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Authenticating Production Origin of Gilthead Sea Bream (*Sparus Aurata)* by Chemical and Isotopic Fingerprinting

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ABSTRACT

Recent EU legislation (EC/2065/2001) requires that fish products, of wild and farmed origin, must provide consumer information that describes geographical origin and production method. The aim of the present study was to establish methods that could reliably differentiate between wild and farmed European gilthead sea bream (*Sparus aurata)*. The methods that were chosen were based on chemical and stable isotopic analysis of the readily accessible lipid fraction.

This study examined fatty acid profiles by capillary gas chromatography and the isotopic composition of fish oil ($\delta^{13}C$, $\delta^{18}O$), phospholipid choline nitrogen ($\delta^{15}N$) and compound specific analysis of fatty acids ($\delta^{13}C$) by isotope ratio mass spectroscopy as parameters that could reliably discriminate samples of wild and farmed sea bream. The sample set comprised of 15 farmed and 15 wild gilthead sea bream (*Sparus aurata)*, obtained from Greece and Spain, respectively.

Discrimination was achieved using fatty acid compositions, with linoleic acid (18:2n-6), arachidonic acid (20:4n-6), stearic acid (18:0), vaccenic acid (18:1n-7) and docosapentaenoic acid (22:5n-3) providing the highest contributions for discrimination. Principle components analysis of the data set highlighted good discrimination between wild and farmed fish. Factor 1 and factor 2 accounted for >70% of the variation in the data. The variables contributing to this discrimination were: the fatty acids 14:0, 16:0, 18:0, 18:1n-9, 18:1n-7, 22:1n-11, 18:2n-6 and 22:5n-3; $\delta^{13}C$ of the fatty acids 16:0, 18:0, 16:1n-7, 18:1n-9, 20:5n-3 and 22:6n-3; Bulk oil fraction $\delta^{13}C$; glycerol/choline fraction bulk $\delta^{13}C$; $\delta^{15}N$; % N; % lipid.

INTRODUCTION

The global demand for finfish and shellfish is increasing in line with the demands of increasing global populations. However, the food-grade capture fisheries have reached, or exceeded, their sustainable limits and increasingly the shortfall in supply must be met by aquaculture produce (1,2). While landings from capture fisheries are static, aquaculture production worldwide is increasing at around 10%/annum (2) and for this reason much more aquaculture produce is currently available to consumers. Currently, more than 30% of world seafood production is derived from aquaculture and this is likely to increase in the future (2).

Within the production chain, similar fish products can arise from different points of origin and there is consequently potential for fraud due to product mislabelling. As a result, in October 2002, the EC issued Commission regulation no. 2065/2001 to ensure that more details on labelling, packaging and traceability of fishery and aquaculture products would be available to retailers and consumers. The reason for this additional legislation is to provide more 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.

A number of recent reports have suggested that farmed salmon may contain higher levels of persistent organic contaminants, such as dioxins and PCBs, (3) although the levels were well within the accepted range (4). However, there is considerable evidence that suggests the benefits of eating fish, particularly oily fish, significantly outweigh any perceived risks (5, 6). Highly unsaturated fish oils, and eicosapentaenoic acid (20:5n-3; EPA) in particular, are under clinical investigation to determine their therapeutic benefit in immunomodulated disease (7-10). However, while the anti-inflammatory effects of EPA are well documented the functional activity of docosahexaenoic acid (22:6n-3; DHA) is also vital for normal cellular function. DHA is required for the normal growth and development of neural tissue in infants and is also essential for maintaining normal brain function in adults (11). The importance of DHA is evidenced by the fact that over 20% of the brain dry weight is DHA and this is the most abundant fatty acid in neural tissues (12, 13). As well as being linked with brain function, DHA deficiencies are also linked to reduced visual acuity, attention deficit hyperactivity disorder, cystic fibrosis, unipolar and bipolar depression, aggression and dysfunctions of the immune system (4, 14, 15).

In addition, the fishmeal and fish oil which have been used by the aquaculture industry as the basis of aquafeed formulations for over 30 years have reached limits of sustainable production (16) and new sustainable raw materials are now being tested and introduced in commercial aquafeeds. However, while the use of plant-derived raw materials can reduce the concentrations of organic contaminants in fish (17, 18) the concentrations of beneficial n-3 highly unsaturated fatty acids (HUFA), particularly EPA and docosahexaenoic acids (22:6n-3; DHA), are reduced as the cheaper vegetable oils likely to be used do not contain these n-3 HUFA (19). Thus, farmed fish, cultured on diets containing lower levels of marine-derived raw materials, may have different lipid compositions compared with fish from wild capture fisheries (20, 21).

The present study examined whether farmed and wild gilthead sea bream (*Sparus aurata)* could be discriminated, in terms of their production origin, using a range of analytical measurements that have the potential to discriminate source terms, on flesh samples including fatty acid composition, $\delta^{13}C$ of individual fatty acids, $\delta^{13}C$ and $\delta^{18}O$ of total flesh oil and $\delta^{15}N$ of the choline fraction of phospholipid.

METHODS

Samples

Farmed sea bream from Greece were supplied by Bernard Corrigan Ltd, Glasgow. Samples of wild sea bream were caught by gill net in the Bay of Cadiz in Spain and were supplied by Prof. Gabriel Mourente of the University of Cadiz. Details of the fish analysed in this survey are given in Table 1.

Sample preparation and extraction of lipids

The compositional and isotopic analyses were performed on the oil fraction obtained from sea bream flesh after evisceration using 0.88% KCl, filtration and extraction using isohexane:isopropanol (3:2 v/v). The lipid content (% lipid) was determined by the weight of oil extracted from a known weight of fish flesh. The flesh oil fraction was used to determine stable isotope ratios for $^{18}O/^{16}O$ and $^{13}C/^{12}C$ by elemental analyser-pyrolysis-isotope ratio mass spectrometry (EA-Py-IRMS) and elemental analyser-combustion-isotope ratio mass spectrometry (EA-IRMS (22)), respectively. $^{15}N/^{14}N$ was determined on a concentrated

glycerol/choline fraction by EA-IRMS. The glycerol/choline fraction was prepared by mixing 2-5g of flesh oil with 50 ml of 1M KOH in ethanol followed by reflux extraction for 2h at 100^{o}C. After cooling and addition of 25 ml distilled water the solution was acidified to ~pH 1 by dropwise addition of 37% (w/v) HCl. Twenty ml of distilled water was added to dissolve KCl salts and following 4 washes with 25 ml of cyclohexane the aqueous phase was dried by rotary evaporation at 50^{o}C. The resulting glycerol/choline was dissolved in 30 ml ethanol, filtered and washed with small amounts of ethanol and dried first by rotary evaporation and then for 1h at 70^{o}C under vacuum. The sample was further dried under a stream of nitrogen for 1h before the weight of the glycerol/choline fraction was determined. A portion of the oil fraction was saponified and the free fatty acids transmethylated to produce fatty acid methyl esters (FAMEs), which were analysed for fatty acid content by GC and ^{13}C abundance by GC combustion IRMS (GC-C-IRMS).

Fatty acid analysis by GC

Fatty acid methyl esters (FAMEs) were prepared from a small quantity (50-100mg) of the dried flesh oil by alkali-catalysed transmethylation. Briefly, the oil was placed in a test tube with 2 ml of iso-hexane and 0.2 ml of 2M KOH in methanol. After shaking for 2 min the tube was centrifuged for 5 min at 1000 x g. One ml of the upper phase was removed and made to 10 ml with methanol in a volumetric flask. One ml of this diluted solution was mixed with 4 µl of 200g/ml butylated hydroxytoluene (internal standard) in iso-hexane in a GC vial. The sample was then ready for injection on the GC. FAMEs were separated and quantified by gas chromatography (GC) in the presence of an internal standard. Separation of fatty acids and detection by flame ionisation detection (FID) was developed to quantify the composition of FAME in the fish lipid extracts on a percentage weight basis. FAMEs were separated and quantified by GC using a Thermo Finnigan Trace 2000 GC (Thermoquest, Hemel Hempstead, UK) equipped with a fused silica capillary column (Chrompack CPWAX52CB, 30 m x 0.32 µm x 0.25mm i.d.; Chrompak, London, UK) using hydrogen as carrier gas (2.0ml/min constant flow mode) and detected by FID at 250^{o}C. The GC temperature program was: initial temp: 50^{o}C, ramp 1: 40^{o}C/min to 150^{o}C, ramp 2: 2^{o}C/min to 225^{o}C, hold for 5 mins at 225^{o}C. Cold on column injection was used (1µl of 1mg/ml in iso-hexane). 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. The identification was carried out in comparison to a standard solution composed of

12 of the above FAME (without 20:4n-6) in equal weights. The standard solution was a custom preparation from Supelco Inc., (Bellafonte, PA, USA).

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 content of an international standard, R_{std}. The primary references standards are VSMOW (Vienna – Standard Mean Ocean Water) for $\delta^{18}O$ ‰, PDB (Pee Dee Belemnite, a calcium carbonate) for $\delta^{13}C$ ‰, and Air for $\delta^{15}N$ ‰. These international standards, or secondary standards calibrated against the primary standards, are produced and certified by the International Atomic Energy Agency (IAEA) in Vienna. This 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:

$$\delta_{\,i}\,[‰] = \left[\frac{R_i}{R_{std}} - 1\right] \times 1000$$

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

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

$\delta^{13}C$ and $\delta^{15}N$ determinations

$\delta^{13}C$ (‰) and $\delta^{15}N$ (‰) were measured separately using an elemental analyser (Carlo Erba 1500N) coupled to a isotope ratio mass spectrometer (IRMSr; ThermoFinnigan, TracerMAT). Samples were weighed into tin capsules (4 × 8 mm; Elemental Microanalysis, Oakhampton, UK) and these were dropped automatically into the "hot-zone" of the reactor where they were oxidised 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, held at 650°C and water vapour 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 $\delta^{13}C$ 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 (23)) by comparison with a calibrated reference of known $\delta^{13}C$ value. The working standard used was menhaden oil ($\delta^{13}C$ = -24.96‰; Sigma-Aldrich, Poole, UK) which was span-calibrated against international reference materials IAEA CH6 and CH7 (24).

For $\delta^{15}N$ 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 $\delta^{15}N$ value. The working standard used was ammonium sulphate ($\delta^{15}N$ = -0.86‰; BDH, UK) which had been span calibrated against the international reference materials IAEA N1 and N2 (24). 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 sulphate standard of known nitrogen content) to the amount of sample.

$\delta^{18}O$ IRMS determinations

Samples were weighed into silver capsules (4 × 6 mm; Elemental Microanalysis, Okehampton, UK) and these are dropped automatically into the "hot-zone" of the reactor where they were pyrolysed 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 nickelised 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 50cm x 6mm ID 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 $\delta^{18}O$. 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, $\delta^{18}O$ 16.85‰ vs. VSMOW) which had been calibrated against a secondary international standard (IAEA CH6, $\delta^{18}O$ 36.4

vs. VSMOW). Preliminary work revealed a strong matrix specific effect which resulted in drift of the measured $\delta^{18}O$ values throughout the analytical cycle, which was not apparent and could not be easily corrected for when using a carbohydrate standard (glucose $\delta^{18}O$, 29.32 ‰ vs. VSMOW). 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 (25).

$\delta^{13}C$ (‰) GC-C-IRMS determinations of individual fatty acids

FAMEs were analysed by GC-C-IRMS to derive $\delta^{13}C$ 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 analysed on an Isochrom III GC-C-IRMS system (GV Instruments, Manchester, UK). 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 capilliary column (Zebron ZB-Wax, 30m × 0.32 ID 0.25 μm; Phenomenex, UK) 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 mins, 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 similar materials as the oxidative furnace for bulk ^{13}C analysis but of 0.5mm 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, UK) 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, Dorest) was used as both chemical and isotopic standard. A portion of the standard was derivatised using acid

catalysed transmethylation and the free fatty acid (FFA) and FAME form were analysed by bulk EA-IRMS (Costech EA - ThermoFinnigan, Delta XP) to determine $\delta^{13}C$ and from the data, $\delta^{13}C$ of the methyl carbon added by derivatisation by mass balance. Span calibration (24) against the international standards IAEA CH6 and IAEA CH7 resulted in $\delta^{13}C$ FFA = -34.3 ‰ and $\delta^{13}C$ FAME = -34.94 ‰ producing a derived $\delta^{13}C$ = -44.53 ‰ for methanol used in derivatisation. The methanol used for this calibration was also the same methanol used in all transmethylation reactions. 10 µL of 20 mM internal standard in isooctane were added to each 100 µL of sample and samples were diluted to attain 1mg FAME/mL solvent. 0.5 µL of analyte mix was injected, equivalent to 200 pmoles of C15:0 on column. Over a mean area ratio range of sample to internal standard of 1.1 - 0.4 (max 1.62, min 0.22), a precision of 0.3 ‰ was attained (n=12). Over the area ratio range 0.3 – 0.1 (max 0.4, min 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 (25).

Statistical analysis: Parameters were analysed by ANOVA with post-hoc analysis to determine parameters of significant difference between wild and farmed sea bream. Principal Components was conducted using the XLSTAT add-in for Microsoft Excel (Addinsoft France, Paris, France) .

RESULTS

Wild sea bream had a significantly lower lipid content than farmed sea bream ($p< 0.01$) with a concomitant higher nitrogen content in wild sea bream compared with farmed sea bream ($p<0.01$). A bivariate plot of the compositional data yielded discrimination simply on lipid and nitrogen content (**Figure 1**). In terms of the fatty acid profiles, farmed sea bream contained significantly more 14:0, 16:1n-7, 20:1n-9, 22:1n-11, 18:2n-6, 20:5n-3 and 22:6n-3 but significantly less 16:0, 18:0, 18:1n-9, 18:1n-7 and 20:4n-6 compared with wild sea bream (**Table 2**). Wild sea bream contained significantly higher levels of 20:4n-6 and significantly lower levels of 18:2n-6 compared with their farmed counterparts. In addition, the wild fish contained higher 16:0 and 18:1n-9. The isotopic data showed that free fatty acid $\delta^{13}C$ exhibited significant differences in $\delta^{13}C$ values in 16:0, 18:0, 16:1n-7, 18:1n-9 and 18:1n-7 between farmed and wild sea bream (**Figure 2**). Bulk $\delta^{13}C$ analysis of the total oil fraction (**Figure 3**) and glycerol/choline fraction (**Figure 4**) yielded highly significant differences

with farmed fish lighter than wild fish. Significant differences were also observed in the nitrogen content (**Figure 5**) and $\delta^{15}N$ (**Figure 6**) of farmed versus wild sea bream. Analysis of $\delta^{18}O$ from total oil extracted from flesh lipid of sea bream exhibited significant differences between farmed and wild sea bream (**Figure 7**).

Principal Components Analysis (PCA) was used to examine the multivariate structure of the bream data set. **Table 3** demonstrates that the first two factors account for over 70% of the variability within the data, with **Table 4** indicating the contribution of the variables to the selected factors. Plots of Factor 1 vs. Factors 2, 3 & 4 all demonstrate clear separation of wild and farmed bream with factor 1 providing greatest discrimination (**Figure 8**).

DISCUSSION

Recent EU legislation has dictated that the production origin of food must be clearly and verifiably defined for the consumer. The increase in aquaculture production to meet consumer demand for finfish has resulted in a number of farmed species entering the marketplace in the last decade. This includes gilthead sea bream (*Sparus aurata*). Gilthead sea bream were first cultured in Italy in 1970. Production has grown steadily over the past two decades with 2004 production reaching 91,000 metric tonnes with Greece, Turkey and Spain being the major producers (2). The temptation to label farmed fish as wild fish by unscrupulous fish merchants, retailers and restauranteurs is significant because of the price premium commanded by wild fish. In order to combat such mislabelling and conform with the legislation, verifiable methods of distinguishing farmed from wild fish are required for consumer confidence and for local authority enforcement purposes. This study establishes the utility of chemical and isotopic fingerprinting as robust methodologies for distinguishing the production origin of gilthead seam bream (*Sparus aurata*).

Analysis of the fatty acid composition showed marked differences in the lipid profiles and lipid content of the fish. Farmed fish contained significantly more lipid (~5 fold more lipid) as a fraction of flesh composition. This may partly be due to the larger average weight of the farmed bream, although, while larger fish generally accumulate more flesh lipid it is also the case that wild fish are generally leaner than their farmed counterparts (26, 27). Farmed fish also exhibited 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

a lower phosphatidylcholine:triacylglycerol ratio in farmed fish and a higher protein content of wild fish due to greater muscle mass. The fatty acid composition reflected the dietary sources of, particularly polyunsaturated fatty acids. The n-3/n-6 ratio was higher for wild fish compared with farmed fish (4.0 (0.4) vs 3.4 (0.1)) although there were marked differences in the n-3 and n-6 profiles between production origin reflecting dietary intake of plant-derived 18:2n-6 and marine fish oil-derived 22:6n-3 in farmed fish and marine derived 20:4n-6 in wild fish. The commercial diet formulations for sea bream utilise a range of marine and terrestrial products. The terrestrial products can include soybean, wheat, maize, sunflower, pea, lupin and rapeseed meals which can contain a significant lipid component (28). The plant lipid, either from the plant meals described above or added directly as vegetable oil, explains the higher levels of 18:2n-6 in the farmed bream flesh compared to wild fish (29). Wild fish also contained higher 16:0, 18:1n-9 and 20:4n-6 and evidence suggests that bream that tend to feed in inshore and estuarine waters often have fatty acid compositions that reflect the dietary fauna from those areas, whereas aquafeeds contain fish products obtained from the open oceans, which are quite different in their fatty acid compositions (30, 31).

Bulk $\delta^{13}C$ analysis of the total oil fraction and glycerol/choline fraction yielded highly significant differences with farmed fish isotopically lighter than wild fish reflecting the lighter ^{13}C content of farmed fish diets, probably containing significant terrestrial carbon input from vegetable meals and oils, as also evidenced by their fatty acid compositions as described above. Marine fish diets 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 ($\delta^{13}C \sim -7.8‰$). Furthermore, marine vertebrates, through their trophic sequestration of zooplankton and crustacea, further enrich the isotopic signature to produce heavier $\delta^{13}C$ values. The source of dietary lipids in farmed feed 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 likely to be used in aquaculture feeds (32).

The compound specific data highlight differences in the carbon isotope signatures of the major fatty acids between wild and farmed species. Whatever the route to C16:0 in wild fish, dietary 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 de-saturation of the major saturated fatty acids appears to reflect dietary origin of the saturated fatty acid. Significant differences were observed with the most abundant fatty acids probably reflecting the increased precision of analysis of the major analytes. Lower abundance analytes have poorer precision of analysis because they suffer to a greater proportion from baseline perturbations and present less CO_2 to the ion source, which has a significant effect on precision. The choice of the internal standard method for quantitation ensured that accuracy was maintained (against an externally calibrated isotopic standard) and furthermore allowed the quantitation of analyte concentration because of the excellent area ratio response of the IRMSr to CO_2 concentration (33). 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 yields 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 heavier than their farmed counterparts was observed in $\delta^{15}N$. This heavier signal reflects the higher trophic level of wild sea bream within marine food webs and, for farmed fish, $\delta^{15}N$ reflects probably significant input of plant protein from terrestrial sources that are commonly included in aquafeed formulations (28). However, extrapolating these observations across species should be undertaken with care because $\delta^{15}N$ may be influenced by other factors including, maturity or growth rate, seasonal variations in $\delta^{15}N$ in coastal marine environments and possible spoilage of the fish sample between collection and analysis. The sample fractions processed for choline analysis did no undergo a thorough 'clean-up' and may as a result be prone to contamination by spoilage amines of varying ^{15}N natural abundance. The differences observed between farmed and wild sea bream $\delta^{18}O$ 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 sea bream in the present study where farmed bream were from the Mediterranean while wild bream were from the Atlantic. Furthermore, although the geographical location of farmed fish is controlled, wild fish may migrate over large geographical regions, over the

course of their life cycles, and the utility of using $\delta^{18}O$ as a discriminatory factor between farmed and wild fish across species must be carefully considered.

These analytical methodologies provide the basis for discriminating between wild and farmed fish and have the potential to be used across species, particularly the isotopic fingerprinting of individual fatty acids. The decreasing stocks of marine derived feedstuffs for the aquaculture industry entails that there will be an ever increasing terrestrial $\delta^{13}C$ input to farmed fish diets, providing greater isotopic discrimination. As the industry strives to deliver a farmed product that reflects the wild product, sources and blends of terrestrial plants may be produced that mimic the fatty acid composition of wild fish more closely although the isotopic signature would still provide firm discriminatory evidence. The utility of nitrogen and oxygen isotopes, whilst useful, may be complicated by maturity, growth rate and regional variations in fish. The application of compound specific isotope analysis appears to provide robust and confirmatory data which may be used in addition to, or in place of, simple fatty acid composition analysis. However, it is noteworthy that the present study is based on a relatively small sample set and future studies should seek to assess the usefulness of the described methods on fish samples derived from different species and geographical locations, from both the farmed and wild production sectors.

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TABLES AND FIGURE LEGENDS

Table 1. Source and average weight gilthead sea bream.

Species	Source	Country of origin	Average weight (g)
Farmed sea bream	Bernard Corrigan Ltd[1]	Greece	545 g
Wild sea bream	University of Cadiz[1]	Spain	192 g

[1]Bernard Corrigan Ltd., Glasgow (Fish Wholesaler) www.bernardcorrigan.com . [2]Prof. Gabriel Mourente, Dept. de Biologia, University of Cadiz, caught by gill netting in the Bay of Cadiz.

Table 2. Selected fatty acid compositions (weight % of total fatty acids) of farmed and wild gilthead sea bream flesh.

Fatty acid	Farmed	Wild	P value
14:0	5.5 ± 0.1	2.7 ± 0.3	<0.0001
16:0	17.4 ± 0.2	24.9 ± 0.7	<0.0001
18:0	3.2 ± 0.2	8.4 ± 0.3	<0.0001
16:1n-7	8.3 ± 0.1	7.7 ± 0.6	0.0007
18:1n-9	18.8 ± 0.6	25.3 ± 3.0	<0.0001
18:1n-7	3.2 ± 0.1	3.5 ± 0.3	0.001
20:1n-9	2.8 ± 0.1	1.1 ± 0.1	<0.0001
22:1n-11	2.6 ± 0.1	0.2 ± 0.1	<0.0001
18:2n-6	8.0 ± 0.0	1.0 ± 0.2	<0.0001
20:4n-6	0.8 ± 0.0	4.3 ± 1.0	<0.0001
20:5n-3	9.4 ± 0.1	6.5 ± 1.1	<0.0001
22:5n-3	4.2 ± 0.1	4.0 ± 0.7	NS
22:6n-3	15.9 ± 0.4	10.3 ± 1.5	<0.0001

Values are mean ± SD, n = 15. NS = not significant ($P > 0.05$) as determined by Student's t-test (2 tailed, paired samples).

Table 3. Table of Eigenvalues for Principle Components Analysis (PCA) of chemical and isotopic data from sea bream.

	Eigenvalue	Variability (%)	Cumulative Eigenvalue	Cumulative %
F1	15.63	55.84	15.63	55.84
F2	5.21	18.60	20.84	74.44
F3	1.81	6.45	22.65	80.89
F4	1.23	4.38	23.88	85.27
F5	0.99	3.52	24.86	88.79
F6	0.92	3.28	25.78	92.07
F7	0.49	1.76	26.27	93.84

Table 4. Factor loadings for bream PCA – bold type indicates loadings > 0.7

Variable	F1	F2
14:0	**-0.946**	0.149
16:0	**0.914**	-0.337
18:0	**0.955**	-0.260
18:1n-9	**0.705**	-0.665
18:1n-7	**0.805**	0.079
22:1n-11	**-0.969**	0.229
18:2n-6	**-0.925**	0.340
22:5n-3	0.347	**0.797**
16:0 $\delta^{13}C$	**0.923**	0.315
18:0 $\delta^{13}C$	**0.826**	0.401
16:1n-7 $\delta^{13}C$	**0.966**	0.163
18:1n-9 $\delta^{13}C$	**0.867**	0.360
20:5n-3 $\delta^{13}C$	0.227	**0.796**
22:6n-3 $\delta^{13}C$	0.300	**0.804**
Bulk $\delta^{13}C$	**0.931**	0.240
G/C bulk $\delta^{13}C$	**0.982**	0.019
$\delta^{15}N$	**0.706**	-0.396
% N	**0.949**	-0.049
% lipid	**-0.964**	0.125

Figure 1. Bivariate plot of mean (and standard deviation) lipid versus nitrogen (choline) content of wild and framed sea bream.

Figure 2. Compound specific $\delta^{13}C$ analysis of fatty acids from farmed and wild sea bream determined by GC-C-IRMS of flesh lipid.

Figure 3. $\delta^{13}C$ of the bulk oil fraction from total lipid extracted from flesh of wild and farmed sea bream determined by EA-IRMS.

Figure 4. $\delta^{13}C$ of glycerol/choline concentrated from flesh lipid extract in wild and farmed sea bream determined by EA-IRMS.

Figure 5. Percentage nitrogen (% N) determined by weight from N analysis of the concentrated glycerol/choline fraction of wild and farmed sea bream by EA-IRMS.

Figure 6. $\delta^{15}N$ of the glycerol/choline fraction concentrated from flesh lipid extract of wild and farmed sea bream determined by EA-IRMS.

Figure 7. $\delta^{18}O$ of total lipid extract from flesh of wild and farmed sea bream determined by EA-pyrolysis-IRMS.

Figure 8. Principle component analysis (PCA) plot of combined chemical and isotopic data measured from farmed and wild sea bream. Factor 1 and factor 2 combined account for 74.4% of the variation between data sets and afford discrimination of production origin.

Figure 1

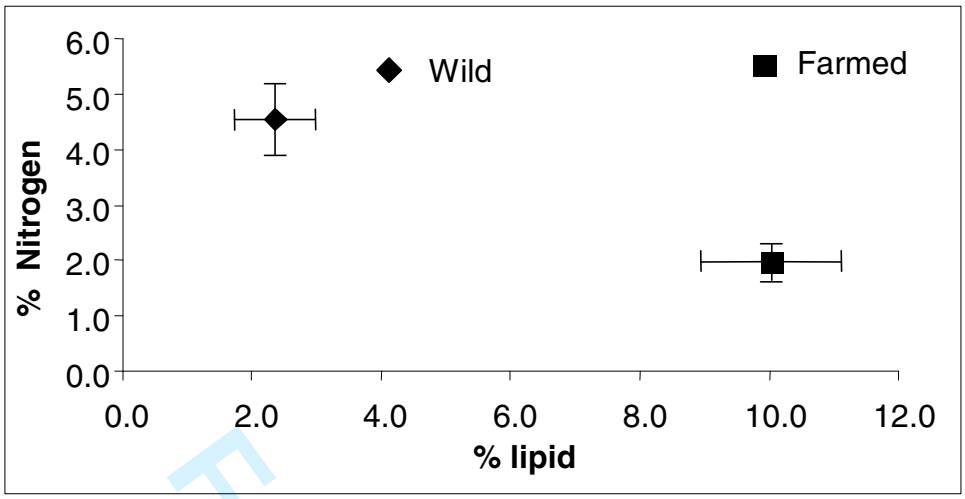
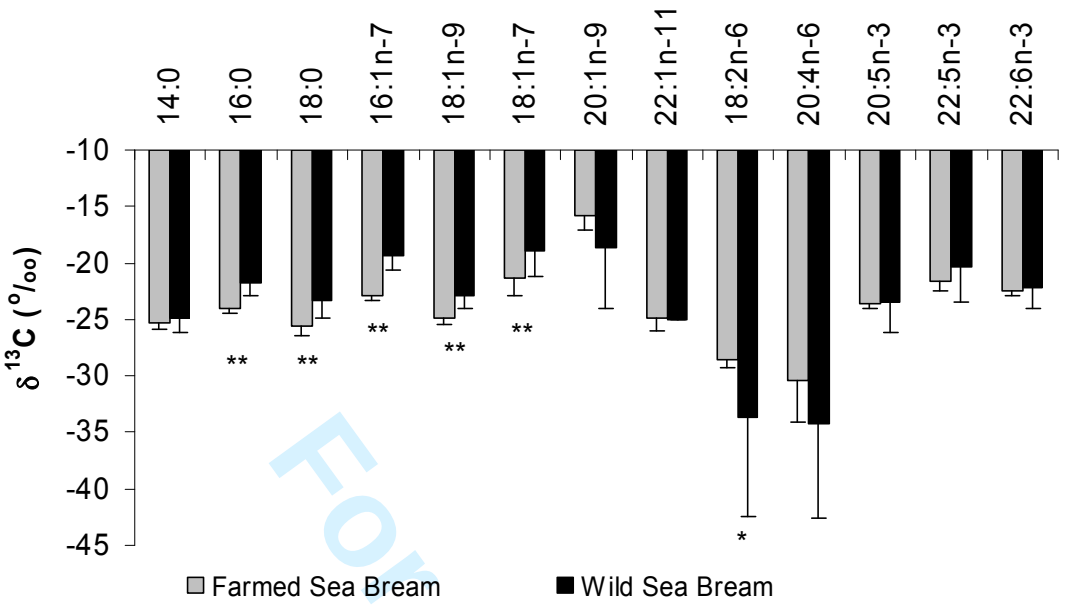
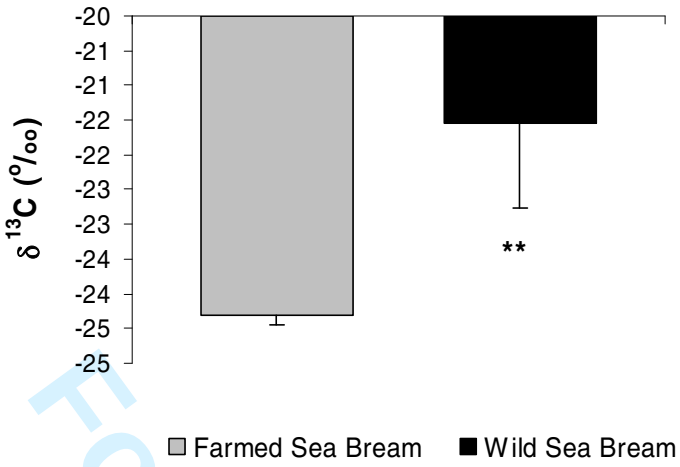


Figure 2



Columns assigned either one or two asterisks are significantly different between farmed and wild samples ($P < 0.05$ and 0.01, respectively).

Figure 3

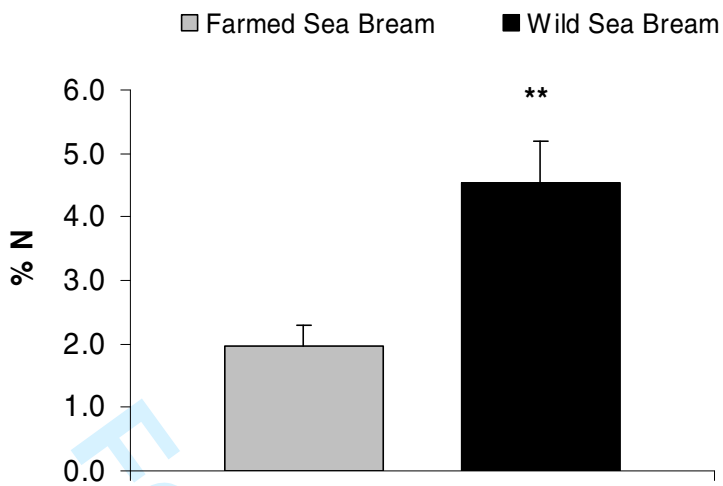


Column assigned ** signifies a significant difference between farmed and wild samples (P < 0. 05).

Figure 4

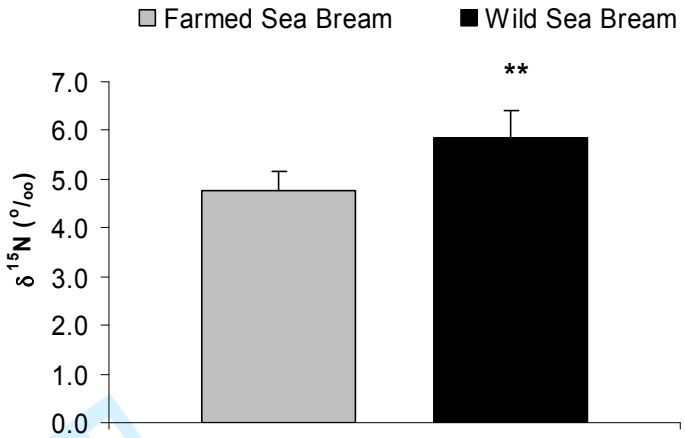
Column assigned ** signifies a significant difference between farmed and wild samples ($P < 0.05$).

Figure 5



Column assigned ** signifies a significant difference between farmed and wild samples (P < 0. 05).

Figure 6



Column assigned ** signifies a significant difference between farmed and wild samples (P < 0. 05).

Figure 7

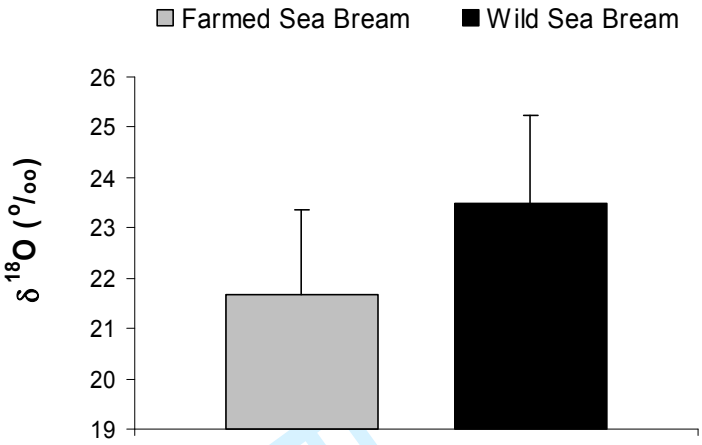


Figure 8