available at: http://www.boldsystems.org/.

[10] iBOL Working Group – Fish Barcode of Life (FISH-BOL). Available at: http://www.fishbol.org/.

ribosomal DNA or type II (ITS1-rDNA, ITS2-rDNA)[11]. The markers are amplified with PCR from universal primers, and the amplicons are then sequenced for comparison with the data base[12].

Different references of DNA based methods were reviewed according to the SUREFISH species:

- **Tuna**: using mitocondrial DNA and liver morphology [13], PCR and direct sequence analysis of the mitocondrial cytochrome b genes[14], PCR assay in bonito[15], by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR in canned tuna[16], by mtDNA direct polymerase chain reaction (PCR) sequencing and PCR− restriction fragment length polymorphism methodologies in canned tuna [17], by microsatellite and mitochondrial DNA analyses of Atlantic bluefin tuna [18], by a cytochrome b gene analysis [19], validated methodology for genetic identification of tuna[20], by minor groove binder probe real-time polymerase chain reaction analysis of mitochondrial DNA sequences [21], by PCR-RFLP [22], by multiplex PCR–ELISA method [23], by FINS [24], Use of the mitochondrial control region as a potential DNA mini-barcoding target for the identification of canned tuna species [25] and finally, the study of Quinteiro [26] about molecular phylogeny, population structure and genetic traceability of scombrids (*Pisces: Scombridae*) from the University of Santiago de Compostela.

Bold Systems – Barcode of life data system v4. Available at: http://www.boldsystems.org/. [11] Bhattacharya M., Sharma A.R., Patra B.C., Sharma G., Seo E.M., Nam J.S., Chakraborty C. & Lee S.S. (2015). – DNA barcoding to fishes: current status and future directions. *Mitochondrial DNA*, , 1–9. doi:10.3109/19401736.2015.1046175.

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[13] Pedrosa-Gerasmio, I. R., Babaran, R. P., & Santos, M. D. (2012). Discrimination of juvenile yellowfin (*Thunnus albacares*) and bigeye (*T. obesus*) tunas using mitochondrial DNA control region and liver morphology. PLoS One, 7(4), e35604.

[14] Bartlett, S. E., & Davidson, W. S. (1991). Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome b genes. Canadian Journal of Fisheries and Aquatic Sciences, 48(2), 309-317.

[15] Lin, W. F., & Hwang, D. F. (2008). A multiplex PCR assay for species identification of raw and cooked bonito. Food Control, 19(9), 879-885.

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[26] Quinteiro J (2011) Filogenia molecular, estructura poblacional y trazabilidad genética de escómbridos (*Pisces: Scombridae*), Universidad de Santiago de Compostela.

- **Anchovies**: method for genetic identification of four species of anchovies: *E. encrasicolus, E. anchoita, E. ringens* and *E. japonicas* [27], by polymerase chain reaction, sequence of their mitochondrial cytochrome b gene, and restriction analysis of polymerase chain reaction products in semipreserves[28], a rapid method for differentiating four species of the *Engraulidae* (Anchovy) family [29], by pyrosequencing [30], by molecular markers and support through a public domain database[31], Development of gene-associated single nucleotide polymorphisms for Japanese anchovy *Engraulis japonicus* through cross-species amplification [32], by COIBar-RFLP[33], by mitochondrial DNA phylogeny and the reconstruction of the population history of a species [34], using nuclear-DNA markers to confirm the presence of two anchovy species in the Mediterranean [35], by mitochondrial DNA variability in European anchovy [36], by PCR-RFLP [37], by FINS[38], by PCR-RFLP[39], by the use of Reduced single nucleotide polymorphism panels for assigning Atlantic albacore and Bay of Biscay anchovy individuals to their geographic origin[40], by BLAST analysis of a highly informative cytochrome b gene fragment[41], A COI nonsynonymous mutation as diagnostic tool for intraspecific discrimination in the European anchovy *Engraulis encrasicolus (Linnaeus*) [42], by mitochondrial DNA and microsatellite

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[40] Montes, I., Laconcha, U., Iriondo, M., Manzano, C., Arrizabalaga, H., & Estonba, A. (2017). Reduced single nucleotide polymorphism panels for assigning Atlantic albacore and Bay of Biscay anchovy individuals to their geographic origin: Toward sustainable fishery management. Journal of agricultural and food chemistry, 65(21), 4351-4358.

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genetic differentiation[43], [44], [45], [46] y [47], by DNA barcoding approaches[48], by recovery and amplification of DNA from fresh and processed sardine[49] and by PCR-RFLP[50].

- **Sardines**: by molecular phylogeny[51], by direct sequencing[52], and other DNA studies [53], [54], by PCR-RFLP[55], by FINS methodology[56] and by real-time PCR[57].
- **Tilapia**: Application of the RAPD technique in tilapia fish [58], [59], by PCR-RFLP of 5S rDNA[60], phylogenetic relationships inferred from mitochondrial DNA sequences [61]

Advantages: The RFLP method is faster and less expensive than the FINS method. These techniques are more suitable compared with protein analysis based techniques, because they are independent on tissue types, age or technology of sample processing.

[43] Borrell, Y. J., Pinera, J. A., Sánchez Prado, J. A., & Blanco, G. (2012). Mitochondrial DNA and microsatellite genetic differentiation in the European anchovy *Engraulis encrasicolus* L. ICES Journal of Marine Science, 69(8), 1357-1371.

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Disadvantages: Expensive, specific equipment and materials needed. Specialised technicians needed.

Morphological analyses:

Methods of fish species identification based on external characteristics are applicable to whole or slightly processed fish. Species identification becomes much more difficult in fish that have undergone processing by the food industry and morphological criteria cannot be used[62], [63], [64].

Advantages: simplicity, cheap analysis, not specific equipment required.

Disadvantages: previous expertise needed. in most of the cases, morphological differences between species of fish could not be detected, especially at juvenile stages. Consequently, a large number of sensory analysis techniques have been developed.

Immunological analysis:

A variety of methods based on the protein analysis have been used in fish species identification. Significant protein characteristic employed in the immunological analyses is their ability to react with specific antibodies. These methods are less applicable for an assessment of broad range of fish species, because it is necessary to prepare high numbers of specific antibodies. Various qualitative methods can be used for the detection of antigen-antibody reactions such as precipitation, immunodiffusion, immunoelectrophoresis or ELISA, which can also be used for quantitative assessment. Due to the fact that heat treatment may affect protein immunogenity, antibodies have been developed that can be used for fish species identification in cooked products[65]. Using immunological methods, fish species identification in cooked and dried fish products including among others Alaska Pollack (*Theragra chalcogramma*) was investigated[66].
Most fish species were identified by means of fingerprinting of immunostained patterns, despite protein staining patterns were not sufficiently distinct for species identification.
Methods based on the analysis of thermostable proteins [67] or the use of specific chemical substances (urea, SDS), which make the denaturized proteins more distinguishable, provide much better solution.

Advantages: simplicity, accuracy, and the ability to analyse high numbers of samples simultaneously.

Disadvantages: their drawback is potential cross-reaction between proteins of closely related fish species. Besides, these methods often have a limited applicability for the highly processed fish products due to the protein denaturation

62 Sotelo C.G., Pineiro C., Gallardo J.M., Perez-Martin R.I. (1993): Fish species identification in seafood products. Trends in Food Science and Technology, 4, 395–401.

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analysis and lab-on-a-chip capillary electrophoresis: Application to detect white fish species in food products and an interlaboratory study. Journal of Agricultural and Food Chemistry, 53, 3348–3357.

65 Sotelo C.G., Pineiro C., Gallardo J.M., Perez-Martin R.I. (1993): Fish species identification in seafood products. Trends in Food Science and Technology, 4, 395–401.

66 Ochiai Y., Watabe S. (2003): Identification of fish species in dried fish products by immunostaining using anti myosin light chain antiserum. Food Research International, 36, 1029–1035.

67 Asensio L., Gonzalez I., Rodriguez M.A., Hernandez P.E., Garcia T., Martin R. (2003): Development of a monoclonal antibody for grouper (*Epinephelus marginatus*) and wreck fish (*Polyprion americanus*) authentication using an indirect ELISA. Journal of Food Science, 68, 1900–1903.

Spectroscopic methods (NIR):

NIR spectroscopy and portable NIR devices demonstrated the good performances in the authentication of fillets and patties of *Gadus morhua* and *Melanogrammus aeglefinus*. In particular, LDA and SIMCA models developed with MicroNIR spectra proved to be as reliable as those calculated using spectra acquired by a benchtop FT-NIR spectrometer. This is an important finding, because the use of a simple, portable and cost-effective tool such as the handheld NIR device can help in fighting commercial frauds in fish market, increasing the number of controlled samples and giving the possibility to make control throughout the entire commercial chain, directly in-situ. The results of this study are of outmost importance because they were obtained both on fish fillets and patties, meaning that there is the possibility to identify fish species even when the morphology is no more evident, thus allowing fish authentication also in processed products [68].

Other studies also demonstrated the potential of NIR in differentiating superior from lower quality fish species [69]. In this case Red Mullet and Mullet; Winter Cod and Cod, and Samlet and Salmon Trout were differentiated.

Advantages: cheap, non destructive and rapid analysis.

Disadvantages: it's important to calculate robust models in order to reduce the error in determination. Usually, calibrations are device dependent.

3.2 Origin mislabelling of fish

Mislabelling is a common problem for fish, and seafood in general. This has been evidenced in many studies across the world, particularly using methods based on DNA analysis for identification of species and its origin.

Trace elements analysis

Coastal marine environments represent high value eco-socio-systems, but they are also discharge and accumulation areas of anthropogenic compounds, such as trace elements[70], [71]. Such chemical contamination leads to the alteration of marine ecosystems, with an impact at individual, species, population and community levels[72], [73]; [74]. The Mediterranean Sea being a semi-enclosed sea, it is predicted to be a

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particularly sensitive system to any change in its hydrographic conditions due to either climatic or anthropogenic forcing[75], [76].

Contaminants can bio accumulate during the life of organisms (increases with size or age) and bio magnify within creasing trophic level, but different patterns are observed depending on the contaminant and the organism [77]. While the contamination of fish by trace elements can come directly from the water through respiration or directly through the skin and membranes, the main way of trace elements inputs into fish is food through the consumption of contaminated prey [78]. Bioaccumulation in fish is a complex process that depends on various biological parameters (species, size, age, sex, diet, trophic level and metabolism)[79], and differs among tissues due to differences in trace elements absorption, detoxification and storage mechanisms[80], [81], [82].

Contaminants, such as trace elements, emerged as useful ecotracers of trophic patterns and environmental parameters when used in combination with other chemical tracers, such as C and N isotopic compositions[83], [84], [85], as they provide complementary time-and space-integrated information on bioaccumulation events and trophic positions of fish in the food web.

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Some examples of studies based on trace elements are the study on Mediterranean scorpaenid fish[86] and croaker[87].

Advantages: precise analytical techniques that provide robust data.

Disadvantages: these techniques require specific analytical equipment and expertise.

Stable isotope analysis:

At present, many methods have been applied to identify the geographical origin and authentication of seafood, among them, stable isotope analysis is considered to be the more accurate and preferable technique and has become the most widely-used method for assessing authenticity and traceability of seafood.

The use of this technique to infer the geographical origin of seafood is mainly focused on fish [88],[89],[90],[91],[92],[93],[94] and shrimp [95], [96], and also in bivalve[97].

Advantages: important tool in the fight against fraud in the food products industry. They provide information on the geographical origin, important characteristics for the consumer that are highly taken into account in national and international legislation. In the UE and north of America, they are considered official methods to fight against food fraud.

Disadvantages: they need specific expertise and equipment to be performed and the results correctly interpreted.

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DNA analysis:

Analyses which can be of use in ascertaining the geographical origin can be based on DNA markers, if the local populations of fish have distinctive features. See section

Otherwise, chemical analyses for elements and trace elements, stable isotopes, fatty acids can be used[98], [99], [100].

Advantages: DNA analysis is in constant development and has become one of the most precise molecular tools. Its use has spread to many and diverse areas, such as checking the authenticity of fish. DNA provides more information and has many more advantages compared to proteins, although it can also be altered during food processing. It is much more heat stable than a protein, so it is possible to amplify small regions of DNA to allow identification

Disadvantages: There are a wide variety of DNA-based methods for identifying fish species. The main differences between them, in addition to the methodology, are their range of application, complexity and cost.

Fish microbiota/microbioma analysis:

The study of microbial communities diversity associated to food products, as well as its linkage to a particular geographic origin, has already been applied in traceability issues, namely the molecular approach employing polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE)[101], [102], [103], [104], [105], [106]. This molecular method relies on DNA amplification of microbial communities associated to a food product. The amplicons produced are after used as samples on the DGGE and the result is an electrophoretic profile composed of several bands that can be assumed as a genetic fingerprint of microbial communities related with the origin of any given food item[107]. PCR-DGGE is recommended as an effective biotechnological tool

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for seafood traceability since it can provide unique biological signatures that can be assigned to specific geographic origins[108], [109], [110].

Nonetheless, this method does not provide an in-depth representation of microbial communities composition[111]. In this way, it is necessary to employ subsequent molecular approaches based on large scale comparative analysis of 16S ribosomal RNA with the potential to profile microbial communities at a higher resolution. Next generation Sequencing (NGS), such as 454 pyrosequencing technique, allows to understand in detail the microbial composition associated with a specific community and provides a simple and cost-effective mechanism for characterizing the composition of bacterial communities[112],[113],[114]. This NGS method allows to recognize the microbial profile associated to each PCR-DGGE fingerprint and thus offers the possibility to identify taxonomically any given microbiological signature[115].

This technique demonstrates that specific bacterial communities present in the skin mucus of fish yield unique signatures that allow to trace each fish to its respective geographic origin.

Advantages: The combined use of PCR-DGGE and NGS are effective molecular tools that can make possible to pinpoint which bacterial taxa hold the potential to be used as natural and unique barcodes of farmed fish.

Disadvantages: it is necessary 1 week to know the final results, it is an expensive analysis (around 200 €/sample) and the analysis must be performed by experimented technicians, it is not simple.

Spectroscopic methods (NIR):

Near infrared reflectance spectroscopy (NIRS) analysis was used to predict proximate chemical composition of Chinese export tilapia fillets from four geographical origins (Guangdong Province, Hainan Province, Guangxi Province and Fujian Province, respectively)[116]. NIRS provided good reliability in the prediction of chemical composition of tilapia fillets but weak results in crude protein prediction. Origin traceability is an important part of food safety traceability system. The tilapia origin traceability model was developed by near infrared reflectance (NIR) spectroscopy coupled with soft independent modelling of class analogy (SIMCA).

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Other references using NIR spectroscopy to analyse origin of fish are Lv et al., that classified freshwater fish species by linear discriminant analyses[117], Ghidini et al., that developed a method based on NIRS for a rapid authentication of European sea bass (*Dicentrarchus labrax L.*) and Alamprese and Casiragui that applied FT-NIR and FT-IR spectroscopy in fish fillet authentication[118]. Besides, Cozzolino *et al*. used NIR spectroscopy to assess chemical composition of fish, also related to geographical origin[119].

Advantages: cheap, non-destructive and rapid analysis.

Disadvantages: it's important to calculate robust models in order to reduce the error in determination. Usually, calibrations are device dependent.

3.3 Farmed fish – vs- wild fish

Few markers can be used efficiently to unequivocally establish specimens as the production method (wild / farmed), some of them are:

Fatty acids composition:

Total fat contents and long-chain fatty acids including saturated, monounsaturated, and polyunsaturated fatty acids, in extracted fish oil have been used for authentication purposes [120]. In order to differentiate between wild and cultured species, Tritt *et al.* (2005) [121] examined fatty acid composition of juvenile largemouth bass, white crappies, and black crappies. By applying a series of chemometric tools, namely, analysis of variance, PCA, and QDA to 4 fatty acids (linoleic acid: 18:2n-6, linolenic acid: 18:3n-3, arachidonic acid: 20:4n-6, and docosahexaenoic acid: 22:6n3), linoleic acid was found to be the primary fatty acid that could be used to differentiate juvenile wild from cultured fishes. In addition, the use of the 4 fatty acids allowed to classify correctly 90 of 91 juvenile fishes as wild or cultured; 32 of 37 wild juvenile fishes originating from the same reservoir were differentiated by species.

Advantages: physicochemical analyses are considered as reference methods, providing accurate and reliable results on quality and authenticity of fish samples.

Disadvantages: they are time-consuming and need a lot of polluting reagents, are relatively expensive, cannot be utilized at/on-line, and involve the use of chemicals and trained technicians.

Morphological and biometric differences:

[117] Lv H, Xu W, You J, Xiong S (2017). Classification of freshwater fish species by linear discriminant analysis based on near infrared reflectance spectroscopy. Journal of Near Infrared Spectroscopy 25(1), 54-62.

[118] Alamprese C and Casiraghi E (2015). Application of FT-NIR and FT-IR spectroscopy to fish fillet authentication. Food Science and Technology 63, 720-725.

[119] Cozzolino D, Chree A, Murray I, Scaife JR (2002). The assessment of the chemical composition of fishmeal by near infrared reflectance spectroscopy. Aquaculture Nutrition 8, 149-155.

[120] Lavilla, I., Costas-Rod ıguez, M., and Bendicho, C. (2013). Authentication of fishery products. In: De la Guardia M, Gonzálvez A, editors. *Comprehensive analytical chemistry*. Oxford: Elsevier. pp. 657-717.

[121] Tritt , K. L., O'Ba a, C. ., and Wells, M. J. M. (2005). Chemometric discrimination among wild and cultured age-0 largemouth bass, black crappies, and white crappies based on fatty acid composition. *J Agric Food Chem*. 53: 5304–5312.

Morphological variations have been shown to be a valuable tool to describe changes occurring in shape features. Thus, Alasalvar et al. (2002)[122] noticed fundamental differences in morphology between farmed and wild sea bream, since the appearance of the latter was described to be more bleached greenish, had sharper dorsal fins, more scales, sharper teeth with greater height and conical edge, smaller bellies, and shorter tails compared to the former one. Wild sea bream had also a golden tape between the eyes and a reddish patch on the surface of the gill cover.

Advantages: easy and cheap analyses to be performed by experienced people.

Disadvantages: in most of the cases, morphological differences between species of fish could not be detected, especially at juvenile stages. Consequently, a large number of sensory analysis techniques have been developed.

Stable isotope analysis:

Methods for establishing the compliance with declarations about wild or farmed fish have been developed in order to fight frauds connected with provenance and processing which could also impact on health. Following on from early studies that had shown that the content of stable isotopes reflects both the environment in which the fish is grown and the composition of its diet, a major project known as COFAWS1 was set up to further develop these techniques.

There are several correlations between the content of isotopes and the geo/climatic environment of a food product. The content in ^{13}C and ^{15}N are related to diet; ^{18}O and ^{2}H are influenced by the origin of the water in the product. To differentiate the farmed and wild origin of salmon, isotope ratios $^{18}O/^{16}O$ (expressed as $\delta^{18}O$) and $^{15}N/^{14}N$ (expressed as $\delta^{15}N$) are measured by IRMS (isotope ratio mass spectrometry) on the fish oil and choline from the lipid fraction extracted from the fish muscle[123]. These parameters successfully separated wild and farmed salmon both from known origins and unknown market samples. The technique has since been used to check mislabelling in the UK market. It has since been extended to other fish such as bream, cod, bass.

Other studies have been reported in the literature including a chemometric approach addressing the global chemical composition (trace elements, stable isotopes, fatty acids)[124], [125]. Stable isotope ratios for carbon, nitrogen and oxygen have also been suggested as a means for discriminating wild from farmed fish, and organic from intensive production, based on differences in the feed origin [126], [127]. A combination of isotope determination and other profiling methods, e.g. trace elements or fatty acids, could be more effective.

[122] Alasalvar, C., Anthony Taylor, K. D., and Shahidi, F. (2002). Comparative quality assessment of cultured and wild sea bream (*Sparus aurata*) stored in ice. *J Agric Food Chem.* 50: 2039-2045.

[123] Thomas F., Jamin E., Wietzerbin K., Guérin R., Lees M., Morvan E., Billault I., Derrien S., Moreno Rojas J.M., Serra F., Guillou C., Aursand M., McEvoy L., Prael A. & Robins R.J. (2008). – Determination of Origin of Atlantic Salmon (Salmo salar): The Use of Multiprobe and Multielement Isotopic Analyses in Combination with Fatty Acid Composition To Assess Wild or Farmed Origin. *J. Agric. Food Chem.*, 56 (3), 989–997. doi:10.1021/jf072370d.

[124] Wang Y.V., Wan A.H.L., Lock E.J., Andersen N., Winter-Schuh C. & Larsen T. (2018). – Know your fish: A novel compound-specific isotope approach for tracing wild and farmed salmon. *Food Chem.*, 256, 380–389. doi:10.1016/j.foodchem.2018.02.095.

[125] Chaguri M.P., Maulvault A.L., Costa S., Gonçalves A., Nunes M.L., Carvalho M.L., Sant'ana L.S., Bandarra N. & Marques A. (2017). – Chemometrics tools to distinguish wild and farmed meagre (*Argyrosomus regius*). *J. Food Process. Preserv.*, 41 (6), e13312. doi:10.1111/jfpp.13312.

[126] Li L., Boyd C.E. & Sun Z. (2016). – Authentication of fishery and aquaculture products by multi-element and stable isotope analysis. *Food Chem.*, 194, 1238–1244. doi:10.1016/j.foodchem.2015.08.123.

[127] Camin F., Bontempo L., Perini M. & Piasentier E. (2016). – Stable Isotope Ratio Analysis for Assessing the Authenticity of Food of Animal Origin. *Compr. Rev. Food Sci. Food Saf.*, **15** (5), 868–877. doi:10.1111/1541-4337.12219.

Isotopes of Strontium could be indicative of geographic provenance, since this element is present together with calcium in bones and calcified materials of seafood[128].

Some studies in farmed and wild European eel[129], Atlantic salmon[130], gilthead sea bream[131] and European sea bass[132] corroborate the utility of the analytical method.

Advantages: important tool in the fight against fraud in the food products industry. They provide information on the geographical origin, important characteristics for the consumer that are highly taken into account in national and international legislation. In the UE and north of America, they are considered official methods to fight against food fraud.

Disadvantages: they need specific expertise and equipment to be performed and the results correctly interpreted.

Accumulative pollutants (organochlorine and heavy metals):

Multi-elemental analysis has been used to trace the origins of clams, shrimp, and crabs[133]. Sometimes, combining multielement analysis with isotope analysis and chemometrics, improvements in identifying fish origin is gained. This study hypothesized that a combination of multi-element analysis and stable isotope ratio analysis based on non-lethal sample material taken from the third pereiopods, as well as multivariate statistical methodology, can provide a non-conventional approach for tracing the origins of *E. sinensis* within China[134]. In other study performed by Ziket *et al.* (2010) [135] same approach combining multielement and isotope data was used to discriminate wild and hatchery trout.

Other related references using multielemental analysis for the detection of wild/farmed fish fraud are the following: shrimps[136], salmon [137] and carp [138].

[128] Baffi C. & Trincherini P.R. (2016). – Food traceability using the 87Sr/86Sr isotopic ratio mass spectrometry. *Eur. Food Res. Technol.*, 242 (9), 1411–1439. doi:10.1007/s00217-016-2712-2.

[129] Vasconi M, Lopez A, Galimberti C, Moreno Rojas JM, Muñoz Redondob JM, Bellagamba F, Morettia VM (2019) . Authentication of farmed and wild european eel (*Anguilla anguilla*) by fatty acid profile and carbon and nitrogen isotopic analyses. Food Control 102, 112-121.

[130] Dempson JB, Power M (2004). Use of stable isotopes to distinguish farmed from wild Atlantic salmon, Salmo salar. Ecology of Freshwater Fish 13, 176–184.

[131] Moreno Rojas JM, Serra F, Giani I, Moretti VM, Reniero F, Guillou C (2007). The use of stable isotope ratio analyses to discriminate wild and farmed gilthead sea bream (*Sparus aurata*). Rapid Communications in Mass Spectrometry, 21 (2), 207-211.

[132] Tulli F, Moreno-Rojas JM, Messina CM, et al. The Use of Stable Isotope Ratio Analysis to Trace European Sea Bass (*D. labrax*) Originating from Different Farming Systems. Animals: an Open Access Journal From MDPI. 2020 Nov;10(11). DOI: 10.3390/ani10112042.

[133] Iguchi, J., Isshiki, M., Takashima, Y., Yamashita, Y., & Yamashita, M. (2014). Identifying the origin of Corbicula clams using trace element analysis. Fisheries Science, 80(5),1089–1096.

[134] Luo R, Jiang T, Chen X, Zheng C, Liu H, Yang J (2019). Determination of geographic origin of Chinese mitten crab (*Eriocheir sinensis*) using integrated stable isotope and multi-element analyses. Food Chemistry 274, 1-7.

[135] Zitek A., Sturm M., Waidbacher H., Prohaska T. (2010). Discrimination of wild and hatchery trout by natural chronological patterns of elements and isotopes in otoliths using LA-ICP-MS. Fisheries Management and Ecology 17, 435-445.

[136] Ortea I, Gallardo JM (2015). Investigation of production method, geographical origin and species authentication in commercially relevant shrimps using stable isotope ratio and/or multi-element analyses combined with chemometrics: An exploratory analysis. Food Chemistry 170, 145-153.

[137] Anderson KA, Hobbie KA, Smith BW (2010). Chemical Profiling with Modeling Differentiates Wild and Farm-Raised Salmon. Journal of Agricultural and Food Chemistry 58, 11768–11774.

[138] Liu Z, Yuana Y, Yan Zhao Y, Zhang Y, Nie J, Shao S, Rogers KM (2020). Differentiating wild, lake-farmed and pond-farmed carp using stable isotope and multi-element analysis of fish scales with chemometrics. Food Chemistry 328, 127115.

Advantages: specific and robust methods that give accurate information about contaminant profiles linking the sample with its origin.

Disadvantages: quite complex analysis that requires specialised equipment and technicians. Data analysis and chemometrics are also needed to correctly interpret results.

Spectroscopic analysis (NIR):

Several studies have been performed to assess the potential of NIR spectroscopy to authenticate fish and other seafoods. For example, Ottavian *et al*. (2012)[139] succeeded in differentiating between wild and farmed European sea bass samples. By applying a series of statistical analyses such as PCA and PLS-DA, the authors indicated that CH, CH_2, CH_3, and H_2O groups, which are related to fat, fatty acids, and water content, were the most interesting spectral regions.

Advantages: NIRS is particularly favourable because it is simpler, more economical, environmentally safer, and faster than many other techniques. It is a non-destructive and fast method that can give information at real-time. The success of spectroscopic depends on the reliability of the reference method employed and the sample presentation.

Disadvantages: there are some limitations concerning the applications of these spectral techniques since the complexity of NIR spectra and the need to develop calibration models based on the use of chemometrics to predict unknown samples

3.4 Previously frozen fish sold as fresh fish

A standard method for establishing if the fish has been thawed from frozen is based on microscopy analysis of muscle, by the Italian accreditation body ACCREDIA[140]. Other methods based on physical and chemical parameters are being developed[141], [142], [143].

Spectroscopic analysis (NIR):

Another application related to authentication issues of red sea bream was performed by Uddin *et al.* (2005)[144] who attempted to determine whether VIS/NIR spectroscopy in a reflection mode could differentiate fresh fish samples from frozen-thawed ones. PCA was applied to 108 samples (54 were used soon after arrival, while the second lot of 54 fish was kept at −40°C for 30 days), and a clear discrimination of frozen-thawed samples from the fresh ones was observed. This difference has been attributed to the fact that the freeze-thawing treatment altered the physical structure of at least the surface layer of frozen-thawed fish, inducing

[139] Ottavian, M., Facco, P., Fasolato, L., Novelli, E., Mirisola, M., Perini, M., and Massimiliano, B. (2012). Use of near-infrared spectroscopy for fast fraud detection in seafood: application to the authentication of wild European sea bass (*Dicentrarchus labrax*). J Agric Food Chem. 60: 639−648.

[140] Bozzetta E., Pezzolato M., Cencetti E., Varello K., Abramo F., Mutinelli F., Ingravalle F. & Teneggi E. (2012). – Histology as a Valid and Reliable Tool To Differentiate Fresh from Frozen-Thawed Fish. J. Food Prot., 75 (8), 1536–1541. doi:10.4315/0362-028X.JFP-12-035.

[141] Karoui R., Hassoun A. & Ethuin P. (2017). – Front face fluorescence spectroscopy enables rapid differentiation of fresh and frozen-thawed sea bass *(Dicentrarchus labrax*) fillets. J. Food Eng., 202, 89–98. doi:10.1016/j.jfoodeng.2017.01.018.

[142] Qu J.H., Liu D., Cheng J.H., Sun D.W., Ma J., Pu H. & Zeng X.A. (2015). – Applications of Near-infrared Spectroscopy in Food Safety Evaluation and Control: A Review of Recent Research Advances. Crit. Rev. Food Sci. Nutr., 55 (13), 1939–1954. doi:10.1080/10408398.2013.871693.

[143] Kamruzzaman M., Makino Y. & Oshita S. (2015). – Non-invasive analytical technology for the detection of contamination, adulteration, and authenticity of meat, poultry, and fish: A review. Anal. Chim. Acta, 853, 19–29. doi:10.1016/j.aca.2014.08.043

[144] Uddin, M., Okazaki, E., Turza, S., Yumiko, Y., Tanaka, M., and Fukuda, Y. (2005). Non-destructive Visible/NIR spectroscopy for differentiation of fresh and frozen-thawed fish. J Food Sci. 70: 506–510.

changes in the shape of spectra. The authors claimed the use of VIS/NIR spectroscopy as a rapid tool for online or at-line processing control of fresh and frozen/thawed fish samples.

Hyperspectral imaging (HIS):

In the past decade, a new technique, referred to as imaging spectroscopy, has been developed in the VIS/NIR region, namely hyperspectral imaging (HSI). In addition to the spectral information, this technique also gives spatial information, which means that a full spectrum is recorded at different locations of the fish sample. Recently, the differentiation between fresh, fast frozen-thawed and slow frozen-thawed fish samples has been achieved using a push-broom HIS system in the 380-1030 nm region[145]. The authors observed differences in the shape of the spectra and ascribed them to the: i) alterations of physical structure, at least in the surface layer of fish; and ii) biochemical and textural changes in fish during freezing and frozen-thawed operations.

Advantages: NIR and HIS are non-destructive and fast methods that can give information at real-time. The success of spectroscopic depends on the reliability of the reference method employed and the sample presentation.

Disadvantages: there are some limitations concerning the applications of these spectral techniques since the complexity of NIR spectra and HIS hypercube, and the need to develop calibration models based on the use of chemometrics to predict unknown samples.

[145] Zhu, F., Zhang, D., He, Y., Liu, F., and Sun, D.W. (2012). Application of visible and near infrared hyperspectral imaging to differentiate between fresh and frozen–thawed fish fillets. Food Bioprocess Technol. 6: 2931-293.

3.5 Undeclared use of food additives.

Chemical analysis:

For each of this food additives, the corresponding chemical analysis method must be applied based mainly in the nature of the corresponding food additive.

In the Regulation (EC) No 1333/2008 of the European Parliament and of the Council of 16 December 2008 on food additives (Text with EEA relevance) in Annex II there is the list with the authorized additives and the corresponding doses or limit in processed fish, raw and frozen fish.

4. Summary table

In the next Table, the different analytical techniques mentioned in this document are classified according to the markers: Time consuming, cost, robustness and simplicity of method. Each characteristic will be identified with a qualitative mark: Green 🟢 means it completely meets the characteristic named in the corresponding column of the table, yellow 🟡 if it partially meets the characteristic and red 🔴 if it does not meet the corresponding characteristic.

Table 5: Summary of advantages and disadvantages of the selected analytical methods to detect the main fraud causes in fish and fish products. Colours code: explained above.

Fraud	Analytical Technique	Time	Cost	Robustness	Simplicity
Species substitution	*DNA based*	🔴	🔴	🟢	🔴
	Morphological Analyses	🟢	🟢	🔴	🟡
	Immunological analysis	🟡	🟡	🟡	🔴
	Spectroscopic analysis (NIR)	🟢	🟢	🟡	🟢
Origin mislabelling	*Trace element analysis*	🔴	🔴	🟢	🔴
	Stable isotope analysis	🔴	🔴	🟢	🔴
	DNA analysis	🔴	🔴	🟢	🔴
	Fish microbiota/microbioma analysis	🔴	🔴	🟢	🔴
	Spectroscopic analysis (NIR)	🟢	🟢	🟡	🟢
Farmed/wild fish	*Fatty-acids composition*	🔴	🟡	🟢	🟡
	Morphological and biometric differences	🟢	🟢	🔴	🟡
	Stable isotope analysis	🔴	🔴	🟢	🔴
	Accumulative pollutants (trace elements, organochlorine and heavy metals)	🔴	🔴	🟢	🔴
	Spectroscopic analysis (NIR)	🟢	🟢	🟡	🟢
Frozen/Fresh fish	*Spectroscopic analysis (NIR)*	🟢	🟢	🟡	🟢
	Hyperspectral imaging (HIS)	🟢	🟢	🟡	🟢
Undeclared use of additives	*Chemical analysis*	🔴	🔴	🟢	🔴

5. Conclusions

FAO[146] has identified the main needs to combat food fraud in the seafood sector as the following ones:

(i) reaching agreements on names of products and species;
(ii) introducing mandatory labelling;
(iii) improving the systems for official control of food;
(iv) improving systems for food safety in production;
(v) adding new Codex guidelines

The transportation of fish and fish product from the capture area to consumers, in long supply chains, provides logistic challenges and risks for health. Consumers nowadays require innovative ways for chilling, preserving, delivering seafood, and in this area authenticity or fraud issues might arise.

New technologies emerge due to the need of giving transparency to the supply chain, as is the case of blockchain technology, a system that allows consumers to check the traceability of the fish they are going to buy through their smartphone, and that they are already working on tests pilot.

Regarding the improving of systems for official control of fish (iii), the main analytical methods identified to detect the most frequent causes of fraud in fish are:

- DNA based methods for species substitution and origin mislabelling.
- Stable isotope methods for origin mislabelling and farmed/wild fish identification.
- Morphological /biometric methods for species substitution and farmed/wild fish identification.
- Inmunological methods for species substitution.
- Spectroscopic methods (NIRS and HIS) for species substitution, origin mislabelling, farmed/wild fish identification, frozen/fresh fish differentiation.
- Some specific nutritional compounds (fatty acids profile) for farmed/wild fish identification.
- Multielement analysis (trace elements, heavy metals and organochlorine compounds) for origin mislabelling and farmed/wild fish identification.
- Fish microbiota/midrobioma analysis for origin mislabelling.

The extensive bibliographic review performed (145 references) will serve as the basis for the setting and harmonization of the different analytical methods based on the selected analytical techniques.

[146] FAO, ed. (2018). – *The state of world fisheries and aquaculture - Meeting the sustainable development goals*. Rome.