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Discrimination of Wild and Cultured European Sea Bass (*Dicentrarchus labrax*) Using Chemical and Isotopic Analyses

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Recent legislation in the European Union (EC/2065/2001) requires that seafood must provide the consumer with information that describes geographical origin and production method. The present studies aimed to establish methods, based on chemical and stable isotopic analysis, that could reliably differentiate between wild and farmed European sea bass (*Dicentrarchus labrax*). The study measured fatty acid and isotopic compositions (δ^{13}C and δ^{18}O) of total flesh oil, δ^{15}N of the glycerol/choline fraction, and compound-specific analysis of fatty acids (δ^{13}C) by isotope ratio mass spectrometry. The sample set comprised 10 wild and 10 farmed sea bass from England (wild) and Scotland or Greece (farmed). Discrimination was achieved using fatty acid composition with 18:0, 18:2n-6, 20:4n-6, and 22:6n-3 providing the highest contributions for discrimination. Principal component analysis of the data set provided good discrimination between farmed and wild sea bass where factor 1 and factor 2 accounted for 60% of the variation in the data.

KEYWORDS: Sea bass; product authentication; fatty acid compositions; isotope ratio mass spectrometry (IRMS); flesh oil δ^{13}C; flesh oil δ^{18}O; glycerol choline fraction δ^{15}N; principal component analysis

INTRODUCTION

The global demand for seafood is increasing in response to increasing global populations (*1*). However, the traditional capture fisheries that have historically provided the bulk of production in the past have reached, or exceeded, sustainable limits due to overfishing and/or habitat decline. Therefore, to offset the shortfall in supply aquaculture production has increased such that >30% of world seafood production is currently derived from aquaculture, and this is likely to increase in the future (*2*, *3*).

Due to the global nature of production, similar fish products can be sourced from variable points of origin, and this can lead to instances of mislabeling, both unintentional and fraudulent. To tighten the traceability in the production chain, in 2002, the European Commission introduced regulation 2065/2001 to ensure that more details on labeling, packaging, and origin of wild capture and aquaculture products would be accessible by retailers and consumers. This legislation was intended to provide additional and clearer information to retailers and consumers who are currently more aware of the food they eat and the consequences of different food production methods on nutritional quality and safety (*1*).

There is increasing public interest and awareness of the health benefits of consuming fish, in particular, but not exclusively, due to the high levels of n-3 highly unsaturated fatty acids (HUFA) present in fish flesh (*2*, *4*). The HUFA, especially eicosapentaenoic acid (20:5n-3; EPA), have shown clinical benefits in coronary heart disease and immunomodulated conditions with an inflammatory pathology (*5*−*7*). However, whereas the anti-inflammatory effects of EPA are well-known, the functional activities of docosahexaenoic acid (22:6n-3; DHA) are also vital for normal cellular function. DHA is essential for normal growth and development of neural tissue in infants and is also required to maintain optimal brain function in adults (*8*). The vital role of DHA is apparent by the fact that >20% of brain dry weight is DHA, and this is the most abundant fatty acid in neural tissues (*9*, *10*). In addition to brain function, suboptimal DHA has been linked to reduced visual acuity, attention deficit hyperactivity disorder, cystic fibrosis, and unipolar and bipolar depression and aggression (*8*, *11*).

In contrast, recent studies have suggested that farmed salmon may contain higher levels of persistent organic pollutants (POPs), such as dioxins and PCBs, compared to the wild product (*12*), although concentrations in all cases were well within permitted ranges (*13*). However, there is considerable evidence that the benefits of eating fish, particularly oily fish, significantly outweigh any perceived risks (*14*, *15*).

Over the past decade, the fishmeal and fish oil that have been the basis of aquafeed formulations for over 30 years have

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Table 1. Source and Average Weight of European Sea Bass

species	source	country of origin	av wt (g)
farmed sea bass	MMERL[a]	Scotland	270
farmed sea bass	Bernard Corrigan Ltd.[b]	Greece	522
wild sea bass	Bernard Corrigan Ltd.[b]	England	1690

[a] Machrihanish Marine Environmental Research Laboratory, University of Stirling. [b] Bernard Corrigan Ltd., Glasgow (fish wholesaler), www.bernardcorrigan.com.

reached limits of sustainable production (*16*), and new sustainable raw materials are being introduced in commercial aquafeeds. However, whereas the use of plant-derived raw materials can reduce concentrations of POPs in fish (*17*, *18*), the concentrations of beneficial EPA and DHA are reduced as the vegetable oils used do not contain these n-3 HUFA (*19*). Therefore, farmed fish, grown on diets containing lower levels of marine-derived raw materials, are likely to have different lipid compositions compared to wild fish (*20*−*22*).

The aim of the present study was to determine whether farmed and wild European sea bass (*Dicentrarchus labrax*) could be discriminated, in terms of their production origin, using a range of analytical measurements that have the potential to discriminate production source. Thus, flesh samples were analyzed for fatty acid compositions, δ^{13}C of individual fatty acids, δ^{13}C and δ^{18}O of total flesh oil, and δ^{15}N of the glycerol choline fraction of flesh phospholipids.

MATERIALS AND METHODS

Fish Samples. Authentic samples of farmed sea bass were obtained from the Machrihanish Marine Environmental Research Laboratory in Kintyre, Scotland, and from a wholesaler, Bernard Corrigan Ltd. of Glasgow, who sourced farmed sea bass from Greece. Wild sea bass from southern England were also supplied by Bernard Corrigan Ltd. Details of the fish analyzed in this survey are given in **Table 1**.

Lipid Extraction and Sample Preparation. The fatty acid compositions and isotopic analyses were performed on the oil fraction obtained from 150 g of sea bass fillets using a modified Bligh and Dyer extraction (addition of 0.88% KCl after filtration) with isohexane/isopropanol (3:2 v/v). The lipid content (% lipid) was determined from the weight of oil extracted from a known weight of fish flesh. The flesh oil fraction was used to determine stable isotope ratios for ^{18}O/^{16}O by elemental analyzer−pyrolysis−isotope ratio mass spectrometry (EA-Py-IRMS) and ^{13}C/^{12}C by elemental analyzer−combustion−isotope ratio mass spectrometry [EA-IRMS (*23*)], respectively. ^{15}N/^{14}N was determined on a concentrated glycerol/choline fraction by EA-IRMS. The glycerol/choline fraction was prepared by mixing 2−5 g of flesh oil with 50 mL of 1 M KOH in ethanol followed by reflux extraction for 2 h at 100 °C. After cooling and the addition of 25 mL of distilled water, the solution was acidified to pH ∼1 by dropwise addition of 37% (w/v) HCl. Twenty milliliters of distilled water was added to dissolve KCl salts, and following four washes with 25 mL of cyclohexane, the aqueous phase was dried by rotary evaporation at 50 °C. The resulting glycerol/choline was dissolved in 30 mL of ethanol, filtered, and washed with small amounts of ethanol and dried first by rotary evaporation and then for 1 h at 70 °C under vacuum. The sample was further dried under a stream of nitrogen for 1 h before the weight of the glycerol/choline fraction was determined. A portion of the oil fraction was saponified, and the free fatty acids were transmethylated to produce fatty acid methyl esters (FAMEs), which were analyzed for fatty acid content by GC and ^{13}C abundance by GC combustion IRMS (GC-C-IRMS).

Fatty Acid Analysis. FAMEs were prepared from 50−100 mg of the dried flesh oil by alkali-catalyzed transmethylation. Briefly, the oil was placed in a stoppered test tube with 2 mL of isohexane and 0.2 mL of 2 M KOH in methanol. After 2 min of shaking, the tube was centrifuged for 5 min at 1000*g*. One milliliter of the upper phase was removed and made up to 10 mL with methanol in a volumetric flask. One milliliter of this diluted solution was mixed with 4 μL of 200 mg/mL butylated hydroxytoluene (internal standard) in isohexane, in a GC vial. The sample was then injected on the GC. FAMEs were separated and quantified by gas−liquid chromatography in the presence of an internal standard. FAMEs were separated and quantified by GC using a Thermo Finnegan Trace 2000 GC (Thermoquest, Hemel Hempstead, U.K.) equipped with a fused silica capillary column [Chrompack CPWAX 52CB, 30 m × 0.32 mm i.d. × 0.25 μm film thickness (Df), Chrompak, London, U.K.] using hydrogen as carrier gas (2.0 mL/min constant flow mode) and detected by flame ionization detection (FID) at 250 °C. The GC temperature program was as follows: initial temperature, 50 °C; ramp 1, 40 °C/min to 150 °C; ramp 2, 2 °C/min to 225 °C; hold for 5 min at 225 °C. Cold on-column injection was used (1 μL of 1 mg of FAME/mL in isohexane). Thirteen peaks, identified as contributing to >95% of the FAME weight, were used. These were 14:0, 16:0, 16:1, 18:0, 18:1n-7, 18:1n-9, 18:2n-6, 20:1n-9, 20:4n-6, 20:5n-3, 22:1n-11, 22:5n-3, and 22:6n-3. Fatty acid values are expressed as percent by weight of total fatty acids. The identification was carried out in comparison to a standard solution composed of 12 of the above FAMEs (without 20:4n-6) in equal weights. The standard solution was a custom preparation from Supelco Inc., (Bellefonte, PA).

Bulk IRMS Analysis of Fish Lipid Components. Isotope ratios (^{13}C/^{12}C, ^{18}O/^{16}O, ^{15}N/^{14}N) determined by IRMS are expressed on a relative scale as the deviation, referred to in delta (δ) units with the notation ‰, parts per thousand, or per mil with respect to the isotope ratio of an international standard, R_{std}. The primary reference standards are VSMOW (Vienna−Standard Mean Ocean Water) for δ^{18}O ‰, VPDB (Pee Dee Belemnite, a calcium carbonate) for δ^{13}C ‰, and air for δ^{15}N ‰. These international standards, or in the case of VPDB secondary standards calibrated against the primary standard, are produced and certified by the International Atomic Energy Agency (IAEA) in Vienna. The δ notation is routinely used by laboratories working in food and beverage authenticity using isotopic measurements by IRMS. The deviation of a measured isotope ratio from the ratio of a calibrated standard is given by

$$\delta_i\,(‰) = \left[\frac{R_i}{R_{\text{std}}} - 1\right] \times 1000$$

where $R_i = {}^{13}\text{C}/{}^{12}\text{C}$, ^{18}O/^{16}O, or ^{15}N/^{14}N.

Fish oil (δ^{13}C and δ^{18}O), glycerol/choline (δ^{13}C and δ^{15}N), and free fatty acids (δ^{13}C) produced from the oil fraction were analyzed for their isotopic fingerprint. δ^{13}C and δ^{15}N provide information on the diet of the fish, and the δ^{18}O affords information on the geographical environment of the fish.

***δ*^{13}C and *δ*^{15}N Determinations.** δ^{13}C (‰) and δ^{15}N (‰) were measured separately using an elemental analyzer (Carlo Erba 1500N, Milan, Italy) coupled to an isotope ratio mass spectrometer (IRMSr; ThermoFinnigan, TracerMAT, Hemel Hempstead, U.K.). Samples were weighed into tin capsules (4 × 8 mm; Elemental Microanalysis, Oakhampton, U.K.), and these were dropped automatically into the "hot-zone" of the reactor, where they were oxidized at a temperature of 1060 °C in a quartz reactor. The combustion gases (CO_2, H_2O, NO_x) were swept by a flow of helium carrier gas through a bed of chromium oxide and silvered cobalt oxide granules. Nitrogen oxides were reduced to N_2 over a bed of reduced copper wires and held at 650 °C, and water vapor was removed by a chemical trap containing magnesium perchlorate, and, for nitrogen analysis, CO_2 was removed using a carbosorb trap. The combustion gases then passed through a packed GC column filled with Porapak Q to separate N_2 from CO_2. A portion of the effluent was allowed to flow into the ion source of the IRMSr. For δ^{13}C determinations, the ratio of the ions at mass to charge ratio (*m*/*z*) 45 (^{13}C^{16}O^{16}O) to the ions at *m*/*z* 44 (^{12}C^{16}O^{16}O) in CO_2 was determined [including correction for the contribution of ^{17}O at *m*/*z* 45; the Craig correction (*24*)] by comparison with a calibrated reference of known δ^{13}C value. The working standard used was menhaden oil (δ^{13}C = −24.96‰; Sigma-Aldrich, Poole, U.K.), which was span-calibrated against international reference materials IAEA CH6 and CH7 (*25*).

For δ^{15}N determinations the ratio of the ions at *m*/*z* 29 ($^{15}N^{14}N$) to the ions at *m*/*z* 28 ($^{14}N^{14}N$) in N_2 was determined by comparison with a calibrated reference of known δ^{15}N value. The working standard used was ammonium sulfate ($\delta^{15}N = -0.86‰$; BDH), which had been span-calibrated against the international reference materials IAEA N1 and N2 (*25*). The nitrogen content of the sample (% nitrogen) was determined by weighing the samples when dispensed into tin capsules and relating the absolute amount of nitrogen (calculated from the nitrogen peak area relative to the ammonium sulfate standard of known nitrogen content) to the amount of sample.

δ^{18}O IRMS Determinations. Samples were weighed into silver capsules (4−6 mm; Elemental Microanalysis), and these were dropped automatically into the hot-zone of the reactor, where they were pyrolyzed at a temperature of 1080 °C in a quartz reactor (Carlo Erba, 1500N). The pyrolysis gases (CO, H_2, N_2) were swept by a flow of helium carrier gas through a bed of nickelized carbon grit (50% nickel). The pyrolysis gases then passed through a carbosorb/magnesium perchlorate trap to remove traces of CO_2 and water, respectively, and thereafter through a packed 50 cm × 6 mm i.d. GC column filled with molecular sieves of 5 Å held at 30 °C to separate H_2, N_2, and CO. A portion of the effluent was allowed to flow into the ion source of the IRMSr (ThermoFinnigan, TracerMAT), and the $^{18}O/^{16}O$ ratio of CO was used to determine δ^{18}O. N_2 was clearly resolved from CO.

The ratio of the ions at *m*/*z* 30 ($^{12}C^{18}O$) to the ions at *m*/*z* 28 ($^{12}C^{16}O$) was determined by comparison with a calibrated working standard (menhaden oil, δ^{18}O 16.85‰ vs VSMOW), which had been calibrated against a secondary international standard (IAEA CH6, δ^{18}O 36.4 vs VSMOW). Preliminary work revealed a strong matrix-specific effect that resulted in drift of the measured δ^{18}O values throughout the analytical cycle, which was not apparent and could not be easily corrected for when a carbohydrate standard (glucose δ^{18}O, 29.32‰ vs VSMOW) was used. The use of a matrix standard allowed correction for this drift in addition to the normal drift correction, specific to the IRMSr. Samples were drift and linearity corrected against laboratory standards that were interspersed throughout the analytical cycle (*26*).

δ^{13}C (‰) GC-C-IRMS Determinations of Individual Fatty Acids. FAMEs were analyzed by GC-C-IRMS to derive δ^{13}C of the free fatty acids. Briefly, this technique uses gas chromatography to separate individual analytes in a continuous stream of helium, which passes through a combustion interface (to convert all analyte carbon to CO_2), and subsequent analysis of ions *m*/*z* 44, 45, and 46 in an IRMSr. Samples were analyzed on an Isochrom III GC-C-IRMS system (GV Instruments, Manchester, U.K.). Briefly, the instrument consisted of an Agilent 6890 gas chromatograph coupled to an isotope ratio mass spectrometer, through a combustion interface. The gas chromatograph was operated in splitless injection mode and was installed with a capillary column (Zebron ZB-Wax, 30 m × 0.32 i.d., 0.25 μm; Phenomenex) to effect analyte separation. The injector temperature was 250 °C, and the carrier gas (helium) was controlled to maintain a constant column flow of 2 mL/min. The GC operating conditions were as follows: initial temperature of 80 °C held for 4 min; ramp, 7.5 °C/min to 150 °C; ramp, 2 °C/min to 225 °C; hold, 5 min. The column flow was directed to a FID, via the heart-split valve (a pressure-balanced microneedle valve for directing column flow to either FID or IRMS), until the bulk of the solvent peak had eluted. The heart-split valve was closed to direct the column flow through the combustion interface, held at 350 °C, and through the combustion furnace. The combustion furnace was made of materials similar to those of the oxidative furnace for bulk ^{13}C analysis but of 0.5 mm bore. It was held at 800 °C. Downstream from the combustion furnace was the open split, where a portion of the gas stream was allowed to enter the IRMSr capilliary. A cryogenic trap was operated (−100 °C, liquid N_2), between the open split and the IRMS, to remove water from the carrier stream. The IRMS continuously monitored ions of *m*/*z* 44, 45, and 46, and the proprietary software (Isochrom, GV Instruments) was used to integrate the major and minor peak areas with appropriate corrections for background and isotopic shift.

An internal standard [pentadecanoic acid (C15:0); Sigma-Aldrich, Poole, Dorset, U.K.] was used as both chemical and isotopic standard. A portion of the standard was derivatized using acid-catalyzed transmethylation, and the free fatty acid (FFA) and FAME forms were analyzed by bulk EA-IRMS (Costech EA−ThermoFinnigan, Delta XP) to determine δ^{13}C and, from the data, δ^{13}C of the methyl carbon added by derivatization by mass balance. Span calibration (*25*) against the international standards IAEA CH6 and IAEA CH7 resulted in δ^{13}C FFA = −34.3‰ and δ^{13}C FAME = −34.94‰, producing a derived $\delta^{13}C = -44.53‰$ for methanol used in derivatization. The methanol used for this calibration was also the same methanol used in all transmethylation reactions. Ten microliters of 20 mM internal standard in isooctane was added to each 100 μL of sample, and samples were diluted to attain 1 mg of FAME/mL of solvent. Half a microliter of analyte mix was injected, equivalent to 200 pmol of C15:0 on column. Over a mean area ratio range of sample to internal standard of 1.1−0.4 (maximum = 1.62, minimum = 0.22), a precision of 0.3‰ was attained ($n = 12$). Over the area ratio range 0.3 −0.1 (maximum = 0.4, minimum = 0.1), a precision of 1.2‰ was attained. In practice, peaks with an area ratio of <0.2 were not reliably quantifiable in terms of peak area and therefore in terms of isotopic composition. Samples were linearity corrected as previously described (*26*).

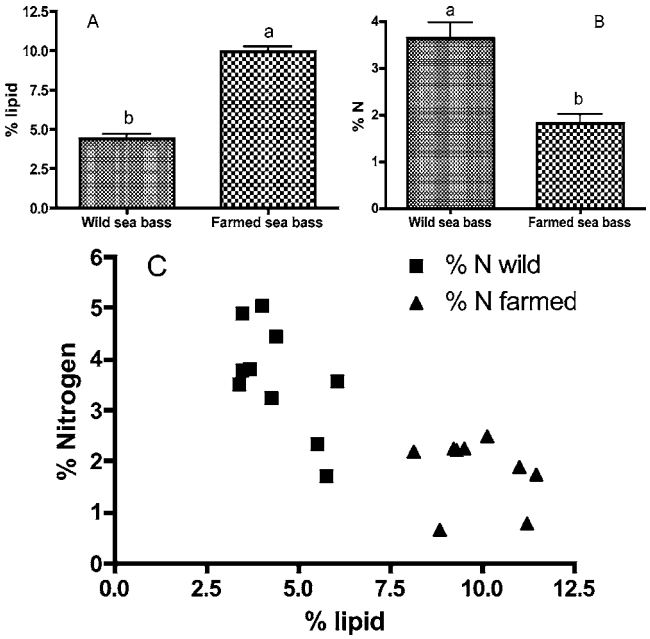

Figure 1. Individual plots of flesh lipid (**A**) and choline nitrogen (**B**) concentration (%) of farmed and wild sea bass and a bivariate plot (**C**) showing discrimination of wild and farmed bass using these two parameters. Columns assigned different letters are significantly different ($P < 0.05$).

Statistical Analysis. Parameters were analyzed by Student's *t* test or ANOVA with post-hoc analysis to determine significant differences between wild and farmed sea bass. Principal component analysis (PCA) was conducted using the XLSTAT add-in for Microsoft Excel (Addinsoft France, Paris, France). The purpose of PCA is to express the main information in the variables using a smaller number of variables, the so-called principal components (PC1, PC2, etc). A high positive or negative loading reveals a significant variable in the actual PCA model. Score plots from the PCA explore the main trends in the data, and their respective loading reveals variables with a significant loading. Samples showing similar chemical and isotopic attributes are located in the same area of the score plot. Samples showing similar attributes are indicated on the PCA plot by name (farmed or wild), and the two sources of farmed fish are indicated by the two circles (**Figure 6**).

RESULTS

Wild sea bass had a significantly lower flesh lipid content than farmed sea bass ($P < 0.0001$), whereas wild bass had a higher choline nitrogen content than farmed bass ($P = 0.0002$). A bivariate plot of the lipid and nitrogen content data provided discrimination based on only these two parameters (**Figure 1**).

Table 2. Major Fatty Acid Compositions (Weight Percent of Total Fatty Acids) of Farmed and Wild Sea Bass Flesh[a]

fatty acid	farmed[b]	wild
14:0	4.8 ± 0.3*	3.8 ± 0.7
16:0	17.6 ± 1.1*	19.0 ± 1.5
18:0	3.1 ± 0.5*	4.7 ± 0.4
16:1n-7	7.3 ± 1.1	6.6 ± 1.0
18:1n-9	18.4 ± 1.0	18.6 ± 2.4
18:1n-7	3.2 ± 0.3	3.5 ± 0.4
20:1n-9	6.1 ± 3.1*	3.9 ± 1.0
22:1n-11	4.9 ± 2.6	4.0 ± 1.8
18:2n-6	7.5 ± 3.7*	1.2 ± 0.3
20:4n-6	0.7 ± 0.1*	1.6 ± 0.6
20:5n-3	9.1 ± 1.1*	8.6 ± 0.5
22:5n-3	1.6 ± 0.3*	2.1 ± 0.2
22:6n-3	15.7 ± 0.9*	22.5 ± 3.5

[a] Values are mean ± SD, $n = 10$. [b] Values are combined Scottish and Greek samples. Values assigned an asterisk are significantly different between wild and farmed bass ($P > 0.05$), as determined by Student's t test.

Table 3. Major Total Lipid Fatty Acid Compositions (Weight Percent of Total Fatty Acids) of Farmed Scottish and Greek and Wild Sea Bass Flesh[a]

fatty acid	farmed, Scotland	farmed, Greece	wild
14:0	5.0 ± 0.1a	4.6 ± 0.7a	3.8 ± 0.7b
16:0	16.6 ± 0.3b	18.6 ± 0.3a	19.0 ± 1.5a
18:0	2.6 ± 0.1c	3.6 ± 0.2b	4.7 ± 0.4a
16:1n-7	8.3 ± 0.3a	6.3 ± 0.3b	6.6 ± 1.0b
18:1n-9	17.9 ± 0.7	18.9 ± 1.1	18.6 ± 2.4
18:1n-7	3.5 ± 0.0a	2.9 ± 0.1b	3.5 ± 0.4a
20:1n-9	9.0 ± 0.1a	3.1 ± 0.4b	3.9 ± 1.0b
22:1n-11	7.3 ± 0.2a	2.5 ± 0.4b	4.0 ± 1.8b
18:2n-6	4.1 ± 0.3b	10.8 ± 1.4a	1.2 ± 0.3c
20:4n-6	0.6 ± 0.1b	0.8 ± 0.1b	1.6 ± 0.6a
20:5n-3	8.1 ± 0.2b	10.0 ± 0.6a	8.6 ± 0.5b
22:5n-3	1.4 ± 0.1b	1.9 ± 0.1a	2.1 ± 0.2a
22:6n-3	15.6 ± 0.8b	15.9 ± 1.1b	22.5 ± 3.5a

[a] Values are mean ± SD, $n = 5$ for Scottish and Greek farmed bass and $n = 10$ for wild bass. Values in the same row assigned a different letter are significantly different (ANOVA, $P < 0.05$).

In terms of flesh total lipid fatty acids farmed bass contained significantly more 14:0, 20:1n-9, 18:2n-6, and 20:5n-3 and significantly less 16:0, 18:0, 20:4n-6, 22:5n-3, and 22:6n-3 than their wild counterparts (**Table 2**). The farmed sea bass were obtained from two locations, Scotland and Greece, with 14:0 and 18:2n-6 being significantly higher in both compared to wild bass (**Table 3**). Arachidonic acid (20:4n-6; ARA) was significantly lower in both farmed groups compared to wild bass. The Scottish bass had significantly higher 16:1n-7, 20:1n-9, and 22:1n-11 compared to both Greek farmed and wild sea bass, whereas 16:0, 18:0, and 22:5n-3 were lower in Scottish farmed bass compared to Greek farmed bass. The values for 18:0 and 18:2n-6 were significantly different between the three bass groups, with the lowest values in Scottish farmed and the highest values in wild for 18:0 and the lowest for wild and highest for Greek farmed bass for 18:2n-6 (**Table 3**).

The isotopic data showed that free fatty acid δ^{13}C exhibited significant differences in δ^{13}C values for 16:0, 18:0, 16:1n-7, 18:1n-9, 18:1n-7, 20:1n-9, and 20:4n-6 between farmed and wild sea bass (**Figure 2**). Bulk δ^{13}C analysis of the total oil fraction (**Figure 3**) showed highly significant differences between fish production origin, with farmed fish being lighter than wild fish. Significant differences were also observed in the δ^{15}N (**Figure 4**) of the flesh lipid total glycerol/choline fraction in farmed versus wild sea bass. Analysis of δ^{18}O from total oil extracted from flesh lipid of sea bass did not show any significant difference between farmed and wild fish (results not shown). However, a significant difference was observed between the

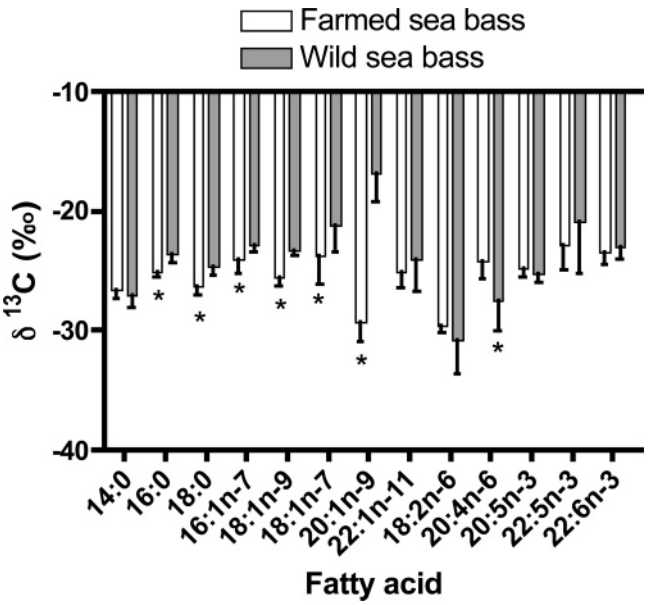

Figure 2. Compound-specific δ^{13}C fatty acids in farmed and wild sea bass flesh total lipid. Columns assigned an asterisk are significantly different between farmed and wild fish ($P < 0.05$).

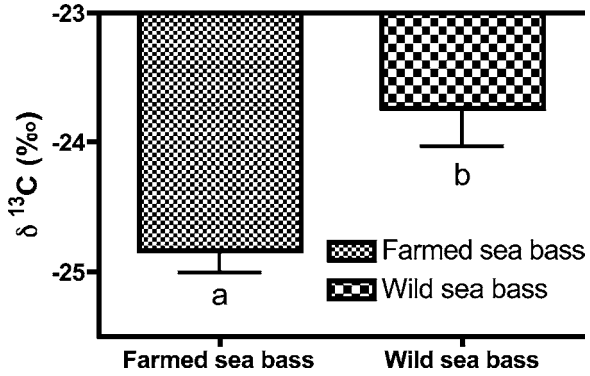

Figure 3. δ^{13}C of the bulk oil fraction of flesh total lipid in farmed and wild sea bass. Columns assigned a different letter are significantly different ($P < 0.05$).

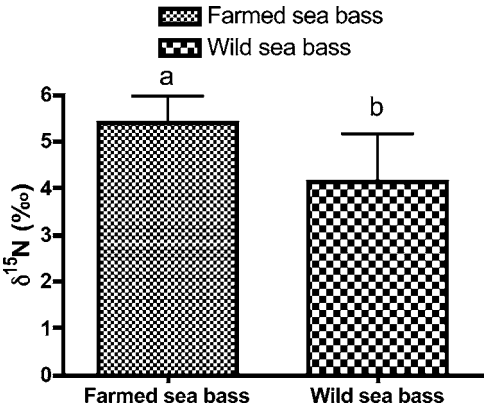

Figure 4. δ^{15}N in the glycerol/choline fraction of total flesh lipid from farmed and wild sea bass. Columns assigned a different letter are significantly different ($P < 0.05$).

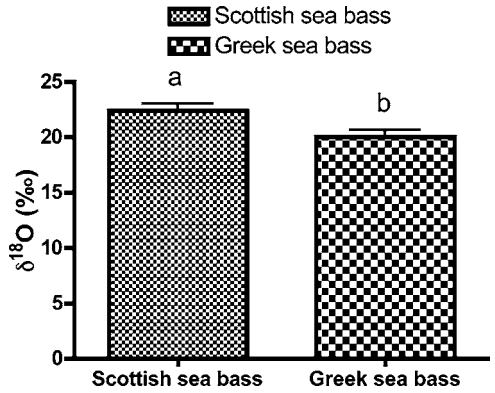

Figure 5. $\delta^{18}O$ in bulk oil fraction of flesh total lipid in farmed Scottish and farmed Greek sea bass. Columns assigned a different letter are significantly different ($P < 0.05$).

Table 4. Eigenvalues for Sea Bass PCA

	eigenvalue	variability (%)	cumulative eigenvalue	cumulative %
F1	10.52	38.94	10.52	38.94
F2	5.49	20.32	16.01	59.26
F3	3.35	12.41	19.36	71.67
F4	2.18	8.07	21.54	79.74
F5	1.39	5.14	22.93	84.89
F6	0.94	3.50	23.87	88.38
F7	0.63	2.33	24.50	90.71

farmed sea bass samples from Scotland compared to the farmed sea bass samples from Greece (**Figure 5**).

PCA was used to examine the multivariate structure of the sea bass data set. **Table 4** indicates that the first three factors account for >70% of the variability within the data, whereas **Table 5** demonstrates the contribution of the variables to the selected factors. Plots of factor 1 versus factors 2, 3, and 4 all demonstrate clear separation of wild and farmed sea bass, as well as farmed sea bass of Scottish or Greek origin, with factor 1 providing greatest discrimination. **Figure 6** shows a plot of factor 1 versus factor 2.

DISCUSSION

Recent European Union (EU) legislation requires that fish products, of wild and farmed origin, must provide consumer information that describes geographical origin and production method. Whereas global food-grade capture fisheries have reached, or exceeded, their sustainable limits, the demand for seafood has continued to expand and the shortfall is increasingly compensated by aquaculture production (*1*, *3*). The increase in aquaculture production, required to meet consumer demand for finfish, has resulted in an increasing number of new farmed species entering the marketplace over the past decade. One of these species is European sea bass (*D. labrax*), which was first cultured in Italy in the early 1970s. Production in Europe and North Africa has grown steadily over the past 30 years, with 2004 production reaching almost 50000 metric tonnes, Greece, Italy, and Spain being the major producers (*3*). As is the case with the majority of fish species, wild fish generally command a price premium over the farmed product and, therefore, there may be a temptation to falsely label farmed produce at the wholesaler, retailer, and restaurant level to obtain a better price. In an attempt to combat false and misleading labeling, robust and verifiable methods to distinguish farmed and wild product need to be developed to maintain consumer confidence as well as for local authority enforcement purposes. This study describes the use of chemical and isotopic methodologies to accurately discriminate the production origin of European sea bass.

Table 5. Factor Loadings for Sea Bass PCA[a]

variable	F1	F2
16:0	0.700	0.196
18:0	**0.968**	0.120
22:1n-11	0.643	–0.435
18:2n-6	–0.614	–0.702
20:4n-6	**0.901**	–0.129
20:5n-3	0.050	**0.745**
22:5n-3	**0.835**	0.306
22:6n-3	0.620	–0.016
16:0 $\delta^{13}C$	**0.849**	–0.196
18:0 $\delta^{13}C$	**0.755**	0.401
16:1n-7 $\delta^{13}C$	**0.966**	–0.257
18:1n-9 $\delta^{13}C$	**0.852**	–0.374
18:1n-7 $\delta^{13}C$	0.625	0.592
20:5n-3 $\delta^{13}C$	–0.205	**0.790**
22:6n-3 $\delta^{13}C$	0.470	0.543
bulk $\delta^{13}C$	**0.920**	0.019
$\delta^{18}O$	–0.275	–0.619
% N	**0.711**	–0.328
% lipid	–0.698	0.397

[a] Bold type indicates loadings >0.7.

The cultured sea bass had significantly higher flesh (>2-fold) lipid levels compared to their wild counterparts. In most fish species, flesh lipid content increases with weight and age of the fish (*27*). However, in this case, the wild fish were >3 times heavier than the farmed fish, yet the lipid was less than half the value in the wild fish compared to the farmed product. This would suggest that the dietary fat is having a greater influence on flesh fat levels than the size and age of the animal. Increased flesh lipid in farmed compared to wild European sea bass has been reported in the literature (*21*, *28*, *29*), although a recent study reported no difference in flesh lipid between farmed and wild fish (*22*). Similarly elevated flesh lipid levels in farmed versus wild fish have also been reported for gilthead sea bream (*Sparus aurata*) (*29*, *30*), red porgy (*Pagrus pagrus*) (*31*), rainbow trout (*Oncoryhchus mykiss*) (*20*), and Atlantic salmon (*Salmo salar*) (*32*). These data suggest that the intensive culture of sea bass using diets with a lipid content of ~20% results in increased lipid deposition in farmed bass compared to wild fish. Thus, in farmed sea bass, as well as in a number of other fish species, flesh lipid content could be used as a factor to discriminate between wild and farmed products. The farmed bass glycerol/choline fraction also had a lower nitrogen content compared with wild fish. The lower nitrogen content cannot be simply explained by the dilution effect of higher lipid content and probably reflects the higher protein content of wild fish due to greater muscle mass.

The flesh fatty acid compositions of wild and farmed bass were different, as were the compositions of farmed bass from Scotland and Greece. This is almost certainly due to different dietary inputs. Farmed bass from Scotland had significantly greater amounts of 16:1n-7, 20:1n-9, and 22:1n-11 than farmed bass from Greece and wild bass. As sea bass feeds are not generally available in Scotland, these fish were fed a commercial Atlantic cod diet, which has a lower lipid content (14−16%) compared to bass diets produced in southern Europe (20−25%). In addition, the three monounsaturated fatty acids described above are characteristic of high-latitude fish oils, for example, capelin, herring, and sand eel, whereas the farmed bass from Greece had higher levels of saturated fatty acids and EPA, which are characteristic of locally sourced fish oils from lower

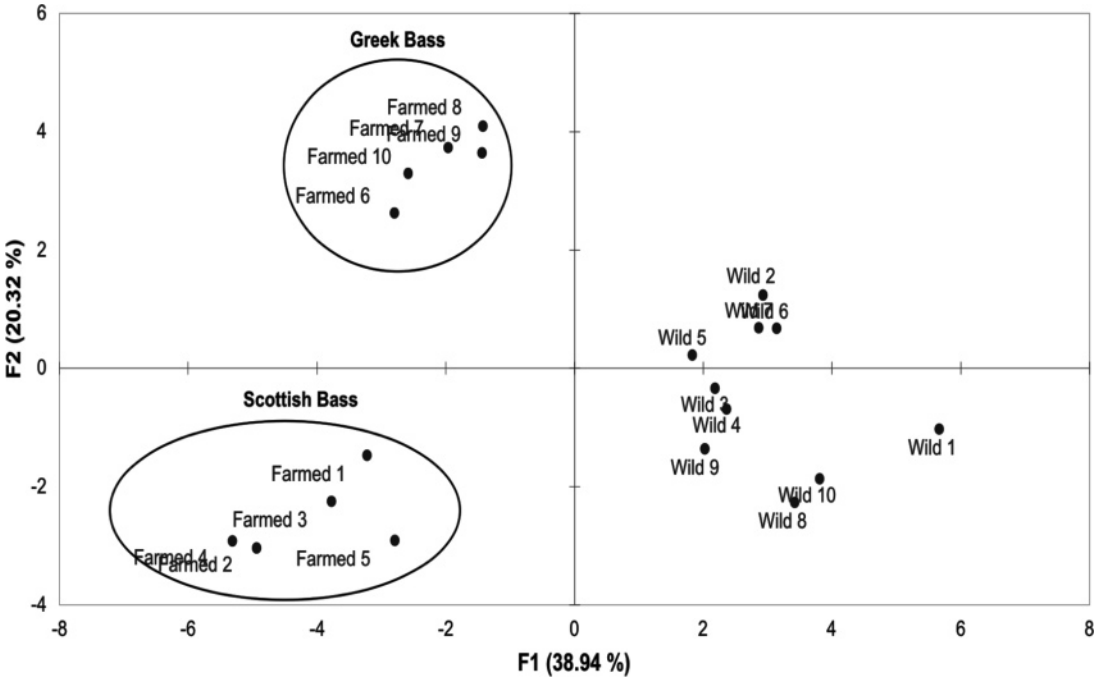

Figure 6. Plot of PCA factor coordinates for the first two principal components of farmed versus wild sea bass data.

latitudes, for example, sardine and anchovy (*33*, *34*). The n-3/n-6 PUFA ratio was significantly higher in wild fish than in farmed fish (11.2 versus 3.2) and was largely due to the higher level of 18:2n-6 in the farmed bass. Commercial aquafeed formulations increasingly contain fewer marine-derived and more terrestrial-derived raw materials, partly due to the increasing cost of marine products and efforts to reduce reliance on marine fish meals and oils. The terrestrial products currently utilized in Mediterranean aquafeeds include soybean, wheat, maize, sunflower, pea, lupin, and rapeseed meals, which can contain a significant lipid component (*35*). The plant lipid, either derived from the plant meals described above or added directly as vegetable oil, explains the higher levels of 18:2n-6 in the farmed bass flesh compared to wild fish (*36*, *37*). Wild bass also contained higher 20:4n-6 and 22:6n-3, and evidence suggests that wild bass tend to feed in inshore and estuarine waters that often have fatty acid compositions similar to the dietary fauna from those areas, whereas aquafeeds contain fish products obtained from the open oceans, which have quite different fatty acid compositions (*38*, *39*). However, the higher levels of ARA and DHA may be related to the relatively lower fat content, and thereby lower triglyceride, in the wild product. Generally, polar lipid is higher in polyunsaturated fatty acids than triglyceride, and this may have influenced the higher flesh levels of these fatty acids in wild bass, in addition to effects due to dietary input (*34*).

Bulk ^{13}C analysis of the total flesh lipid fraction highlighted a highly significant difference between farmed and wild sea bass, with farmed fish being isotopically lighter than wild fish. This is a reflection of the lighter $\delta^{13}C$ input from dietary terrestrial carbon sources used in the commercial feed formulations for farmed sea bass as discussed for the fatty acids above. The natural diets of marine fish are isotopically heavier due to the source of carbon (dissolved inorganic carbon pool, $\delta^{13}C \sim 0‰$) used by macroalgae and phytoplankton, which form the lower trophic levels of the marine food web, compared with terrestrial photosynthesis from atmospheric CO_2 ($\delta^{13}C \sim -7.8‰$). In addition, marine vertebrates, through their trophic sequestration of zooplankton and crustacea, further enrich the isotopic signature to produce heavier $\delta^{13}C$ values. The origin of dietary lipids in commercial feed formulations reflects a significant terrestrial signature of plant oils and appears largely to be of C3 origin, the photosynthetic pathway used by temperate plants, such as cereals and legumes, likely to be used in aquafeeds (*40*).

The measured $\delta^{13}C$ values of individual fatty acids yielded some significant differences such that $\delta^{13}C$ values of 16:0, 18:0, 16:1n-7, 18:1n-9, and 18:1n-7 were "heavier" in wild sea bass than in farmed sea bass. Whether C16:0 in wild fish originated from dietary sources or de novo synthesis, the source will be heavier than farmed fish, which will have a significant dietary input of C16:0 from terrestrial plant origin. The desaturation of the major saturated fatty acids appears to reflect the dietary origin of the saturated fatty acid. Wild sea bass will have fed on, almost exclusively, marine sources, and the $\delta^{13}C$ signature of the major fatty acids will reflect the $\delta^{13}C$ signature of the diet. Farmed sea bass appear, from their fatty acid $\delta^{13}C$ signature, to have a significantly "lighter" terrestrial $\delta^{13}C$ input that reflects accretion of fatty acids from plant sources included in their diets as described earlier. Significant differences were found with the most abundant fatty acids and probably reflect the increased precision of analysis of the major analytes. By contrast, lower abundance analytes have poorer analytical precision because they suffer to a greater extent from baseline perturbations and present less CO_2 to the ion source, which has a significant impact on precision. Thus, the difference observed in 20:1n-9 between farmed and wild sea bass appears to be large, but this is likely an analytical artifact caused by poor resolution of this peak from coeluting peaks and a changing baseline. This peak is also a minor peak, making accurate quantitation more difficult. The choice of the internal standard method for quantitation ensured that accuracy was maintained (against an externally calibrated isotopic standard) and also allowed the quantitation of analyte concentration due to the excellent area ratio response of the IRMSr to CO_2 concentration (*41*). This approach would not have been possible using the reference gas configuration, as is commonly used in GC-C-IRMS, and thus the analysis of compound-specific fatty acid analysis also

provides the fatty acid composition of the sample. Further analysis allows accurate reconstruction of the bulk $\delta^{13}C$ value using mass balance equations (data not shown). Thus, the utility of compound-specific analysis is clear, yielding fatty acid composition, individual fatty acid $\delta^{13}C$, and bulk $\delta^{13}C$.

The expectation that wild fish are isotopically enriched in ^{15}N compared with their farmed counterparts (because of their higher trophic position in the food chain) was not observed in $\delta^{15}N$ of sea bass. This observation may reflect the maturity or growth rate of farmed sea bass (mean weight = 386 g) relative to wild sea bass (mean weight = 1690 g) or seasonal variations in $\delta^{15}N$ in coastal marine environments. It may also reflect inputs from terrestrial N sources in farmed sea bass whereby $\delta^{15}N$ may vary from −5 to 18‰ (*42*). The differences observed in $\delta^{18}O$ from total flesh oil from farmed Scottish bass compared to the product originating from Greece may reflect the latitudinal differences in mean ocean $\delta^{18}O$, which will be in isotopic equilibrium with fish metabolic water and therefore may discriminate between fish caught in different geographical locations. This may have influenced the values in Greek bass in the present study that were cultured in the Mediterranean, whereas Scottish bass were grown on the Scottish west coast and the wild bass were from southern England. In addition, the geographical location of farmed fish is tightly controlled, but wild fish may migrate over large geographical regions, over the course of their seasonal life cycles. Thus, the usefulness of $\delta^{18}O$ as a discriminatory factor between farmed and wild fish between different species must be carefully considered.

PCA was initially conducted using only four components, the $\delta^{13}C$ and $\delta^{18}O$ ratios in bulk oil, the $\delta^{15}N$ ratio in the choline/glycerol fraction, and the 18:2n-6 fatty acid concentration (results not shown). It is possible that these factors alone could provide a relatively rapid discrimination of wild and farmed sea bass in a larger sample set, although this remains to be tested conclusively. However, whereas this effectively discriminated between farmed and wild sea bass in the current data set, it should be remembered that this data set is from a limited number of individuals from only three geographic locations and that commercial products can originate from a wide range of locations with various environmental and dietary inputs. In such instances, where a more heterogeneous range of measured values might be expected, additional discriminant power could be obtained by using other factors including all or some of the flesh fatty acids or the $\delta^{13}C$ ratio of free fatty acids as shown in **Figure 6**. This combination of analytical methodologies can provide the basis for discriminating between wild and farmed fish over a range of species, particularly the isotopic fingerprinting of individual fatty acids. The use of compound-specific isotope analysis appears to provide robust and confirmatory data that may be used as an addition to, or instead of, fatty acid methyl ester analysis. Future studies should investigate the application of the described methods on a wide range of fish samples derived from different species and geographical locations, from both aquaculture and fisheries sectors.

ABBREVIATIONS USED

HUFA, highly unsaturated fatty acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; POPs, persistent organic pollutants; EA-Py-IRMS, elemental analyzer−pyrolysis−isotope ratio mass spectrometer; EA-IRMS, elemental analyzer (combustion oven) coupled to isotope ratio mass spectrometer; FAME, fatty acid methyl ester; GC-C-IRMS, gas chromatography coupled to isotope ratio mass spectrometer via a combustion interface; VSMOW, International Standard Vienna−Standard Mean Ocean Water (IAEA); IAEA, International Atomic Energy Authority; FFA, free fatty acids; ARA, arachidonic acid; PCA, principal component analysis; EU, European Union.

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