

Research Paper

Wild or Farmed Gilthead Seabream (*Sparus aurata*)? How To Distinguish between Them by Two-Dimensional Gel Electrophoresis

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ABSTRACT

Because the world's wild fish stocks are limited and the market demand is increasing, fish farming has become an alternative food source and a way to reduce costs for consumers. The sale of farmed as wild fish is a fraudulent practice; it is, therefore, important to find new and alternative tools that can help in the fight against fraud to protect consumers and to ensure food traceability. The proteomic profiles of farmed and wild fish differ. With this study we wanted to identify liver protein markers via two-dimensional electrophoresis that would allow us to distinguish wild from farmed gilthead seabream. The liver samples from 32 gilthead seabream, wild and farmed, were stored at -80°C before protein extraction. The samples were subjected to two-dimensional electrophoresis to detect qualitative and quantitative differences. Proteomic analysis showed a protein spot (molecular weight of ~ 34 kDa and isoelectric point of ~ 6.9) only in the samples from the wild gilthead seabream; liquid chromatography–tandem mass spectrometry identified the spot as ubiquitin. Ubiquitin could be a valid marker to differentiate wild from farmed gilthead seabream; it could be used to ensure continuous monitoring throughout the entire commercial chain and to fight commercial fraud.

HIGHLIGHTS

- A proteomic approach to differentiate the method of fish production is proposed.
- A 2-DE spot, characterized by MS/MS as ubiquitin, differentiated between samples.
- Liver proteome could be useful to find biological markers.

Key words: Farmed; Food fraud; Gilthead seabream; 2D-electrophoresis; Ubiquitin; Wild

Human societies need to provide food for a growing population, and the nutritional attributes of fish make it an excellent food for human health. Total fish production in 2018 was 179 million tons; $\sim 88\%$ of this was for direct human consumption, with a record high per capita consumption of 20.5 kg. The increase in the global demand for aquatic food products and the decline in fish populations due to overfishing have driven the spread of aquaculture, which produces about half of the fish consumed worldwide and which has a higher growth rate than any other animal food production sector (8). The most commonly farmed fish and shellfish are carp, salmon, gilthead seabream, sea bass, croaker, amberjack, eel, trout, tuna, sturgeon, crustaceans, and mollusks (7). Consumers

need to be informed about the origin and the characteristics of the products they purchase.

Food product traceability and labeling are regulated under Regulations (EC) 178/2002 and 1169/2011. Regulation 1379/2013 covers the requirements for the labeling of seafood products, their origin and species name, and the area and method of production. The sale of farmed fish as wild fish is a fraudulent practice; to protect consumers from commercial fraud and to ensure food traceability, reliable analytical methods need to be developed and applied. Techniques to detect substitution fraud in seafood range from macroscopic examination of morphological traits (4) to analysis of stable isotopes and fatty acids (19).

Farmed fish have the same quantity and quality of protein as wild fish per gross composition. Nevertheless, farming can modify the protein expression profiles of tissues (1, 11). Proteomics can be used to track and detect

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TABLE 1. List of the 32 samples used for the analyses with sample ID, method of production, region of origin, and presence of the ubiquitin protein spot^a

Sample no.	Sample ID	Production method (F or W)	Region of origin	Presence of ubiquitin spot
1	F 1	F	Adriatic	No
2	F 2	F	Adriatic	No
3	F 3	F	Adriatic	No
4	F 4	F	Malta	No
5	F 5	F	Malta	No
6	F 6	F	Malta	No
7	F 7	F	Italy	No
8	F 8	F	Italy	No
9	F 9	F	Italy	No
10	F 10	F	Italy	No
11	F 11	F	Italy	No
12	F 12	F	Italy	No
13	F 13	F	Italy	No
14	F 14	F	Italy	No
15	F 15	F	Italy	No
16	F 16	F	Italy	No
17	W 1	W	France	Yes
18	W 2	W	France	Yes
19	W 3	W	France	Yes
20	W 4	W	France	Yes
21	W 5	W	France	Yes
22	W 6	W	Adriatic	Yes
23	W 8	W	France	Yes
24	W 9	W	France	Yes
25	W 10	W	France	Yes
26	W 14	W	Italy	Yes
27	W 15	W	Italy	Yes
28	W 16	W	Italy	Yes
29	W 17	W	Italy	Yes
30	W 19	W	Italy	Yes
31	W 20	W	Italy	Yes
32	W 21	W	Italy	Yes

^a F, farmed; W, wild.

variations in protein expression and reveal differences between wild and farmed fish. The effect of aquaculture on the liver proteome has been evaluated by studying specific factors. The species investigated for comparative wild and farmed liver protein expression include rainbow trout (*Oncorhynchus mykiss*), in which changes in protein profiles were studied under different feeding regimens (11), and the gilthead seabream (*Sparus aurata*), in which manipulation stress and cold temperature were evaluated (3, 10). Liver proteomics coupled with conventional stress monitors, such as plasma levels of cortisol, glucose, and lactate, have enabled researchers to identify stress-associated proteins in fish, which may hold promise for the development of more practical tests, such as enzyme-linked immunosorbent assay, for evaluating and monitoring chronic stress factors for fish welfare and sustainability, concepts that are increasingly important at the global level (14, 15, 18).

The desirable aroma and quality of gilthead seabream, and its consequent high commercial value, have made farming the fresh product a profitable business, with a

significant increase in gilthead seabream production over the past decade (1). Gilthead seabream is a leading product of Mediterranean aquaculture: 97,151 tons were produced in Europe in 2017, of a total 229,922 tons that were produced globally during the same year, according to the Food and Agriculture Organization of the United Nations, Fisheries and Aquaculture Information and Statistics Service (8).

A simple method based on direct sampling analysis, coupled with a time-of-flight mass spectrometer (DSA-TOF), was developed by our group to discriminate between wild and farmed gilthead seabream muscle on the basis of the docosahexaenoic and arachidonic fatty acid ratio. The method has good precision in repeatability and reproducibility (relative standard deviation, 15%) (17). With the aim of providing additional tools for the DSA-TOF method in the fight against commercial fraud, and based on the potential of proteomic studies, we used gilthead seabream liver to identify liver protein markers via a proteomic approach based on two-dimensional electrophoresis (2-DE) to distinguish wild from farmed gilthead seabream.

MATERIALS AND METHODS

Samples and experimental design. A total of 32 samples of gilthead seabream ($n = 16$ farmed and $n = 16$ wild) were bought from Italian fishmongers at the harbor and from supermarkets between July 2017 and January 2018 and were stored at +4°C until arrival at the laboratory. The samples weighed from 200 to 1,500 g. Weight and geographical origin were recorded for each sample (Table 1). All analyses were performed on the liver, which was harvested from the gilthead seabream immediately after arrival at the laboratory and was frozen at −80°C until analysis. Each sample was analyzed in triplicate.

Protein extraction. Soluble proteins were obtained from 0.3 g of liver tissue placed in five volumes (w/v) of buffer containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate (CHAPS), 60 mM dithiothreitol (DTT), 10 mM Tris, 1 mM EDTA, Complete. After homogenization, the samples were incubated for 3 h at +4°C and then were centrifuged at $100,000 \times g$ for 30 min at +4°C. Lipids were discarded by pipetting, and the supernatant was collected and stored at −20°C until analysis. The protein amount was determined using a Qubit protein assay (Thermo Fisher Scientific, Waltham, MA). Sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.6% ampholytes (100× Biolyte ampholyte pH 3 to 10, pH 4.7 to 5.9, pH 6.3 to 8.3; Bio-Rad, Hercules, CA) was added to reach a volume of 0.24 mg/mL of total protein for each sample. The samples were incubated at room temperature overnight in end-over-end rotation and then were centrifuged at $15,294 \times g$ for 5 min at room temperature before loading on the strip for isoelectric focusing.

2-DE. Each solution was prepared with ultrapure water (18 MO), and all products met the quality requirements for proteomics. Isoelectric focusing was performed with ReadyStrip immobilized pH gradient strips (Bio-Rad), 18 cm in length (pH gradient 3 to 10 NL) according to the manufacturer's instructions. The immobilized pH gradient strips were rehydrated overnight in 750 μ L of rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 10 mM DTT, 0.6% ampholytes (100× Biolyte

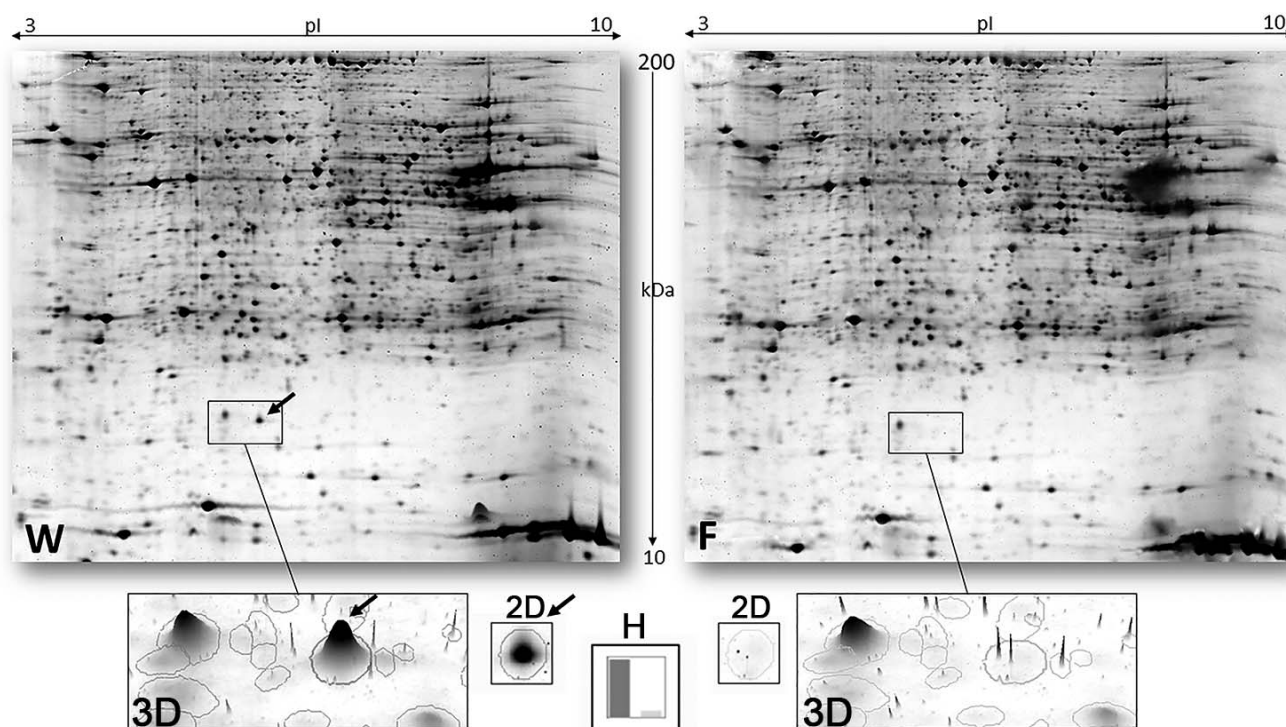


FIGURE 1. 2D maps of tissue samples from gilthead seabream, wild (W) and farmed (F). Identification of ~1,200 protein spots by Oriole fluorescent stain. The arrow indicates a protein spot (molecular weight ~34 kDa and isoelectrical point ~6.9) present only in the wild samples and absent in the farmed gilthead seabream. The boxes show an enlarged 3D view of the area containing the ubiquitin spot (3D), the 2D view (2D), and the histogram (H) of the ubiquitin spot for the wild (left side) and the farmed (right side) samples.

ampholyte pH 3 to 10, pH 4.7 to 5.9, pH 6.3 to 8.3; Bio-Rad) with 0.001% bromophenol blue to track migration.

The rehydrated strips were then positioned in the Protean isoelectric focusing cell (Bio-Rad) on the focusing plate, and 150 μ L of sample buffer containing the proteins was loaded on each strip with the cup-loading method. Proteins were focused for 24 h for a total of 65,000 Vh at a maximum intensity of 50 μ A per strip. After focusing, all strips were incubated in two equilibration buffers for 15 min. The first equilibration buffer contained 6 M urea, 2% sodium dodecyl sulfate (SDS), 50 mM Tris pH 8.8, and 1% DTT. The second equilibration buffer was identical in composition, except 3% iodoacetamide was used instead of DTT.

Equilibrated strips were loaded on 15% SDS gels and underwent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on Protean Plus Dodeca Cell (Bio-Rad) at 200 kV for 6 h and 30 min with a Tris glycine buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3), thermostated to 12°C. After electrophoresis, the gels were stained with Oriole fluorescent gel staining (Bio-Rad) for 90 min, washed quickly, and scanned with Chemi Doc Touch (Bio-Rad).

Image analysis. The images were analyzed with Delta2D software (version 4.7, Decodon, Greifswald, Germany). Briefly, the gel images (16-bit tiff) were imported into Delta2D, warped, and fused (i.e., union setting) to produce a master image. Detection was carried out on this fusion image, and the results were then propagated to all raw individual images to compare the spots in the wild and the farmed samples. The resulting quantification table was analyzed using the Student's *t* test function of the software, with permutation-based *P* value (critical *P* value 0.01) and adjusted Bonferroni correction for determining significance. The spots of interest were excised and analyzed using mass spectrometry (MS) as described below.

MS analysis. Spots from the SDS-PAGE were cut out, and the proteins were digested in-gel with trypsin (Sigma-Aldrich, Saint Louis, MO). The peptide digests were desalted on Discovery DSC-18 solid-phase extraction 96-well plates (25 mg per well) (Sigma-Aldrich) and then were vacuum evaporated before liquid chromatography (LC)-MS analysis.

The digested samples were analyzed on a micro-LC (Eksigent Technologies, Dublin, CA) interfaced to a 5600+ TripleTOF mass spectrometer system (AB Sciex, Concord, Canada) equipped with a DuoSpray ion source and a calibrant delivery system. The LC column was a Halo Fused C18. The mobile phase was a mixture of 0.1% (v/v) formic acid in water (A) and 0.1% (v/v) formic acid in acetonitrile (B), eluting at a flow rate of 15.0 μ L min⁻¹ at an increasing concentration of solvent B from 2 to 40% in 30 min. The injection volume was 4.0 μ L, and the oven was set at 40°C. For identification purposes, the MS analysis was performed at a mass range of 100 to 1,500 Da (TOF scan with an accumulation time of 0.25 s), followed by a tandem MS (MS/MS) product ion scan from 200 to 1,250 Da (accumulation time 5.0 ms) with the abundance threshold set at 30 counts per s (35 candidate ions per cycle can be monitored). The ion source parameters in electrospray positive mode were set as follows: curtain gas (N₂) at 25 lb/in² gauge, nebulizer gas 1 at 25 lb/in² gauge and gas 2 at 20 lb/in² gauge, ion spray floating voltage at 5,000 V, source temperature at 450°C, and declustering potential at 25 V.

The data-dependent acquisition files were searched using Mascot v. 2.4 (Matrix Science Inc., Boston, MA) (Fig. 1). Trypsin as digestion enzyme and one missed cleavage were specified. The instrument was set to electrospray ionization quadrupole TOF, and the following modifications were specified for the search: carbamidomethyl cysteine as fixed modification and oxidized methionine as variable modification. A search tolerance of 0.08 Da

TABLE 2. Protein identified in 2-DE spot of wild gilthead seabream with Mascot score, peptide sequences obtained by μ LC-MS, calculated molecular mass and pI, sequence coverage, and accession number^a

Protein Mascot score	Peptide sequence	Mass (Da) and pI	Sequence coverage	UniProt Consortium accession no.
92	MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQR LIFAGKQLEDGRTLSDYNIQKESTLHLVLRRLRGGMQIFVK TLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLLIFAGK QLEDGRTLSDYNIQKESTLHLVLRRLRGGMQIFVKTLTGKT ITLEVEPSDTIENVKAKIQDKEGIPPDQQRLLIFAGKQLEDG RTLSDYNIQKESTLHLVLRRLRGGMQIFVKTLTGKTITLEVE PSDTIENVKAKIQDKEGIPPDQQRLLIFAGKQLEDGRTLSD YNIQKESTLHLVLRRLRGGN	34,298 and 6.95	8.2%	A0A024CHU0_SPAAU

^a μ LC-MS, microcapillary liquid chromatography–mass spectrometry; pI, isoelectric point. Boldface indicates peptide sequences obtained by μ LC-MS.

was specified for the peptide mass tolerance and 10 ppm for the MS/MS tolerance. The peptide charges to search for were set to 2+, 3+, and 4+, and the search was set on monoisotopic mass. The trEMBL unreviewed database containing *Sparus aurata* proteins (version 12/03/2019, containing 11,217 sequence entries) was used.

RESULTS AND DISCUSSION

Proteomic analysis showed ~1,200 protein spots by Oriole fluorescent stain, and software analysis showed six protein spots with a statistically significant difference. Five spots showed little under- or overexpression, whereas one protein spot (molecular weight ~34 kDa, isoelectric point ~6.9) was present in the wild gilthead seabream samples and absent in all the farmed gilthead seabream samples. We proceeded with the MS identification of the latter spot only; this was mainly because, in the other cases, there was a low difference between the two groups and, furthermore, the spots were in critical positions (acidic area) or showed a high standard deviation within the group for the presence of outliers (data not shown).

LC-MS/MS analysis identified, with a Mascot score of 92 (two peptides), the protein spot of the wild gilthead seabream as ubiquitin (isoelectric point about 6.9, and molecular mass about 34 kDa). Table 2 reports the protein identified in the spot on the 2-DE map (Fig. 1).

Farmed fish are exposed to environmental constraints and stimuli that differ from those of their free-ranging counterparts; diet, crowding, physical activity, and water quality (e.g., temperature, salinity, microbial pathogens) can differ from those experienced in the wild (18). These differences have an impact on the protein expression profiles of tissues, as demonstrated in cod (*Gadus morhua*), rainbow trout (*Oncorhynchus mykiss*), sea bass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*) (1–3, 6, 11–13).

Whereas the majority of previous studies have analyzed muscle tissue, we worked on liver tissue because of its fundamental role in the synthesis and metabolism of proteins (transferrin, fibrinogen, apolipoproteins, globulins), phospholipids and cholesterol, and bile acid production and excretion (9), with over 10,000 estimated biochemical reactions, including carbohydrate, fat, and protein metabo-

lism, storage of vitamins and minerals, and regulatory functions that control blood sugar and hormone levels. Moreover, the liver is exposed to xenobiotics and toxins and is subject to factors such as diet, environment, and stress.

Using 2-DE, we explored the liver proteome and, among all the protein spots, identified ubiquitin as a specific marker of wild gilthead seabream, as determined by LC-MS/MS analysis. Ubiquitin is involved in the ubiquitin proteasomal pathway of protein degradation, which involves two steps: tagging of damaged or misfolded protein by multiple ubiquitin molecules followed by degradation of the tagged protein by the proteasome complex. This pathway is stress activated in fish (5, 16); however, it can also be influenced by diet, as reported for rainbow trout fed with supranutritional dietary selenium yeast, which down-regulates the expression of protein degradation-related (autophagy-lysosome and ubiquitin-proteasome related) genes (20).

Farming conditions may influence ubiquitin expression by down-regulating it or by increasing its use in the pathway, which is why the protein spot was visible only in the wild seabream and not in the farmed samples. These data suggest an influence of the production method on the food product. Although further analyses are needed on a larger number of samples, the fact that the ubiquitin observed on the 2-DE maps of the liver was present only in the wild gilthead seabream means that it could be used, in a screening test, as a biological marker to distinguish the method of production in this species. In a global market with ever-increasing food demands, there are multiple opportunities for fraud. 2-DE proved a useful methodology to identify a potential biomarker able to discriminate between farmed and wild gilthead seabream (*Sparus aurata*); this opens the way to the development of tests that will be useful tools for producers and government agencies.

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