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Authentication of Farmed and Wild Turbot (*Psetta maxima*) by Fatty Acid and Isotopic Analyses Combined with Chemometrics

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Fatty acid composition and stable isotope ratios of carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) were determined in muscle tissue of turbot (*Psetta maxima*). The multivariate analysis of the data was performed to evaluate their utility in discriminating wild and farmed fish. Wild ($n = 30$) and farmed ($n = 30$) turbot of different geographical origins (Denmark, The Netherlands, and Spain) were sampled from March 2006 to February 2007. The application of linear discriminant analysis (LDA) and soft independent modeling of class analogy (SIMCA) to analytical data demonstrated the combination of fatty acids and isotopic measurements to be a promising method to discriminate between wild and farmed fish and between wild fish of different geographical origin. In particular, IRMS (Isotope Ratio Mass Spectrometry) alone did not permit us to separate completely farmed from wild samples, resulting in some overlaps between Danish wild and Spanish farmed turbot. On the other hand, fatty acids alone differentiated between farmed and wild samples by 18:2n-6 but were not able to distinguish between the two groups of wild turbot. When applying LDA isotope ratios, 18:2n-6, 18:3n-3, and 20:4n-6 fatty acids were decisive to distinguish farmed from wild turbot of different geographical origin, while $\delta^{15}N$, 18:2n-6, and 20:1n-11 were chosen to classify wild samples from different fishing zones. In both cases, 18:2n-6 and $\delta^{15}N$ were determinant for classification purposes. We would like to emphasize that IRMS produces rapid results and could be the most promising technique to distinguish wild fish of different origin. Similarly, fatty acid composition could be used to easily distinguish farmed from wild samples.

KEYWORDS: Turbot (*Psetta maxima*); fatty acids; stable isotopes; authentication; multivariate data analyses

INTRODUCTION

Turbot (*Psetta maxima*) is a marine flatfish, naturally distributed in European waters from the Northeast Atlantic to the Arctic Circle including the Baltic, Mediterranean, and Black Seas. Wild and farmed turbot production yields about 6000 t annually. In the last years, a considerable proportion of the total production was derived from aquaculture (3800 and 900 t from Spain and France, respectively) principally along the Atlantic coast. This species has a high commercial value and is appreciated by consumers for its firm, white, and low-fat flesh.

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Turbot lives on sandy, muddy-sandy, and gravel bottoms, most commonly at depths between 1 and 15 m. Adults consume primarily benthic food, such as amphipods, mysid shrimps, and small fish (*1*).

The body of turbot is scaleless and studded with numerous isolated tubercles. Tubercles are small, with mineralized conical plates randomly distributed in the eyed side of the body. The blind side of the body is completely white, while the eyed side is from sandy-brown to gray with minute brown, blackish, or greenish specks. From a morphological point of view, only an accurate inspection done by a skillful operator could lead to distinguish between wild and farmed turbot. The blind side of farmed individuals occasionally presents hypermelanosis in the form of dark spots. Environmental, nutritional, and neurological factors seem to be possible causes for the abnormal pigmentation developed in farmed turbot (*2*). In the recent years, progress in

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hatchery-reared flatfish, such as the enhancement of larval nutrition, has minimized these defects in farmed turbot, which are consequently more similar to their wild counterparts.

International fish trade is nowadays strongly influenced by food authenticity and safety norms, and several European Directives have introduced aspects concerning quality and safety standards into the fish chain. Particularly, the labeling regulation for fishery and aquaculture products that came into force in the European Union in 2001 (*3*) requires the statement of the official commercial and scientific name, the geographical origin (FAO fishery zone for wild fish, country of production for farmed fish), and the production method of fish. This regulation was aimed to provide consumers with a minimum of information on the origin of these products.

On the basis of these considerations, a number of recent studies have been published to investigate the potential of different analytical tools in distinguishing wild and farmed fish. Classically, this was carried out using scale pattern analysis (*4*) and morphological characteristics or a combination of both methods (*5*). More recently, many analytical procedures have been applied for this purpose, ranging from the determination of carotenoid stereoisomers (*6*) to the use of fatty acid profile and compositional analysis (*7–10*), as well as to the quantification of different levels of organic contaminants (*11*), in conjunction with statistical multivariate analysis (*12*).

Wild and farmed fish differ in nutritional (*13*), sensory, chemical, and physical properties, (*14*) and diet is one of the main factors that influence these properties (*15*). Additional factors such as the nature and availability of the food web, catching area, and production technologies are believed to be important contributors to these variations.

The effects of different lipid sources in the diet on growth and tissue fatty acid composition have been investigated in different cultured and wild species. In all cases, farmed fish were found to have much higher lipid content than their wild counterparts, and the fatty acid profile of farmed fish reflected the fatty acid composition of the diet. As a general rule, cultured fish was characterized by higher levels of n-6 fatty acids (especially 18:2n-6), while wild fish showed higher levels of n-3 fatty acids and a higher n-3:n-6 ratio (*16*). Reasonably, the high presence of 18:2n-6 in cultured fish might be explained by the abundant presence of this fatty acid in plant oils used to partially substitute fish oil in fish feed formulation.

Isotope ratio mass spectrometry (IRMS) has been demonstrated to be a technique capable of revealing the origin of fish (*17*). Over the past few years, the determination of stable isotope ratios of light elements, especially carbon and nitrogen, has been applied to authenticity control and origin assessment of food of animal origin (*18*).

The isotope ratio of animals is primarily determined by diet and, to some extent, reflects their origin (*19, 20*). The carbon isotopic composition of a terrestrial or aquatic organism reflects the isotopic composition of its diet, but the organism is usually enriched in δ^{13}C by about 1‰, relative to its diet. Thus, carbon isotope ratio remains relatively unaffected by trophic transfer. Similarly, the isotopic composition of nitrogen reflects the isotopic composition of the diet, but with an enrichment much higher than for carbon. Therefore, the consistency of nitrogen enrichment (3–5‰) at each trophic transfer provides a valuable measure of the position of an organism within the food web (*21*).

Stable isotope analysis has been used as a tool to control the traceability of foodstuff providing good results also for geographical approaches (*22*). In recent studies, it has been also applied to study the dietary habits of aquatic animals and relative food webs (*23, 24*) and the seasonal variation of the isotopic composition in fish tissues (*25*), to distinguish anadromous and nonanadromous populations of *Salmo trutta* (*26*), to establish the rearing location of juvenile salmon (*27*), and to investigate the change in isotopic composition during spawning (*28*). Further and more recently, IRMS has been applied to distinguish wild and farmed gilthead sea bream (*29, 30*).

The aim of the present work was to study the potential application of fatty acid analysis and IRMS of carbon and nitrogen in the muscle tissue of wild and farmed turbot to discriminate the production method and the geographical origin of fish. Both linear discriminant analysis (LDA) and soft independent modeling of class analogy (SIMCA) were applied to the data as classification tools. On the basis of analytical results, a reasonable partition of groups was studied and discussed.

MATERIALS AND METHODS

Fish. Wild and farmed turbot from different origins were sampled in March, June, October 2006, and February 2007 (15 fish per month, for a total of 60 fish). Thirty farmed turbot (FT) were from Spain (average weight 666.5 g) and were collected at the wholesale fish market of Milan ($n = 24$) and by local retailers ($n = 6$). Fifteen wild turbot were from Denmark (WT1) (average weight 578.3 g) and were collected from the wholesale fish market of Milan ($n = 9$) and by local retailers ($n = 6$). Fifteen wild turbot were from The Netherlands (WT2) (average weight 637.9 g) and were collected from the wholesale fish market of Milan ($n = 13$) and by local retailers ($n = 2$). Danish and Dutch wild turbot were caught in the NorthEast Atlantic Ocean, FAO fishery zone No 27. Farmed turbot were from farms located in Galicia waters.

Upon arrival in the laboratory, fish were accurately weighed and measured, and Fulton's K condition index (*31*) was calculated using the formula K = weight/length (*3*). This morphometric index assumes that heavier fish for the same length are in better nutritional condition. All fish were then filleted by hand,and fillets were skinned. One aliquot of approximately 50 g of each fillet was grounded and freeze-dried to eliminate the water from the tissues. This method has been recognized as the most reliable method for avoiding shifts in δ^{13}C and δ^{15}N isotopic signatures during storage time (*32*). The freeze-dried tissues were used for isotopic analyses. The remaining fillet samples were vacuum packed and stored at –20 °C until analysis.

Proximate Composition and Fatty Acid Analysis. All assays for proximate composition analysis were performed using standard methods (*33*). The moisture content of fillets was determined by drying samples in an oven at 60 °C to constant weight. Total protein was determined by the Kjeldahl method, by which the concentration of nitrogen is measured. A factor of 6.25 was used to convert total nitrogen to crude protein. For the analysis, an automated distillation unit (Büchi 339, Switzerland) was used. The lipid content was determined by extraction with diethyl ether/petroleum benzene (1/1, v/v) in a Soxhlet system extractor (SER 148, Italy). Ash was determined by incineration of sample in a muffle furnace at 550 °C for 18 h. All analyses were done in duplicate.

For fatty acid analysis, the extraction of total lipids was performed according to Bligh and Dyer (*34*). The preparation of fatty acid methyl esters (FAMEs) was performed according to Christie (*35*). Briefly, the lipid sample (20 mg) was dissolved in 10% methanolic hydrogen chloride (2 mL). A 0.1 mL solution of tricosanoic acid (10 mg/mL) was added as internal standard. The sample was sealed and heated at 50 °C overnight and then 2 mL of a 1 M potassium carbonate solution was added to each sample. The FAMEs were extracted with 2 × 2 mL of hexane and 1 μL was injected into the gas chromatograph, in split mode (split ratio 1:50). Fatty acid analysis was performed on an Agilent gas chromatograph (model 6890), equipped with an automatic sampler (model 7683) and a flame ionization detector (FID). The carrier gas was helium with a flow at 1.0 mL/min and an inlet pressure of 16.9 psi. The column was an HP-Innowax fused silica capillary column (30

Table 1. Country of Origin and Biometric Measurements on Wild and Farmed Turbot Used in This Study[a]

fish group	no. of fish	country of origin	eviscerated weight (g ± SEM)	minimum weight	maximum weight	length (cm)	Fulton's K condition index[e]
WT1[b]	15	Denmark	578.3 ± 38.1	395.0	883.2	32.2 ± 0.6	1.70[a] ± 0.16
WT2[c]	15	The Netherlands	637.9 ± 17.5	527.3	763.3	32.6 ± 0.4	1.85[b] ± 0.19
FT[d]	30	Spain	666.5 ± 15.9	560.9	923.6	32.2 ± 0.4	1.99[c] ± 0.21

[a] Data are reported as mean values ± standard error of the mean (SEM). Means within columns with different superscripts are significantly ($P < 0.05$) different by one-way ANOVA and S−N−K comparison test. [b] Wild turbot from Denmark. [c] Wild turbot from The Netherlands. [d] Farmed turbot from Spain. [e] K = Weight/Length^3.

m × 0.25 mm i.d., film thickness 0.25 μm) (Agilent Technologies). The oven temperature program was from 100 to 180 at 3 °C/min, then from 180 to 250 at 2.5 °C/min, then held for 10 min. Fatty acids were identified using external standards and quantified using tricosanoic acid as internal standard. Peak areas were corrected according to the theoretical relative FID response correction factors (TRFs) published by ref *36*. The results are presented as g/100 g of fatty acids (% by weight). All analyses were done in duplicate.

Isotope Measurements. δ^{13}C (signal for reference peaks = 4000 mV) and δ^{15}N (signal for reference peaks = 4000 mV) values were measured by continuous flow isotope ratio mass spectrometry (CF-EA-IRMS) using an Elemental Analyzer EA 1108 CHN (Carlo Erba, Milan, Italy; oxidation column temperature, 1050 °C; reduction column temperature, 650 °C; and GC-column, 65 °C) coupled to a DeltaPlus mass spectrometer (ThermoFischer, Rodano, Italy). Since fish muscle has a C:N ratio less than 5:1, the CF-EA-IRMS system was operated in the dual isotope mode, allowing δ^{15}N and δ^{13}C to be measured on the same sample. Amounts of 0.60–0.75 mg of sample were weighed into tin capsules for measurements.

Standards and Equations. The results of carbon (δ^{13}C) and nitrogen (δ^{15}N) isotope ratio analyses are reported in per mil (‰) on the relative δ-scale and referred to the international standards V-PDB (Vienna Pee Dee Belemnite) for carbon isotope ratio and Atmospheric Air, for nitrogen isotope ratio. All results were calculated according to the following equation

$$\text{Delta (‰)} = [(R_{\text{sample}}/R_{\text{reference}}) - 1] \times 1000$$

where R is the ratio of the heavy to light stable isotope (i.e., ^{13}C/^{12}C) in the sample (R_{sample}) and in the standard ($R_{\text{reference}}$).

The calibration of the control gases (CO_2, N_2) was performed using the following reference materials:

IAEA−CH7−Polyethylene (δ^{13}C = –32.15 ‰) and IAEA−CH6−sucrose (δ^{13}C = –10.4 ‰) for CO_2 gas cylinder calibration (used for δ^{13}C measurements).

IAEA−N1−Ammonium sulfate (δ^{15}N = 0.4 ‰) and IAEA−N2−ammonium sulfate (δ^{15}N = 20.3 ‰) for N_2 gas cylinder calibration (used for δ^{15}N measurements).

The precision (standard deviation) for analysis of laboratory standard (urea) for δ^{13}C is ± 0.2 ‰ (n = 10) and for δ^{15}N is ± 0.15 ‰ (n = 10).

The standard deviations of the measurements (n = 10) determined using the respective reference gas were ± 0.05 ‰ for δ^{13}C and ± 0.08 ‰ for δ^{15}N. Each sample was analyzed in triplicate, and the standard deviations of these analyses were <0.18 ‰ for δ^{13}C and <0.23 ‰ for δ^{15}N.

International standard, USGS-40 (δ^{13}C = –26.24 ‰ and $\delta^{15}N$ = –4.52 ‰), was used as the reference material and was analyzed, at intervals in each run, to monitor possible instrumental drift.

One muscle sample of turbot was calibrated with the international reference materials mentioned before and used as a working standard. The working standard was analyzed at regular intervals to control the repeatability of the measurements and to correct possible deviations in the measurements.

Statistical Analysis. First, basic statistics, both ANOVA and Principal Component Analysis (PCA), were performed. The homogeneity of variance was confirmed, and a comparison between means was performed by one-way ANOVA. Student–Newman–Keuls was used as the post hoc test for comparison of the means among different groups of fish. Significance was accepted at probabilities of 0.05 or less. PCA was performed to study the structure of the data and to detect the most important variables to be submitted to the following Linear Discriminant Analysis (LDA) and soft independent modeling of class analogy (SIMCA).

The supervised LDA was applied to classify samples in clusters according to their characteristics and to identify variables able to distinguish the origin groups. LDA is a statistical method used to distinguish in groups a collection of objects, having a set of cases whose group membership is known a priori (*37*). The variables were first transformed into natural logarithms, in such a way to have a normal data distribution. In the LDA, the algorithm chosen to select the variables was stepwise selection, which combines forward selection and backward elimination using the minimization of Wilks' lambda. Wilks' lambda is a measure of a variable's potential, and smaller values indicate which variable is better at discriminating between groups. The F significance level was chosen as the variable entry (less than 0.05) and removal (greater than 0.10) criterion. The number of functions obtained by LDA is equal to the number of groups minus one. So, the discriminant equations, which are a linear combination of the independent variables selected by the stepwise method, are expressed as

$$Dj = B0j + BijXi + \cdots + BnjXn$$

where Dj is the discriminant score (j = 1, . . . ,m-1, where m is the number of groups), B0j is a constant term, and Bij and Xi (i = 1,. . .,n) are the coefficients estimated from the data and the values of each independent variable chosen by stepwise LDA, respectively.

A leaving-one-out cross validation procedure was performed to assess the accuracy of the classification rule. In this procedure, the sample data minus one observation were used for calculating discriminant functions, then the omitted observation was classified from them. This procedure was repeated a number of times equal to the number of samples (n = 60). Consequently, each sample was classified from discriminant functions which were estimated without its contribution.

SIMCA classification is based on constructing separately a PC model for each data class. Every considered sample is then assigned to one class according to its distance from the class model. Farmed and wild turbot purchased at the fish market of Milan (n = 46) were used as the training set, while samples purchased by local retailers (n = 14) were used as the testing set, to evaluate the discrimination power of the SIMCA model.

All the statistical analyses were performed by SPSS version 15.0 (SPSS Inc., Chicago, Illinois) and The Unscrambler version 9.7 (Camo, Norway). Data in the tables are reported as mean values ± the standard error of the mean (SEM).

RESULTS AND DISCUSSION

Biometric Measurements and Proximate Composition. As presented in **Table 1**, farmed turbot were lightly heavier than wild turbot. The Fulton's K condition index in wild turbot was lower than in farmed counterparts ($p < 0.05$). These findings are rather frequent when comparing wild and farmed fish.

The proximate composition of fillets of wild and farmed turbot is presented in **Table 2**. The lipid content of farmed turbot was higher when compared to wild turbot. These results are in agreement with those obtained by ref *38*. Higher lipid content in cultured species is influenced by different factors, including type of feed, dietary ingredients, and higher energy consumption. Usually commercial feed for turbot contains fish meal and marine oil, wheat and wheat gluten, and vegetable oils (*39*). Its

Table 2. Proximate Composition of Muscle of Wild and Farmed Turbot[a]

	WT1[b] ($n = 15$)	WT2[c] ($n = 15$)	FT[d] ($n = 30$)
moisture	79.25[c] ± 0.43	78.40[b] ± 0.25	77.42[a] ± 0.18
protein	19.16[a] ± 0.37	19.35[a] ± 0.23	20.16[b] ± 0.17
lipid	0.60[a] ± 0.10	1.12[b] ± 0.20	1.36[b] ± 0.10
ash	0.99[a] ± 0.04	1.14[b] ± 0.04	1.07[ab] ± 0.03

[a] Data are reported as mean values ± standard error of the mean (SEM). Means within rows with different superscripts are significantly ($P < 0.05$) different by one-way ANOVA and S−N−K comparison test. [b] Wild turbot from Denmark. [c] Wild turbot from The Netherlands. [d] Farmed turbot from Spain.

proximate composition is 14–16% of lipid and 53–55% of crude protein. The use of this type of diet produces an increase of lipid level in farmed fish when compared to wild fish. Lipid-rich diets have been demonstrated to increase both visceral and fillet fat content in turbot (*40*).

Similarly to lipid, the protein content of farmed turbot was significantly higher when compared to wild turbot. Protein is considered to be a rather stable component of the fish body, depending on fish size and genetic factors. The weight of the individual fish used in our experiment was rather homogeneous (**Table 1**), thus the differences in protein content were presumably due to the age difference between farmed (younger) and wild (older) turbot or to unknown genetic factors.

The moisture level was higher in wild than in cultured turbot, and indeed it is generally recognized that an inverse relationship exists between water and lipid content in fish (*41*).

Fatty Acid Composition. As a general rule, depending on the species, 18:2n-6 and 18:3n-3 are essential fatty acids for fish and are required for the production of C_{20} and C_{22} polyunsatured fatty acids, as precursors of biologically active eicosanoids. In most marine species, including turbot, also 20:5n-3 and 22:6n-3 are required for survival and growth (*42*). Commercial feed used for farmed turbot contain fish meal and marine oil, wheat and wheat gluten, and vegetable oils. Among these ingredients, fish oils are characterized by a high content of EPA and DHA and by the presence of 22:1n-11 (cetoleic acid) and 20:1n-9 (gadoleic acid) fatty acids. These last fatty acids derive from the corresponding fatty alcohols in the wax esters of the zooplankton (*43*) and are present particularly in the triacylglycerols of pelagic fish caught in the North Sea and used for fish meal production.

Vegetable oils (soybean, corn, linseed, and rapeseed oils) are generally used in fish feed formulations for partial substitution of fish oil, as alternative and economically sustainable feed ingredients (*44*). These oils, in particular soybean oil, are characterized by a high proportion of n-6 polyunsaturated fatty acids, especially linoleic acid (18:2n-6).

The fatty acid composition of total lipids of muscle of wild and farmed turbot of this study is presented in **Table 3**. Theoretically and as shown in data from other species (*8, 45*), the fatty acid composition of muscle should reflect and depend on the fatty acid composition of the diet.

Among saturated fatty acids (SFA), farmed turbot showed a lower percentage of 16:0 and 18:0 and a higher percentage of 14:0 than their wild counterparts. The sum of SFA did not present statistically significant differences between groups. The saturated fatty acids 16:0 and 18:0 can be biosynthesized in fish (*46*) and can be desaturated by a Δ^9 desaturase to 16:1n-7 and 18:1n-9, respectively. These activities are presumably regulated by water temperature and by the presence of essential fatty acids in the diet. In marine wild fish, the final metabolic pathway is not well studied, but it is possible that this enzyme is involved in enhancing monounsaturated fatty acid production

Table 3. Fatty Acid Composition of Muscle of Wild and Farmed Turbot (G/100 G of Fatty Acids)[a]

	WT1[b] ($n = 15$)	WT2[c] ($n = 15$)	FT[d] ($n = 30$)
14:0	2.22[a] ± 0.16	2.51[a] ± 0.23	3.84[b] ± 0.16
16:0	18.88[b] ± 0.22	19.19[b] ± 0.38	17.47[a] ± 0.31
18:0	6.70[b] ± 0.37	6.37[b] ± 0.52	5.25[a] ± 0.26
Σ SFA	27.79 ± 0.44	28.07 ± 0.67	26.57 ± 0.42
16:1n-7	4.28 ± 0.34	4.84 ± 0.49	4.72 ± 0.21
18:1n-7	3.91 ± 0.36	3.41 ± 0.16	3.35 ± 0.26
18:1n-9	11.95 ± 0.34	12.23 ± 0.66	11.67 ± 0.24
20:1n-9	1.02[a] ± 0.11	1.07[a] ± 0.11	1.59[b] ± 0.12
20:1n-11	0.34[ab] ± 0.05	0.42[b] ± 0.07	0.25[a] ± 0.02
22:1n-9	0.21 ± 0.02	0.26 ± 0.04	0.26 ± 0.02
22:1n-11	0.28[a] ± 0.16	0.47[a] ± 0.11	1.19[b] ± 0.14
Σ MUFA	21.98 ± 1.01	22.70 ± 1.43	23.05 ± 0.71
18:2n-6	1.59[a] ± 0.12	0.97[a] ± 0.08	8.96[b] ± 0.37
18:3n-6	0.17 ± 0.02	0.15 ± 0.02	0.20 ± 0.01
20:2n-6	0.47[b] ± 0.02	0.34[a] ± 0.03	0.64[c] ± 0.02
20:3n-6	0.15 ± 0.02	0.14 ± 0.01	0.17 ± 0.00
20:4n-6	4.34[c] ± 0.35	2.99[b] ± 0.24	1.81[a] ± 0.08
Σ n-6	6.70[b] ± 0.32	4.59[a] ± 0.24	11.79[c] ± 0.37
18:3n-3	0.77[b] ± 0.13	0.48[a] ± 0.06	1.09[c] ± 0.05
18:4n-3	0.67[a] ± 0.12	0.76[a] ± 0.14	1.31[b] ± 0.08
20:5n-3	9.74[a] ± 0.32	10.12[a] ± 0.31	11.14[b] ± 0.22
22:5n-3	5.85[b] ± 0.35	6.66[b] ± 0.56	4.22[a] ± 0.08
22:6n-3	26.83[b] ± 0.81	26.61[b] ± 1.20	21.28[a] ± 0.64
Σ n-3	43.85[b] ± 0.79	44.64[b] ± 0.94	39.04[a] ± 0.54
Σ PUFA	50.56 ± 0.83	49.23 ± 0.96	50.83 ± 0.46
Σ HUFA	47.42[b] ± 0.84	47.15[b] ± 1.04	39.76[a] ± 0.60
n-3/n-6	6.80[b] ± 0.38	10.21[c] ± 0.64	3.44[a] ± 0.13

[a] Data are reported as mean values ± standard error of the mean (SEM). Means within rows with different superscripts are significantly ($P < 0.05$) different by one-way ANOVA and S−N−K comparison test. [b] Wild turbot from Denmark. [c] Wild turbot from The Netherlands. [d] Farmed turbot from Spain.

in response to a lowered water temperature so as to maintain membrane fluidity, as demonstrated in carp (*47*).

Monounsaturated fatty acids (MUFA), in particular 20:1n-9 and 20:1n-11, were higher in farmed than in wild animals. These are typical fatty acids of fish oils contained in feed and are transferred to the muscle of farmed fish. These results are consistent with those published by Sérot (*46*), who compared the fatty acid composition of muscle from farmed and wild turbot.

All polyunsaturated fatty acids (PUFA) of the n-6 series, except of 20:4n-6, were higher in farmed fish than in other groups. Interestingly, 18:2n-6 was much higher in farmed fish (8.96% vs 0.82%) than in wild fish, while the end product of its desaturation and elongation (20:4n-6) was significantly lower in farmed fish. Also, the intermediates of 20:4n-6 synthesis, 18:3n-6 and 20:3n-6, were at very low levels in farmed groups. Other studies have shown higher percentages of 20:4n-6 in wild fish when compared to its farmed counterpart (*10, 45, 46*).

This fact could be explained with the reduced capacity of turbot to synthesize highly unsaturated fatty acids (HUFA) from C_{18} precursors (*42*). Tocher et al. (*48*) demonstrated that cultured cells from turbot were unable to elongate C_{18} into C_{20}. Furthermore, desaturases are known to have a better affinity for n-3 fatty acids than for n-6 fatty acids in turbot (*49*). Reasonably the bioconversion of 18:2n-6 into 20:4n-6 is more effective when the diet is deficient in n-3 PUFA (*50*).

Concentrations of 18:3n-3, 18:4n-3, and 20:5n-3 were higher in farmed fish than in wild fish. In contrast, 22:5n-3 and 22:6n-3 were significantly higher in wild than in farmed turbot. It could be possible that the commercial feed contained a high percentage of EPA, which resulted in a higher amount of these fatty acids in farmed turbot. When comparing the fatty acid composition within wild turbot, the WT1 group showed a higher

Table 4. Stable Isotope Ratios of Carbon ($\delta^{13}C$) and Nitrogen $\delta^{15}N$ in Wild and Farmed Turbot[a]

	$\delta^{13}C$				$\delta^{15}N$			
group	mean	min.	max.	SEM	mean	min.	max.	SEM
FT[b] ($n = 30$)	−19.27[a]	−20.01	−18.75	0.06	13.82[a]	12.18	15.04	0.13
WT1[c] ($n = 15$)	−18.98[a]	−21.58	−15.88	0.41	14.48[b]	12.66	17.74	0.34
WT2[d] ($n = 15$)	−17.33[b]	−19.56	−16.50	0.21	16.81[c]	14.14	17.87	0.24
total WT[e]	−18.15	−21.58	−15.88	0.28	15.64	12.66	17.87	0.30

[a] Means within columns with different letters are significantly ($P < 0.05$) different by one-way ANOVA and S−N−K comparison test. [b] Farmed turbot from Spain. [c] Wild turbot from Denmark. [d] Wild turbot from The Netherlands. [e] Total wild turbot.

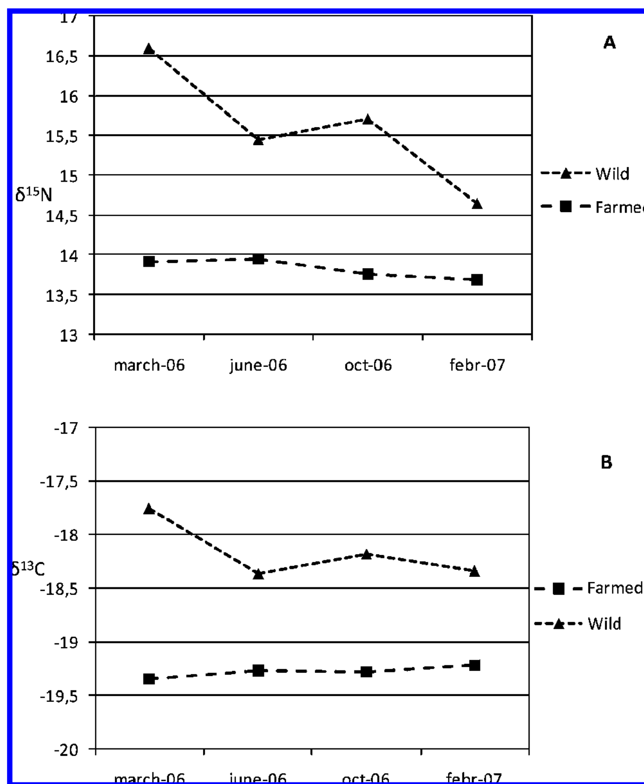

Figure 1. Monthly variation of $\delta^{15}N$ **(A)** and $\delta^{13}C$ **(B)** values in wild (WT1 and WT2) and farmed (FT) turbot.

amount of 20:4n-6 than the WT2 group.

Isotope Measurements. The results of isotopic analysis of the three groups of wild and farmed turbot are presented in **Table 4**. Carbon isotopic ratios were statistically different between farmed and wild turbot ($p < 0.01$). When considering separately Danish and Dutch samples, $\delta^{13}C$ of farmed turbot were statistically different from wild Danish but not from wild Dutch fish, whereas nitrogen isotopic ratios had a high variability between groups, showing a significant increment from farmed to wild Danish turbot ($p < 0.01$). In **Figure 1**, the isotopic measurements of wild and farmed turbot grouped according to the month of sampling are presented. The isotopic composition of the farmed samples remains relatively constant during the feeding period, due to the application of the same process of production (diet composition and dose). On the other hand, small differences can be observed on the carbon and nitrogen isotopic ratios for wild samples, probably arising from different availability of food during the year and other additional parameters (metabolism). During the periods of scarcity of food, the fish uses the reserves accumulated in its body. Consequently, more positive values in the case of nitrogen and less negative values in the case of carbon are observed. An additional factor that can influence in the same way is the reproductive period of the turbot (spring time) during which the fish does not eat any food for a long time (**Figures 1** and **2**, significant difference, point march-06). On the other hand, for the farmed turbot, the type and the quantity of feed received is the same during all the feeding periods, and in addition, the farmed turbot does not reproduce during that period.

As evidenced in **Figure 2**, isotopes permitted a clear discrimination between Spanish farmed turbot and Dutch wild turbot. There are two homogeneous groups with differences in $\delta^{13}C$ and $\delta^{15}N$ ratios. These differences between farmed and wild fish can be related to the diet of the two groups of fish. Muscle from farmed fish has more negative values of $\delta^{13}C$ than from wild ones, due to a diet less variable and richer in fat. The high-fat diet of farmed fish produces tissues with a higher lipid content, inducing a larger isotopic fractionation of ^{13}C than the one found for wild fish, for which the scarcity of food induces a higher metabolic turnover, resulting in less accumulation of fat in the tissues. For this reason, farmed fish should have lighter $\delta^{13}C$ ratios (values more negatives) than wild ones.

The differences in $\delta^{15}N$ values are not as well defined because the values for nitrogen isotope ratios for both groups depend on protein content and mostly on the origin and type of protein of the diets of both types of fish. The natural diet of wild turbot consists mainly of benthic food, such as amphipods, mysid shrimps, and small fish, and its composition could vary depending on the availability of preys and on the geographical origin of fishing areas. Whereas for farmed fish, the diet formulation can change depending on the percentage or the origin of the protein added to the diet (animal or vegetable sources). For this reason, the variation in $\delta^{15}N$ values in our case may be due to the differences between "natural diet" and feed administered.

On the other hand, the values obtained for Danish wild turbot are widely spread in **Figure 2**. The reasons for this behavior are not simple to address. One of the things that would affect the isotopic results obtained in this way could be the different geographical origin of the samples and the different sources and availability of food in places well separated geographically. The fact that the fishery in FAO fishery zone 27 can be done in such different places like Bornholm Island (southeast, between Sweden and Poland), the Faroe Islands (northwest, between Scotland and Iceland), and Greenland (American continent) would support the previously mentioned hypothesis, and a more extensive sampling in all these specific areas should be done to perform an isotopic map of the samples and to better explain the differences given by our results.

Principal Component Analysis. PCA was used to provide an overview of the capacity of the variables (chromatographic and isotopic measurements) to discriminate wild from farmed samples and to find the discriminating power of the variables. After applying PCA to our data set, three PCs were extracted. The percentage of variance explained by each PC was 36.9, 18.2, and 9.8%, respectively. According to the loading of the

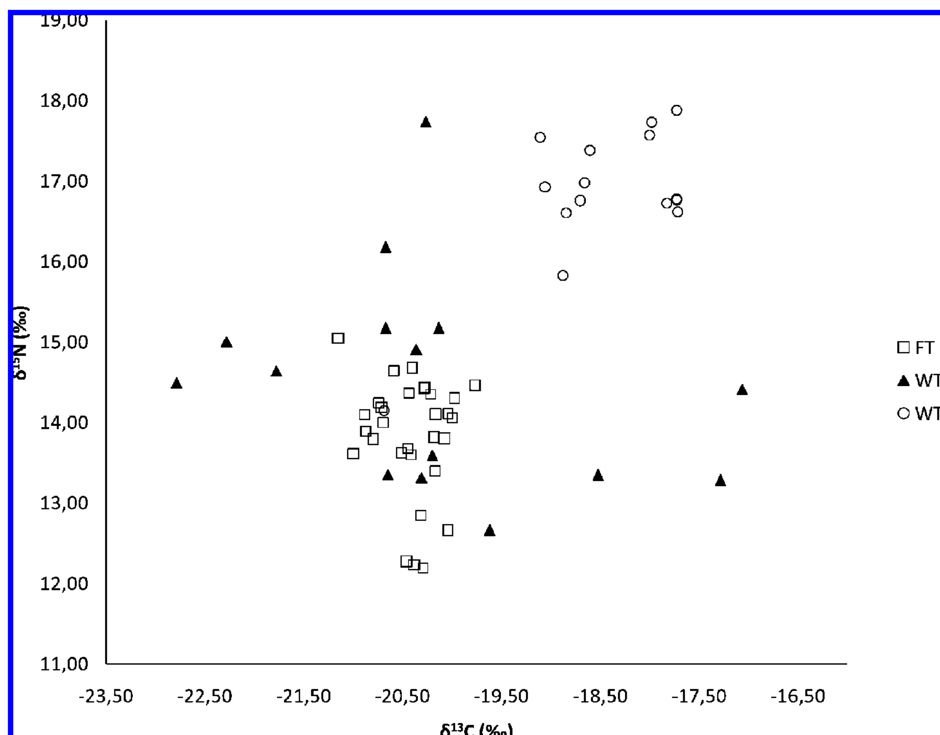

Figure 2. ^{15}N vs ^{13}C values of muscle of wild (WT1 and WT2) and farmed (FT) turbot.

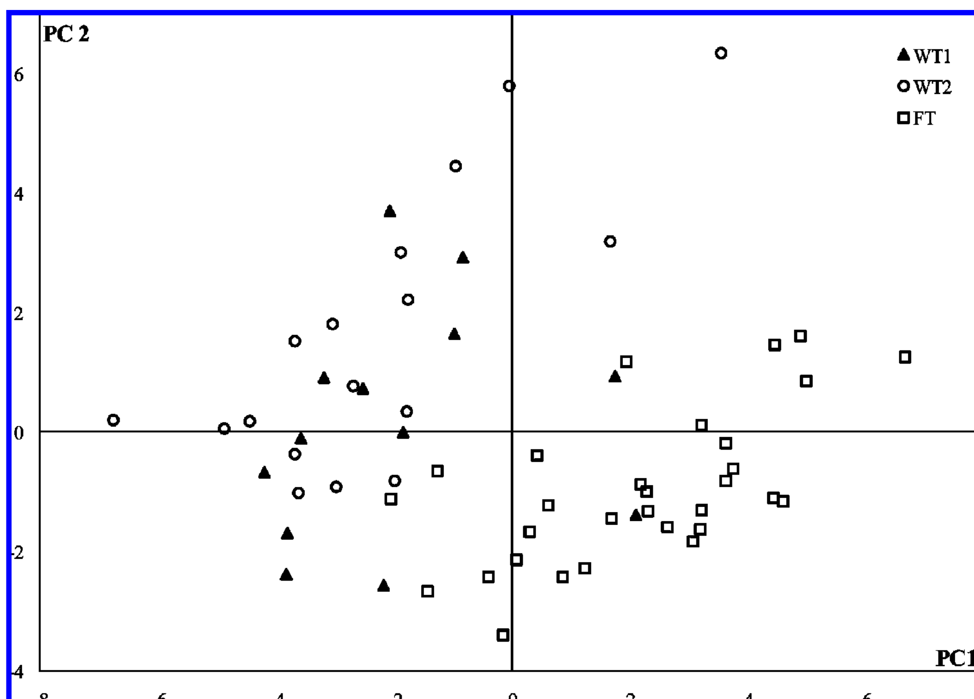

Figure 3. Principal component analysis: score plot of the turbot samples in the bidimensional space of the first two PCs.

variables in the first PC, the most contributing descriptors were 14:0, 16:0, 18:4n-3, 22:6n-3, and 20:4n-6. Furthermore, the correlation loadings showed strong correlation between 16:0, 18:0, 20:4n-6, 22:6n-3, and between 14:0 and 18:4-n3. When representing the scores of the turbot samples on the two-dimensional space defined by the calculated PCs (**Figure 3**), farmed samples appeared well distinguished from wild samples. Wild samples coming from Denmark do not seem to separate from those coming from the Netherlands.

Linear Discriminant Analysis. First, LDA was used to classify the two groups of turbot (farmed and wild) without considering the different origins of wild samples. After applying LDA, one discriminant function was obtained. **Table 5** shows independent variables selected and the calculated discriminate factors. Interestingly, the variables selected by stepwise statistics demonstrated that isotope ratios and n-6 fatty acids gave the most important contribution to discriminate between wild and farmed fish. A complete separation of the two groups was achieved. The recognition ability was 100% for each class. The leaving-one-out cross validation procedure used to evaluate the classification performance confirmed a prediction ability of 100% for the two classes.

Table 5. First Factor (LDA1) Coefficient, The Wilks' Lambda, and the Percentage of Correctly Classified Samples of Linear Discriminant Analysis Applied on Fatty Acid and Isotopic Measurements of Turbot Grouped According to the Production Method (Farmed Turbot vs Wild Turbot)

variable	function coefficients
δ^{15}N	0.446
δ^{13}C	−0.482
18:2n-6	−4.287
18:3n-3	1.977
20:4n-6	2.316
constant	−12.075
Willks' lamba	0.046
original samples correctly classified	100%
cross validated samples correctly classified	100%

Table 6. First and Second Factor (LDA1 and LDA2) Coefficients, The Explained Percentage of Variance, The Wilks' Lambda, and the Percentage of Correctly Classified Samples of Linear Discriminant Analysis Applied on Fatty Acid and Isotopic Measurements of Wild and Farmed Turbot Grouped According to the Geographical Origin (Denmark vs the Netherlands vs Spain)

variable	function 1	function 2
δ^{15}N	−0.700	−0.373
δ^{13}C	−0.346	−0.501
18:1n-9	−3.962	0.059
18:2n-6	4.704	−0.200
18:3n-3	−0.905	1.543
20:1n-11	1.131	0.804
20:4n-6	−1.653	3.119
constant	23.761	−5.074
explained of variance (%)	95.3	4.7
Willks' lamba	0.013	0.401
original samples correctly classified	96.7%	
cross validated samples correctly classified	95.0%	

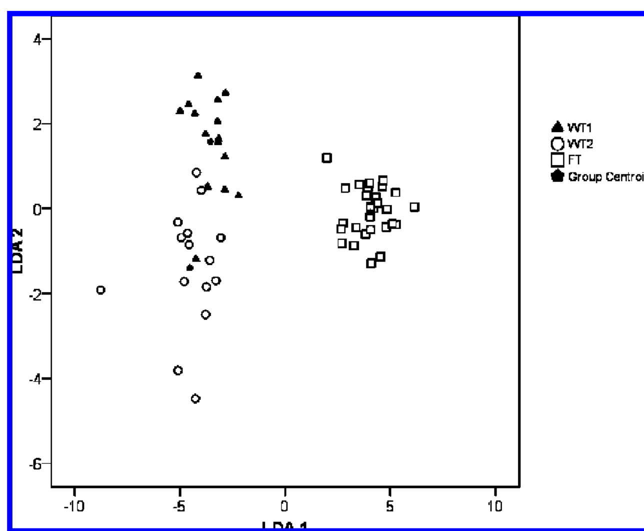

Figure 4. Canonical discriminant functions of wild turbot (WT1 and WT2) and farmed turbot (FT).

When applying LDA to distinguish farmed turbot from wild turbot of different geographical origin (three classes), two discriminant functions were obtained (**Table 6**). Also, in this case, isotope ratios, 18:2n-6 and 20:4n-6 were selected.

One Danish wild sample was classified as Dutch, and two Dutch wild samples were classified as Danish. All farmed Spanish samples were classified correctly. The recognition ability for all groups was 96.7%, while the leave-one-out cross validation method showed a prediction ability of the functions of 95.0%.

Table 7. First Factor (LDA1) Coefficient, the Wilks' Lambda and the Percentage of Correctly Classified Samples of Linear Discriminant Analysis Applied on Fatty Acid and Isotopic Measurements of Wild Turbot Grouped According to the Country of Origin (Denmark vs the Netherlands)

LDA1	function coefficients
δ^{15}N	−0.851
18:2n-6	1.969
20:1n-11	0.910
constant	14.077
Willks' lamba	0.319
original samples correctly classified	93.3%
cross validated samples correctly classified	93.3%

Table 8. SIMCA Sensitivity and Specificity

SIMCA	sensitivity	specificity
training set (46)	100	100
testing set (14)	87.5	100

Figure 4 shows the plot given by the first and the second discriminant functions accounting for 95.3% and 4.7% of variance of the total between-groups variability, respectively. The discrimination between farmed turbot and wild turbot was clearly displayed along the first linear discriminant function. The second function led, to a minor extent, to the separation of WT1 from WT2 wild samples.

When applying LDA to classify wild samples from different catching zones, the classification results were 93.3% for the two classes, and the leave-one-out cross validation method showed a prediction ability of the functions of 93.3%. Among 30 samples analyzed, only two samples were incorrectly classified. The discriminate function obtained is presented in **Table 7**. The independent variables selected were δ^{15}N, 18:2n-6, and 20:1n-11. These could be considered the most discriminate variables in distinguishing the two groups of wild turbot.

Soft Independent Modeling of Class Analogy (SIMCA). To apply SIMCA procedure to the samples, the data set was split into training (46 samples: 22 wild and 24 farmed) and testing (14 samples: 8 wild and 6 farmed) sets. The training set consisted of samples purchased at the wholesale fish market of Milan, while the testing set consisted of samples collected from the local retailers.

The separation of the studied groups can be easily shown by using the Cooman plot, as can be seen in **Figure 5**. The Cooman plot shows the samples to model distances both for training and for testing samples. If a sample truly belongs to a class, it should fall within the membership limit, that is, to the left of the vertical line and below the horizontal line. The sensitivity and the specificity of the SIMCA model were calculated (**Table 8**). The sensitivity represents the percentage of samples belonging to a class that are correctly classified by the class model, while specificity is the percentage of samples not belonging to a class that are correctly rejected by the class model. As shown in **Figure 5**, only one wild sample was incorrectly classified.

This study showed that fatty acid composition and isotopic analysis of carbon and nitrogen allowed, if applied together, to discriminate between farmed turbot and wild turbot of different origin. IRMS alone did not permit us to separate completely farmed from wild samples. In fact, there were overlaps between samples, especially between Danish wild and Spanish farmed turbot. Interestingly, Dutch and Danish wild fish showed different behavior in isotopic composition both for carbon and for nitrogen isotopes, reasonably due to the different living environment. Furthermore, seasonal variation of isotopic com-

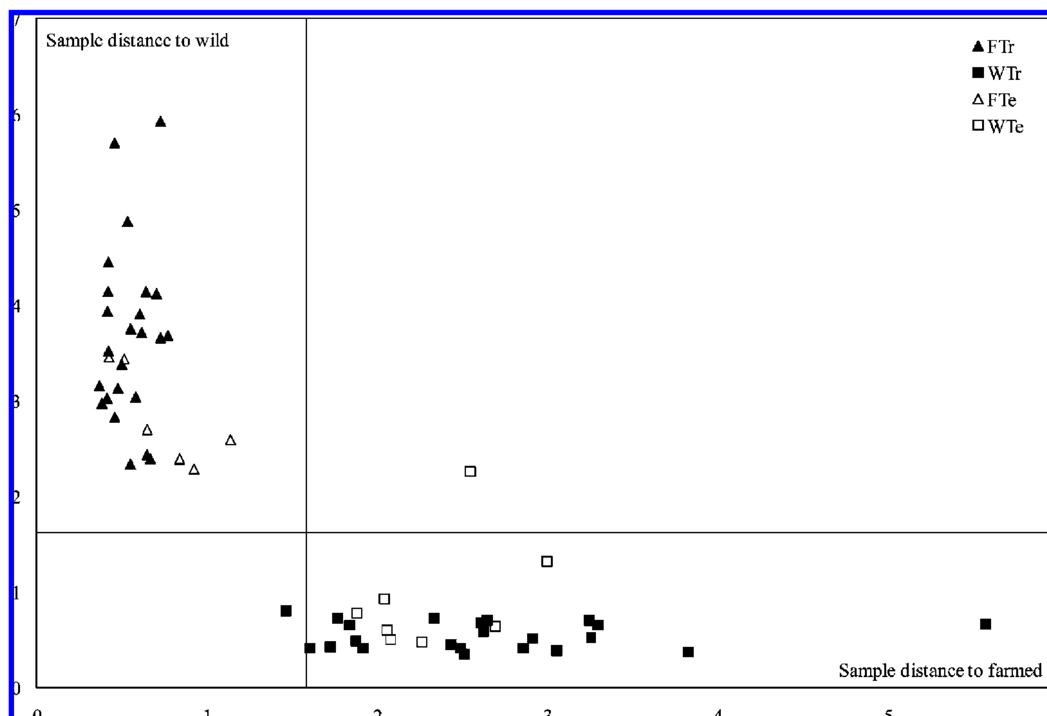

Figure 5. Cooman's plot of the SIMCA model calculated on wild and farmed samples. Letters F and W indicate farmed and wild samples, respectively. Letters Tr and Te indicate training and testing samples, respectively. Horizontal and vertical lines represent the critical distance ($p = 0.05$) of the sample from the wild and farmed models, respectively. As evidenced, one wild sample is not correctly classified.

position was found according to the month of sampling, reflecting different growth rates of fish and food consumed during these periods.

On the other hand, fatty acids alone differentiated between farmed and wild samples by 18:2n-6 but were not able to distinguish between the two groups of wild turbot. The application of linear discriminant analysis and soft independent modeling of class analogy to various combinations of analytical data demonstrated that the combination of fatty acids and isotopic measurements led to a promising method to discriminate between wild and farmed fish and between wild fish of different geographical origin. When applying LDA to distinguish farmed turbot from wild turbot of different geographical origin, isotope ratios, 18:2n-6, 18:3n-3, and 20:4n-6 fatty acids were decisive, while to classify wild samples from different zone of catching δ^{15}N, 18:2n-6 and 20:1n-11 were chosen. In both case, 18:2n-6 and δ^{15}N were decisive.

We would like to emphasize that IRMS produces rapid results and could be the most promising technique to distinguish wild fish of different origin. Similarly, fatty acid composition could be simply used to distinguish farmed from wild samples.

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