

Stable isotope and multi-elemental fingerprinting for seafood authenticity: discriminating production type and geographical origin

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ABSTRACT

Stable isotope and multi-element fingerprinting were applied to discriminate seafood by production method and geographical origin. A total of 222 samples were analysed, comprising 162 gilthead seabream (*Sparus aurata*) samples (112 farmed and 50 wild) collected from the Mediterranean, Adriatic, and Atlantic regions, and 60 Mediterranean mussel (*Mytilus galloprovincialis*) samples from Portugal (30 farmed and 30 wild). In addition, gill tissues from selected farmed seabream groups were analysed as an exploratory matrix. Stable isotope values ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^2\text{H}$, $\delta^{18}\text{O}$ and $\delta^{18}\text{O}_{\text{water}}$) were determined by IRMS while elemental concentrations were measured by ICP-MS, and the combined dataset was evaluated using chemometric modelling. For seabream, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ showed clear differences between farmed and wild samples, while an OPLS-DA model based on stable isotope variables achieved 100% classification accuracy within the investigated dataset. $\delta^{34}\text{S}$ provided additional discriminatory information for production type and geographical origin in seabream, and for production type in mussels. The highest classification performance for geographical origin was achieved by combining isotopic and elemental variables, reaching 95.5% overall accuracy for seabream. The DD-SIMCA model was used for class modelling and verification of Portuguese-origin samples, achieving 96% accuracy within the investigated dataset, with high sensitivity (93%) and specificity (98%). These findings demonstrate the potential of complementary isotopic and elemental fingerprints for seafood authenticity, while broader applicability requires validation using independent and geographically diverse datasets.

1. Introduction

Seafood, including finfish, crustaceans, and mollusks, serves as a nutritionally dense source of high-quality proteins, amino acids, vitamins, and various minerals, contributing to a balanced diet (Kawarazuka, 2010; Lund, 2013). Its increasing popularity is evidenced by a substantial global increase in consumption, which averaged 20.7 kg per capita in 2022, compared to 9.1 kg per capita reported in 1961 (Food and Agriculture Organization of the United Nations [FAO], 2024). However, overfishing has led to the depletion of global fish stocks, threatening the long-term availability of wild-caught seafood (Elleby et al., 2025). In this context, aquaculture emerges as a viable solution to ensure a sustainable and reliable seafood supply worldwide (Yue & Shen, 2022).

In 2022, aquaculture production reached a record high, accounting for 94.4 million t (51%) of the 185.4 million t of aquatic production (including fish, crustaceans, and mollusks) for the first time (FAO, 2024). Owing to its tolerance to a wide range of salinities and temperatures, omnivorous feeding habits and high market demand, gilthead seabream (*Sparus aurata*) has become one of the principal finfish species in Mediterranean aquaculture (Elalfy et al., 2021; Mhalhel et al., 2023). Globally, Turkey currently accounts for the largest share of farmed gilthead seabream production, with approximately 150,000 t in 2022 representing about 44% of the estimated 344,000 t produced worldwide. Within the EU, aquaculture output of gilthead seabream reached about 107,000 t in 2022. Greece contributed around 70,000 t (about 65% of the total), while Spain, Italy and Croatia produced approximately 9000, 7900 and 7500 t, respectively (each around 7–8% of EU

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production) (Scientific, Technical and Economic Committee for Fisheries [STECF], 2025). In addition, aquaculture development in Portugal and Malta has also expanded, driven by technological advances and increased investment in the seabass and seabream sector (Evans, 2021). Aquaculture production of gilthead seabream was approximately 3.6 thousand tonnes in Portugal in 2021 and 2.1 thousand tonnes in Malta in 2022 (European Market Observatory for Fisheries and Aquaculture Products [EUMOFA], 2024; 2025). In addition to finfish, the Mediterranean mussel (*Mytilus galloprovincialis*) is one of the principal bivalve aquaculture species in Europe. According to the STECF 2024 Aquaculture Economic Report (data for 2022), sea mussels, mainly represented by *Mytilus galloprovincialis*, accounted for approximately 195,000 t of production, representing about 17% of total EU aquaculture output. Portugal is one of the major shellfish-producing countries in the EU (STECF, 2025).

Despite the growing importance of aquaculture, consumers generally prefer wild-caught fish over farm-raised alternatives and are willing to pay an average premium of €1.29 per kg for wild-caught products (Menozzi et al., 2020). At the same time, increasing demand for clear information on product origin and identity reflects consumers' willingness to preferentially select and pay for seafood with verified provenance, highlighting the need for reliable traceability systems (del Río-Lavín et al., 2022; Santos et al., 2023).

In the EU, Regulation (EU) No 1379/2013 (Articles 35 and 38) requires that fishery and aquaculture products listed in Annex I and sold at retail be labelled with at least the commercial designation and scientific name of the species, the production method ("caught at sea", "caught in freshwater" or "farmed") and the catch or production area. The high value of aquatic products, combined with complex supply chains, makes them highly vulnerable to substitution and mislabeling, i.e., food fraud (Mazarakioti et al., 2022). Together, these factors underscore the need for an effective traceability system for ensuring the accuracy of labelling information (Dimitrakopoulou & Vantarakis, 2023).

Stable isotope ratio analysis (SIRA) of light elements (H, C, N, O, S), combined with multi-elemental profiling, is widely recognised as one of the most promising approaches for determining the geographical origin and production method of seafood (Han et al., 2021). The isotopic signatures of these elements reflect distinct environmental and biological controls. In fish tissues, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ predominantly reflect dietary inputs and trophic position, with small systematic shifts introduced during digestion and metabolism (Thomatou et al., 2022; Tulli et al., 2020). In contrast, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values of ambient water are shaped by local hydrological and oceanographic conditions, such as evaporation–precipitation balance, freshwater inputs, temperature and salinity, and are subsequently reflected in fish tissues, making them useful markers for geographical origin discrimination (Soto et al., 2013; Vander Zanden et al., 2016). $\delta^{34}\text{S}$ primarily reflects the relative contribution of marine sulfate versus locally reduced sulfur produced in sediments and coastal environments, and is therefore more closely linked to redox-driven sulfur cycling rather than trophic level, which makes it an useful marker for geographical origin discrimination as well (Jørgensen et al., 2019).

Several studies have demonstrated the effectiveness of SIRA in differentiating between wild and farmed fish species. For example, Bell et al. (2007) found that $\delta^{13}\text{C}$ of total flesh lipids was significantly lower in farmed European sea bass (*Dicentrarchus labrax*) (mean $\approx -24.8\text{‰}$) than in wild individuals (mean $\approx -23.7\text{‰}$). Similarly, Molkentin et al. (2015) distinguished organically from conventionally farmed salmon and brown trout using $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ in defatted dry matter with organic salmon having higher $\delta^{15}\text{N}$ values ($11.6 \pm 0.8\text{‰}$ vs $8.9 \pm 0.6\text{‰}$) and less negative $\delta^{13}\text{C}$ values ($-19.7 \pm 0.6\text{‰}$ vs $-22.0 \pm 0.6\text{‰}$) than conventional salmon. A similar pattern was observed in brown trout, with organic samples showing higher $\delta^{15}\text{N}$ values than conventional ones ($11.6 \pm 0.9\text{‰}$ vs $9.3 \pm 0.5\text{‰}$) and less negative $\delta^{13}\text{C}$ values ($-20.5 \pm 0.7\text{‰}$ vs $-22.0 \pm 0.6\text{‰}$). For salmon, $\delta^{13}\text{C}$ values of lipids also enabled discrimination between organic and wild fish, with values of $-26.0 \pm$

0.5‰ and $-27.9 \pm 0.6\text{‰}$, respectively. Gopi et al. (2019) also achieved clear separation of farmed and wild Asian seabass (*Lates calcarifer*) from different regions using $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, with wild fish showing higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values than farmed fish, reflecting differences in natural diet versus aquaculture feed.

Combining SIRA with elemental profiling has been shown to enhance discrimination power. Anderson et al. (2010) demonstrated that both isotopic and elemental profiles, together, effectively distinguish between wild and farm-raised salmon, identifying eight elements (As, Cu, Ca, Mg, Na, P, K, and Zn) that are consistently above detection limits as key differentiators. Likewise, del Río-Lavín et al. (2022) found significant differences in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, as well as concentrations of Ba, Mn, Pb, and Al, among mussels from different geographical locations, identifying these as the most informative markers for provenance discrimination.

While most studies focused on carbon and nitrogen isotopic ratios, sulfur isotopes have received comparatively little attention. However, recent work by Thomatou et al. (2022) demonstrated that $\delta^{34}\text{S}$ is effective for distinguishing the geographical origin of Greek bottarga (*Avgotaracho Messolongiou*), showing its potential.

Reliable authentication of geographical origin using SIRA and Multi-elemental composition depends on the availability of authentic and representative reference datasets against which unknown samples can be compared (Camin et al., 2017). For provenance determination, such datasets must be constructed according to the specific analytical objective and include well-characterised authentic samples from the relevant origins (Kelly et al., 2005; Patarnello et al., 2026). A key added value of the present study is the incorporation of newly generated isotopic and elemental data for gilthead seabream and Mediterranean mussel into the publicly available IsoFoodTrack database, thereby strengthening the reference dataset available for future seafood authenticity and traceability studies. The IsoFoodTrack database, established at the Jožef Stefan Institute, serves as a central repository for analytical data related to food authenticity (<http://isofoodtrack.ijs.si>; Terro et al., 2025) and includes various commodities susceptible to fraud, including seafood such as fish, bivalves, cephalopods, crustaceans, and echinoderms. Once such reference datasets are established, chemometric and other multivariate statistical approaches can be used to explore data structure, discriminate among origins or production systems, and support the classification of unknown samples (González-Domínguez et al., 2022; Terro et al., 2025).

This study presents an integrated analytical framework for seafood authenticity and traceability based on the combined use of stable isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^2\text{H}$, $\delta^{18}\text{O}$ and $\delta^{18}\text{O}_{\text{water}}$) and multi-elemental profiles evaluated by chemometric modelling. While previous studies have typically addressed either production method or geographical origin, the present work systematically evaluates both within a single dataset covering multiple seafood species and marine regions. This study evaluates the potential contribution of sulfur isotopes alongside carbon and nitrogen isotopes for production-type and geographical-origin discrimination. The complementary value of isotopic and elemental data for geographical classification is also assessed using OPLS-DA and DD-SIMCA. The resulting authentic data are incorporated into the IsoFoodTrack database to support the development of reference datasets for future seafood authenticity studies.

2. Materials and methods

2.1. Sampling collection

A total of 222 fish and mussel samples were collected between 2023 and 2024 from Malta (Mediterranean Sea) and Portugal and Spain (Atlantic Ocean) (Fig. 1; Table 1). The dataset included authentic wild and farmed gilthead seabream (*Sparus aurata*) and Mediterranean mussels (*Mytilus galloprovincialis*). As all mussel samples originated from Portugal, they were used only for farmed-versus-wild classification and



Fig. 1. Map of the five sampling locations in Europe.

Table 1
Sample collection information.

Origin	Production type	Tissue type	Sample size	Category	Altitude and latitude
Mediterranean Sea (Malta)	Farmed	Seabream fillet	60	Authentic	35°50'10.9"N 14°32'40.8"E
Atlantic Ocean (Spain)	Farmed	Seabream fillet and gills	15	Authentic	42°31'11.5"N 9°02'14.1"W
Atlantic Ocean (Portugal)	Wild	Seabream fillet	20	Authentic	43.3619° N 8.5537° W
	Farmed	Seabream fillet	30	Authentic	37°02'01.9"N 7°49'05.1"W
	Wild	Seabream fillet	30		36°59'24.9"N 7°49'47.9"W
	Farmed	Mussel tissue	30		37°00'59.3"N 8°56'57.9"W
	Wild	Mussel tissue	30		37°00'59.3"N 8°56'57.9"W
Adriatic Sea (Slovenia)	Farmed	Seabream fillet and gills	3	Commercial	
Adriatic Sea (Croatia)	Farmed	Seabream fillet and gills	4	Commercial	

not for geographical origin modelling. In addition, commercial farmed gilthead seabream samples labelled as originating from Slovenia and Croatia (Adriatic Sea) were collected in March 2024. Fish fillets were selected as the primary matrix because they are the edible tissue most relevant for food authentication and traceability studies and integrate long-term dietary and environmental signals. Gill samples were included as an exploratory matrix because they are directly exposed to the surrounding environment and harbour microbial communities that may contain complementary information on farming conditions and geographic origin.

2.2. Sample preparation

Whole fish were dispatched by air within one week of collection and shipped frozen at -30°C in zip-lock plastic bags. Fish fillets, available gill samples and edible parts of mussels were selected for analysis. Upon arrival, fish were first dissected, and the fillets were frozen at -80°C for 24 h. The fillets were thawed, and water extracted from the fillets was filtered through a GF/PVDF 45/25 filter (Macherey-Nagel, Chromafil, Germany) and stored at 4°C for no more than 24 h prior to analysis. Fillets were homogenised in a titanium knife mill blender and then freeze-dried (Liof Gamma 1–16 LSC plus, Christ, Germany), whereas gills and mussels were freeze-dried without prior homogenisation.

2.3. Stable isotope analysis

2.3.1. Isotope ratio mass spectrometry (IRMS)

Stable isotope measurements were performed using an isotope ratio mass spectrometer (IRMS) and reported using the δ -notation in ‰ according to Eq. (1) (Brand et al., 2014):

$$\delta^{i/j}E(\text{‰}) = \left(\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1 \right) \times 1000 \quad (1)$$

Superscripts i and j denote the highest and the lowest atomic mass number of element E, respectively. R_{sample} and $R_{\text{reference}}$ represent the corresponding isotope ratios, ($^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{18}\text{O}/^{16}\text{O}$, $^{15}\text{N}/^{14}\text{N}$, $^{34}\text{S}/^{32}\text{S}$) in the sample and reference material. The isotope values were reported relative to Vienna Standard Mean Ocean Water (VSMOW) for $\delta^2\text{H}$ and $\delta^{18}\text{O}$, Vienna Pee Dee Belemnite (VPDB) for $\delta^{13}\text{C}$, Vienna Cañon Diablo Troilite (VCDT) for $\delta^{34}\text{S}$, and AIR for $\delta^{15}\text{N}$.

2.3.2. Determination of bulk $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ isotope values

Bulk stable isotope compositions were determined in fish gills, defatted fish fillets, and edible mussel tissues. Prior to analysis, approximately 4 g of freeze-dried fish fillet and 0.4 g of freeze-dried mussel tissue were defatted by three consecutive extractions using a 2:1 mixture of petroleum ether (CARLO ERBA Reagents, France) and diethyl ether (Supelco, Merck, Germany). For each extraction, 30 mL of solvent mixture was used for fish samples and 2.5 mL for mussel

samples. Samples were homogenised using an Ultraturrax homogeniser (IKA T18 digital ULTRA-TURRAX, Germany), centrifuged at 3000 rpm for 5 min, washed twice with Milli-Q water, freeze-dried, homogenised, and stored until analysis.

Bulk $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values were measured using an IsoPrime100 isotope ratio mass spectrometer (IRMS; IsoPrime, UK) coupled to a Vario PYRO Cube elemental analyser (Elementar, Germany). Approximately 3.5 mg of sample was weighed into tin capsules together with tungsten trioxide (WO_3), sealed, and introduced into the elemental analyser. Bulk $\delta^2\text{H}_{\text{solid}}$ and $\delta^{18}\text{O}_{\text{solid}}$ values were determined using a DELTA XP IRMS (Thermo Scientific, Germany) coupled to a TC/EA pyrolyser. Approximately 0.2 mg of sample was weighed into silver capsules. To minimise the effect of exchangeable hydrogen, samples and reference materials were equilibrated with ambient moisture in a desiccator for five days prior to analysis. All measurements were performed in duplicate.

The carbon and nitrogen obtained data were normalised against the following international reference materials: USGS89 with $\delta^{13}\text{C} = -18.13 \pm 0.11\text{‰}$ and $\delta^{15}\text{N} = 6.25 \pm 0.12\text{‰}$, IAEA-600 with $\delta^{13}\text{C} = -27.77 \pm 0.04\text{‰}$ and $\delta^{15}\text{N} = 1.02 \pm 0.05\text{‰}$, USGS88 with $\delta^{13}\text{C} = -16.06 \pm 0.07\text{‰}$ and $\delta^{15}\text{N} = 14.96 \pm 0.14\text{‰}$, USGS91 with $\delta^{13}\text{C} = -28.28 \pm 0.08\text{‰}$ and $\delta^{15}\text{N} = 1.02 \pm 0.06\text{‰}$ and USGS43 with $\delta^{13}\text{C} = -21.28 \pm 0.06\text{‰}$ and $\delta^{15}\text{N} = 8.44 \pm 0.10\text{‰}$. Sulfur isotope data were normalised against the international reference materials USGS89 with $\delta^{34}\text{S} = 3.86 \pm 0.56\text{‰}$, USGS88 with $\delta^{34}\text{S} = 17.10 \pm 0.44\text{‰}$, and USGS43 with $\delta^{34}\text{S} = 10.46 \pm 0.22\text{‰}$. CRP-IAEA casein with $\delta^{13}\text{C} = -20.3 \pm 0.09\text{‰}$, $\delta^{15}\text{N} = 5.62 \pm 0.19\text{‰}$ and $\delta^{34}\text{S} = 4.18 \pm 0.74\text{‰}$ was used as laboratory control material. Normalisation of the H and O data was performed based on two reference materials: CBS (Caribou Hoof Standard) with $\delta^2\text{H}$ values of $-157 \pm 0.9\text{‰}$ and $\delta^{18}\text{O}$ values of $2.39 \pm 0.13\text{‰}$, and KHS (Kudu Horn Standard) with an average $\delta^2\text{H}$ value of $-35.3 \pm 1.1\text{‰}$ and $\delta^{18}\text{O}$ value of $21.21 \pm 0.17\text{‰}$. The reproducibility of measurements for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ was $\pm 0.2\text{‰}$, $\pm 0.7\text{‰}$ for $\delta^{34}\text{S}$, $\pm 0.5\text{‰}$ for $\delta^{18}\text{O}$ and $\pm 5\text{‰}$ for $\delta^2\text{H}$.

2.3.3. Determination of $\delta^{18}\text{O}$ in fish water

The oxygen isotope composition of water extracted from fish fillets ($\delta^{18}\text{O}_{\text{water}}$) was determined after equilibration with CO_2 . Briefly, 200 μL of extracted water was purged with CO_2 and analysed using an IsoPrime100 IRMS connected to a Multiflow Bio preparation system (IsoPrime, Cheadle, UK). Data were normalised against two laboratory reference materials: seawater ($\delta^{18}\text{O} = 0.36 \pm 0.04\text{‰}$) and snow ($\delta^{18}\text{O} = -18.91 \pm 0.03\text{‰}$) and one international reference material, USGS53 ($\delta^{18}\text{O} = 5.47 \pm 0.03\text{‰}$). Milli-Q water ($\delta^{18}\text{O} = -9.12 \pm 0.04\text{‰}$) was used as quality control material.

The reproducibility of measurements for $\delta^{18}\text{O}_{\text{water}}$ was $\pm 0.5\text{‰}$.

2.4. Multi-elemental analysis

2.4.1. Sample digestion

A known weight (0.1 g) of freeze-dried fish fillet, fish gill or mussel sample was weighed into a pre-cleaned Teflon digestion vial, followed by the addition of 2 mL HNO_3 (67%, CarloErba, suprapur); in the case of gill samples, 0.5 mL H_2O_2 (30%, Merck, suprapur) was also added. Samples were digested using the ULTRA WAVE, Single Reaction Chamber (SRC) Microwave Digestion System (MILESTONE, Italy). The program was as follows: heating to 240°C in 20 min and holding at 240°C for 15 min, with a maximum pressure of 100 bar and a maximum power of 1500 W. The digested solutions were transferred into measuring tubes and diluted to 10 mL with Milli-Q water. To ensure the quality of the results, blank samples and certified reference materials produced by the National Research Council of Canada (NRC), specifically DOLT-5 Dogfish Liver and DORM-5 Fish Protein, were run in the same batch as the samples. At least every tenth sample was prepared in duplicate. Before analysis, the samples were additionally diluted with 5% HNO_3 in a 1:5 ratio.

2.4.2. ICP-MS analysis

The determination of selected elements was performed using an Agilent 8800 triple quadrupole inductively coupled plasma mass spectrometer (QQQ-ICP-MS; Agilent, Japan). The instrument was equipped with a micro-mist nebuliser, a Scott-type spray chamber, and an ASX-510 autosampler (Cetac). The instrument conditions were as follows: plasma gas flow rate of 15 L/min, carrier gas flow rate of 0.95 L/min, make-up gas flow rate of 0.1 L/min, sample solution uptake flow rate of 0.3 mL/min, RF power of 1500 W, sampling depth 8 mm, nickel sampling and skimmer cones. The internal standards used were ^{45}Sc , ^{89}Y , ^{103}Rh , and ^{157}Gd . To eliminate interference, a collision reaction cell was used with helium, hydrogen, or oxygen gas. External calibration was used for the quantification of the results.

The limits of detection (LoD), calculated as three times the standard deviation of the blank samples were as follows: sulfur (S) 230 $\mu\text{g/g}$, calcium (Ca) 8 $\mu\text{g/g}$, potassium (K) 6 $\mu\text{g/g}$, sodium (Na) 3 $\mu\text{g/g}$, phosphorus (P) 0.6 $\mu\text{g/g}$, magnesium (Mg) 0.3 $\mu\text{g/g}$, zinc (Zn) 200 ng/g, iron (Fe) 90 ng/g, nickel (Ni) 40 ng/g, copper (Cu) 20 ng/g, chromium (Cr) 15 ng/g, selenium (Se) 8 ng/g, manganese (Mn) 7 ng/g, molybdenum (Mo) 1 ng/g, cobalt (Co) 0.9 ng/g, boron (B) 280 ng/g, lithium (Li) 30 ng/g, strontium (Sr) 20 ng/g, arsenic (As) 5 ng/g, barium (Ba) 3 ng/g, rubidium (Rb) 2.5 ng/g, mercury (Hg) 2 ng/g, lead (Pb) 2 ng/g, tin (Sn) 2 ng/g, silver (Ag) 1 ng/g, antimony (Sb) 0.7 ng/g, cadmium (Cd) 0.6 ng/g, vanadium (V) 0.6 ng/g, thallium (Tl) 0.4 ng/g, and uranium (U) 0.2 ng/g.

2.5. Statistical analysis

The final isotope and elemental data were organised in Microsoft Excel spreadsheets and uploaded to the IsoFoodTrack database (www.isofoodtrack.ijs.si; Terro et al., 2025). Descriptive statistics (mean, standard deviation, minimum and maximum) were calculated using Microsoft Excel. Normality and homogeneity of variance were evaluated prior to statistical analysis. Because at least one of these assumptions was not satisfied, group comparisons were performed using the non-parametric Mann–Whitney U test (two-group comparisons) and Kruskal–Wallis test (multiple-group comparisons). These tests were selected because they are robust to deviations from normality and are suitable for comparing independent groups based on ranked observations (Eftimov et al., 2017). These tests were selected because they are robust to deviations from normality and are suitable for comparing independent groups based on ranked observations. Statistical analyses were conducted in R (version 4.2.1; R Core Team, 2022). AI was consciously excluded from the statistical analysis, as some of the fish and mussel samples were stored in aluminium foil during shipment. Additionally, elements with more than 30% of observations below the LoD were excluded from the statistical analysis. In the fish muscle dataset this criterion led to the removal of ten elements (Li, Ba, Ni, Ag, Cd, Sn, Sb, Tl, Pb and U), while in the fish gill dataset only Tl was excluded. For mussels, all elemental concentrations were included in statistical evaluation. In the remaining datasets, values below the detection limit were replaced with one-half of the corresponding LoD before statistical analysis.

Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was used to discriminate between predefined sample classes. Model performance was evaluated using $R^2\text{X}(\text{cum})$, $R^2\text{Y}(\text{cum})$, and $Q^2(\text{cum})$, while model robustness was assessed by permutation testing ($N = 200$) and CV-ANOVA (Fiamegos et al., 2021). Classification performance was evaluated using accuracy, sensitivity, specificity, and F1 score. Discriminant markers were identified based on Variable Importance in Projection (VIP) values, with $\text{VIP} > 1$ considered significant. OPLS-DA models were developed using SIMCA version 18.0 (Sartorius Stedim Data Analytics AB, Umeå, Sweden).

For production-method classification, all seabream samples were included in the OPLS-DA analysis. For geographical origin assessment, only samples from Malta ($n = 60$), Spain ($n = 35$), and Portugal ($n = 60$)

were considered, as these countries provided sufficient sample numbers. Samples from Slovenia ($n = 3$) and Croatia ($n = 4$) were excluded due to insufficient representation.

Data-driven Soft Independent Modelling of Class Analogy (DD-SIMCA) was applied as a one-class classification approach for sample authentication (Pomerantsev & Rodionova, 2014). The method is based on principal component analysis (PCA) and evaluates sample conformity to a predefined target class using score and orthogonal distances. Model performance was assessed using sensitivity and specificity as measures of correct acceptance and rejection of samples, respectively. DD-SIMCA analyses were performed using the modified DD-SIMCA MATLAB GUI tool (Zontov et al., 2017) and the mdatools package in R version 4.0.3 (Kucheryavskiy, 2020), following the procedures described by Strojnik et al. (2022) and Mahynski et al. (2025).

3. Results and discussion

Results of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}_{\text{solid}}$, $\delta^2\text{H}_{\text{solid}}$ and $\delta^{18}\text{O}_{\text{water}}$ are summarised in Table 2, while the complete dataset is provided in Table S1 of the Supplementary Material 2. In our dataset, gills showed lower $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^2\text{H}$ values and higher $\delta^{34}\text{S}$ values than fillets, indicating tissue-specific isotopic patterns.

This pattern is consistent with gills being in continuous contact with seawater and more strongly influenced by dissolved nutrient and sulfate pools in seawater, whereas fillet (muscle) integrates dietary inputs and body-water composition over longer time scales, which results in consistent differences between the two tissues. Similar reasoning underlies the work of Meriggi et al. (2024), who showed that gill-associated microbiota in marine fish closely reflects local environmental conditions. Furthermore, higher element concentrations in gills likely reflect their continuous contact with seawater and their role in ion exchange and metal uptake, whereas muscle is less directly exposed to the water and primarily incorporates elements via diet (exact elemental concentrations are provided in Table S3).

In fillet samples, $\delta^2\text{H}_{\text{solid}}$ and $\delta^{18}\text{O}_{\text{solid}}$ varied within a relatively narrow range and did not show clear clustering by country (Table 2; Figs. S11–S14 in the Supplementary Material 1). This indicates that both isotopes mainly reflect a common marine water baseline rather than strong regional differences. The combined distribution of $\delta^2\text{H}_{\text{solid}}$ and $\delta^{18}\text{O}_{\text{solid}}$ in fillets is therefore consistent with hydrogen and oxygen in tissue being controlled predominantly by the isotopic composition of ambient seawater and body water. Because the hydrological conditions across the sampled regions are relatively similar, these isotopes provide limited additional power for distinguishing geographical origin in this dataset. Extracted water $\delta^{18}\text{O}$ is expected to be closest to the isotopic composition of ambient seawater, whereas bulk $\delta^{18}\text{O}_{\text{solid}}$ additionally reflects incorporation of oxygen into organic and mineral components

through diet and metabolism. Consequently, the $\delta^{18}\text{O}_{\text{water}} - \delta^{18}\text{O}_{\text{solid}}$ relationship in fillets mainly confirms the influence of the local seawater signal, but does not provide strong additional geographical discrimination at the spatial scale considered here.

3.1. Production method (farmed vs wild)

3.1.1. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ in seabream fillet and mussels

The initial evaluation of results involved comparing $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values between farmed and wild seabream fillets from all five countries using a bi-plot presented in Fig. 2. Higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values are typically found in wild marine fish compared to farmed fish. These differences are linked to feeding habits and trophic levels. The commercial feed given to farmed fish, mostly made from vegetable meals and oils, is influenced by terrestrial carbon input resulting in farmed fish being enriched in ^{12}C isotope. Marine fish feed on microalgae, mollusks, crustaceans, small fish and phytoplankton, which utilise dissolved inorganic carbon (DIC) with $\delta^{13}\text{C} = 0\text{‰}$, which contributes to wild fish being enriched in ^{13}C isotope (Morrison et al., 2007). For nitrogen isotopes, fish occupying higher trophic levels have higher $\delta^{15}\text{N}$ values, with an increase of approximately 3‰ for each trophic level above their prey (Camin et al., 2016). Variations in $\delta^{15}\text{N}$ values in farmed fish are also influenced by inputs from terrestrial nitrogen sources, which can range from -5‰ to 18‰ (Bell et al., 2007). It should be noted that several factors known to influence stable isotope compositions, including maturity, growth rate, and seasonal variations in environmental isotopic baselines. Maturity stage and growth rate may influence $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values through ontogenetic dietary shifts, metabolic turnover and tissue-specific isotope incorporation, while seasonal changes in coastal baseline conditions may affect $\delta^{13}\text{C}$, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ through temporal variation in primary production, nutrient sources and sulfur pools (Ke et al., 2022; Rodde et al., 2020; Skinner et al., 2022). Sample condition may also affect $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values if degradation occurs before preservation (Stallings et al., 2015), although this effect was minimised in the present study by frozen transport and storage prior to sample preparation. These factors were not assessed separately and may have contributed to within-group isotopic variability, particularly for geographical origin interpretation; however, they did not obscure the main production-type differences, and geographical classification was evaluated using combined isotopic and elemental fingerprints together with model validation.

Unlike $\delta^{15}\text{N}$ values, which increase with each trophic transfer, $\delta^{34}\text{S}$ is often treated as a conservative tracer because trophic fractionation is very small (typically close to 0‰), so the isotopic signature of the primary production source is largely preserved in consumer tissues (Peterson & Fry, 1987; Raoult et al., 2024). Raoult et al. (2024) showed that sulfur isotopes ($\delta^{34}\text{S}$) have small, taxon-dependent trophic

Table 2

Isotope data of seabream fillet, gills and mussel varying in geographical origin and production method. Results are expressed as mean values and standard deviations of the mean.

Country	Prod. type	Tissue	$\delta^{18}\text{O}_{\text{water}}$		$\delta^{13}\text{C}$		$\delta^{15}\text{N}$		$\delta^{34}\text{S}$		$\delta^{18}\text{O}_{\text{solid}}$		$\delta^2\text{H}_{\text{solid}}$	
			$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD	$\bar{x}$	SD
Malta	F	SBr-f	1.14	±0.22	−22.5	±0.44	8.72	±0.28	6.49	±0.71	17.8	±0.53	−79.3	±2.98
	F	SBr-f	n.d.	n.d.	−21.1	±0.11	9.9	±0.13	10.4	±0.23	17.3	±0.29	−76.3	±1.48
Spain	F	SBr-g	n.a.	n.a.	−24.0	±0.30	7.9	±0.22	11.3	±0.42	n.d.	n.d.	n.d.	n.d.
	W	SBr-f	−0.08	±0.93	−16.9	±0.46	12.5	±0.47	15.9	±0.79	18.3	±0.27	−85.1	±5.82
Portugal	F	SBr-f	1.20	±0.11	−19.4	±0.22	9.3	±0.19	9.0	±0.23	17.2	±0.36	−78.8	±2.33
	W	SBr-f	1.00	±0.16	−18.1	±1.85	12.3	±0.91	15.3	±1.59	17.9	±0.51	−82.2	±5.17
	F	M	n.d.	n.d.	−19.3	±0.42	5.6	±0.34	19.1	±0.44	21.7	±0.53	−97.5	±2.58
	W	M	n.d.	n.d.	−19.5	±0.40	5.8	±0.33	19.9	±0.68	21.0	±0.47	−99.6	±2.88
Slovenia	F	SBr-f	0.32	±0.10	−21.6	±0.18	9.3	±0.27	7.5	±0.26	17.4	±0.30	−80.5	±3.90
	F	SBr-g	n.a.	n.a.	−24.9	±1.41	8.9	±0.32	10.0	±0.53	17.9	±0.24	−110	±9.55
Croatia	F	SBr-f	−0.84	±1.34	−21.3	±0.13	9.0	±0.34	6.9	±0.73	17.0	±0.53	−82.6	±3.11
	F	SBr-g	n.a.	n.a.	−24.9	±0.89	8.3	±0.35	7.9	±1.56	15.4	±0.90	−113	±2.91

$\bar{x} \pm \text{SD}$ = mean $\pm$ standard deviation; F = farmed; W = wild; SBr-f = seabream fillet; SBr-g = seabream gills; M = mussels; n.a. = not applicable; n.d. = not determined. For mussels and Spanish fillets, $\delta^{18}\text{O}_{\text{water}}$ was not determined due to insufficient extractable water. Bulk $\delta^2\text{H}_{\text{solid}}$ and $\delta^{18}\text{O}_{\text{solid}}$ were not determined in gills.

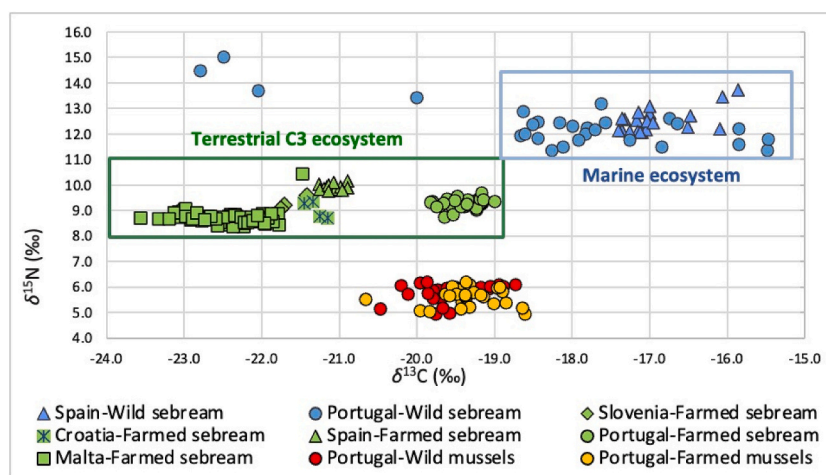


Fig. 2. Relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in farmed and wild fish fillets from all 5 countries (Malta, Spain, Portugal, Slovenia, Croatia) and wild and farmed mussels from Portugal.

discrimination but large contrasts among sulfur sources, making them highly complementary to $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. Including $\delta^{34}\text{S}$ often improves separation of benthic and pelagic pathways and increases ecological resolution compared with using carbon and nitrogen alone.

Fig. 2 shows the relationship between $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in wild and farmed samples. Variable selection was based on discriminatory performance. The combination of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values achieved complete separation of the investigated groups, whereas inclusion of $\delta^{34}\text{S}$ did not provide additional classification power. Therefore, only $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were used for visualization.

The $\delta^{13}\text{C}$ values in our fish samples ranged from -23.6‰ to -19.0‰ for farmed and from -18.7‰ to -15.5‰ for wild fish, with four points having lower $\delta^{13}\text{C}$ values. These were wild fish samples from Portugal. They suggest that the fish were either caught in areas with higher inputs of terrestrial particulate organic matter or may have escaped from aquaculture facilities and subsequently integrated into the wild population. The $\delta^{15}\text{N}$ values ranged from 8.4‰ to 10.2‰ for farmed and from 11.1‰ to 13.7‰ for wild fish. A Mann–Whitney test revealed highly significant differences between farmed and wild seabream for $\delta^{13}\text{C}$ ($p < 0.0001$) and $\delta^{15}\text{N}$ ($p < 0.0001$), showing clear differentiation between farmed and wild seabream within the investigated dataset.

The findings of Serrano et al. (2007) align with those of this paper regarding the successful differentiation of wild-caught and farmed seabream (*Sparus aurata*) from the western Mediterranean. They reported higher $\delta^{15}\text{N}$ values in farmed fish compared to wild-caught fish, with a difference of $1.5 \pm 0.2\text{‰}$, likely due to the aquaculture feed, which contained animal-derived proteins such as fishmeal that are naturally enriched in ^{15}N . Similarly, Varrà et al. (2019) reported significantly higher $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values in wild European seabass compared to farmed individuals, consistent with this study's findings for gilthead seabream. Previous studies that successfully discriminated between wild-caught and farmed seafood using carbon and nitrogen isotope abundances include Morrison et al. (2007) in seabream; Gamboa-Delgado et al. (2014) and Ortea and Gallardo (2015) in shrimp; Dempson and Power (2004) in Atlantic salmon; and Bell et al. (2007) in seabass.

Although $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values clearly differentiate by production method, this approach was less effective for Portuguese mussels. The mean $\delta^{13}\text{C}$ values were -19.3‰ for farmed and -19.5‰ for wild mussels ($p = 0.022$), while the mean $\delta^{15}\text{N}$ values were 5.6‰ for farmed and 5.8‰ for wild mussels ($p = 0.012$). The $\delta^{13}\text{C}$ – $\delta^{15}\text{N}$ biplot (Fig. 2) shows substantial overlap between farmed and wild groups, indicating lower discriminatory power compared to fish samples. These results are consistent with del Rio-Lavín et al. (2022), who reported similar $\delta^{13}\text{C}$ ($-19.00 \pm 0.75\text{‰}$) and $\delta^{15}\text{N}$ ($4.47 \pm 0.39\text{‰}$) values for farmed

Portuguese mussels. The limited separation between farmed and wild mussels is likely due to the proximity of sampling sites, which results in similar environmental and dietary inputs influencing isotopic composition. As filter feeders, mussels rely primarily on planktonic particulate organic matter, resulting in lower trophic variability compared to fish (Kasai et al., 2004). The non-parametric Mann–Whitney test revealed significant differences between wild and farmed mussels for $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, P, and Mo. Among these variables, $\delta^{34}\text{S}$ and P showed the strongest differences between groups (both $p < 0.0001$), followed by Mo ($p = 0.008$). Wild mussels exhibited higher $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values and higher Mo concentrations, whereas farmed mussels showed higher $\delta^{13}\text{C}$ values and P concentrations. The higher P concentrations observed in farmed mussels may reflect nutrient-enriched farming environments and phosphorus-rich suspended food sources.

3.1.2. OPLS-DA analysis of seabream fillet and mussels

OPLS-DA was used to classify fish samples by production method (wild vs. farmed) regardless of their geographical origin using only stable isotopes (Fig. 3). The overall accuracy of the model was 100%, with the most discriminative parameters being $\delta^{34}\text{S} > \delta^{15}\text{N} > \delta^{13}\text{C} > \delta^{18}\text{O}_{\text{water}}$, all of which showed higher values in wild fish as expected. Within the investigated dataset, stable isotope variables provided strong discrimination between wild-caught and farmed seabream. These findings are consistent with those of Varrà et al. (2019), who reported 100% correct classification of farmed and wild-caught Mediterranean seabass using a similar approach.

Importantly, within the present dataset this production-type classification was robust across the different geographic origins considered, meaning that a single isotopic model could be applied without prior knowledge or reliable labelling of origin. However, the model was developed using samples from only five countries (Malta, Portugal, Spain, Slovenia and Croatia), and further work is needed to assess whether isotopic differentiation between production types remains consistent in other regions. OPLS-DA was applied to Portuguese mussel samples to identify the best model for classifying wild and farmed groups (Fig. 4). The model was further optimised, and the most promising variables, ultimately included in the model, were: $\delta^{34}\text{S}$, P, Mo, Tl, K, As, Mg, Na, Zn, V, Rb, Cd, Cu, Pb, and Ag. The overall accuracy of the model was 84.8% (See Fig. 5.).

Sorte et al. (2013) demonstrated that elemental fingerprinting of mussel shells can reliably assign populations to their source regions with 97% accuracy, highlighting its potential for traceability studies. Element accumulation in mussels, however, is tissue-specific: Deudero et al. (2009) found that the digestive gland is a suitable indicator for Pb and Cd, while the foot serves as a reliable proxy for Pb, Cd, Se, and Zn. In

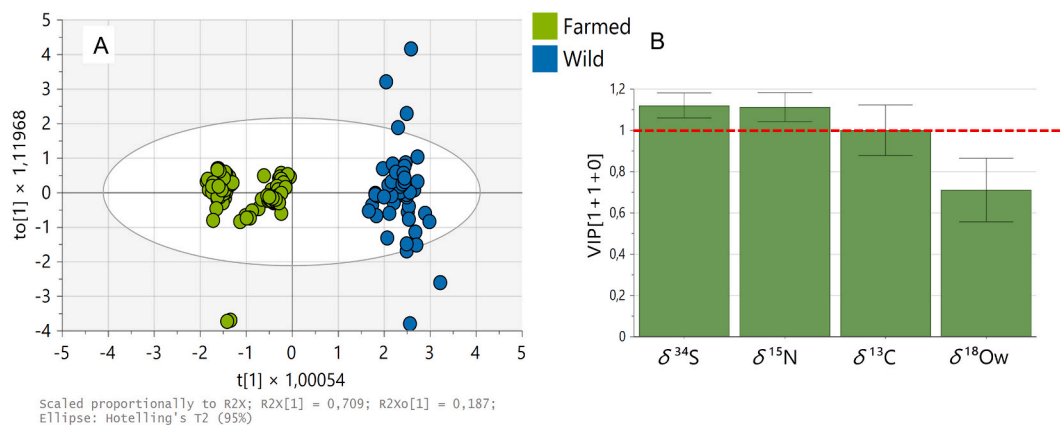


Fig. 3. OPLS-DA score plot A) and VIP values B) in the pairwise comparison between farmed and wild seabream samples from 5 different countries. Samples are coloured according to their production type. The ellipse on the score plot represents the 95% confidence interval. A red-dashed line indicates the criteria used to identify the most important variables. VIP values indicate each variable's contribution to class separation, with values >1 considered influential. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

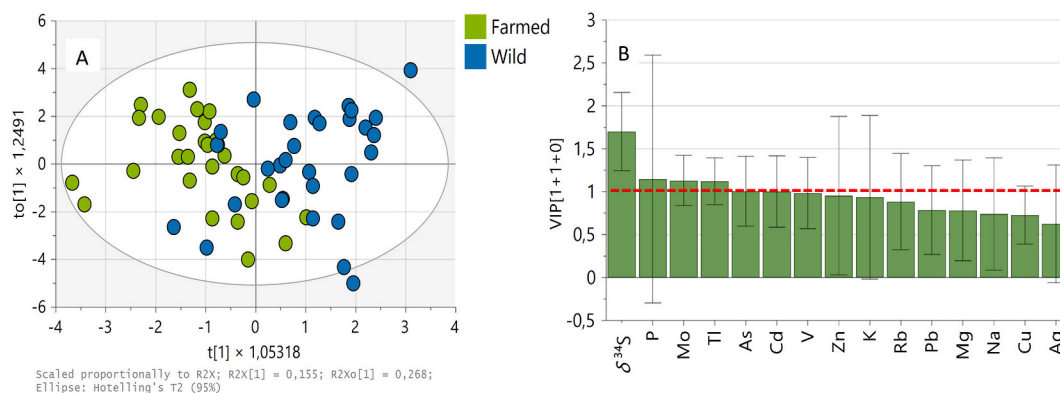


Fig. 4. OPLS-DA score plots A) and VIP values B) in the pairwise comparisons between different types of mussel production, farmed vs. wild. Samples are coloured according to their production type. The ellipse on the score plot represents the 95% confidence interval. A red-dashed line indicates the criteria used to identify the most important variables. VIP values indicate each variable's contribution to class separation, with values >1 considered influential.

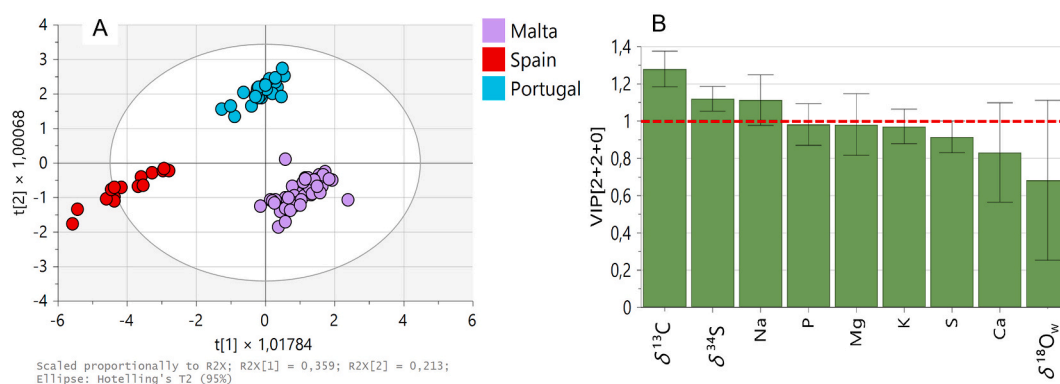


Fig. 5. OPLS-DA score plot A) and VIP values B) in the multiclass comparison between farmed seabream samples from 3 different countries. Samples are coloured based on their country of origin. The ellipse on the score plot represents the 95% confidence interval. A red-dashed line indicates the criteria used to identify the most important variables. VIP values indicate each variable's contribution to class separation, with values >1 considered influential.

contrast, the gills showed no significant correlations with the analysed metals. Unlike biomonitoring, which targets tissue-specific accumulation, food authentication requires analysis of the product as it is consumed; therefore, the whole edible part was analysed.

Bennion et al. (2019) employed trace element fingerprinting to determine the origins of blue mussels (*Mytilus edulis*) from various

harvesting sites in Ireland. They found that As was crucial for accurate classification in soft tissue, while Ni, Fe, and Co were present in the shell, and Mn was present in the periostracum. The study showed that analysing soft tissues alone was not the most accurate for tracing mussels to their harvest locations. However, combining them with the shell and periostracum significantly improved traceability, and individuals could

be traced to their site of harvesting with 100% accuracy.

del Rio-Lavín et al. (2022) reported significant spatial variability in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values, enabling the differentiation of mussel samples from different regions. Mediterranean samples had more negative $\delta^{13}\text{C}$ values compared to those from the Atlantic and South Eastern Pacific. Seasonal variation further influenced isotopic composition, with $\delta^{13}\text{C}$ values exhibiting larger isotopic shifts than $\delta^{15}\text{N}$. While isotopes were measured in the edible part of the mussel, trace element fingerprinting was applied to the shells. Ba, Mn, Pb, and Al were found to have the most discriminatory power. Using random forest classification, shell elemental profiles alone correctly assigned mussels to their harvest regions with 87.8% accuracy, which increased to 97% when combined with stable isotope analysis.

3.2. Discrimination of seabream samples by geographical origin

The dataset used to evaluate the geographical discrimination of gilthead seabream included $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^2\text{H}$, $\delta^{18}\text{O}$, and $\delta^{18}\text{O}_{\text{water}}$, and Na, Mg, P, S, K, Ca, V, Cr, Mn, Fe, Co, Cu, Zn, As, Se, Rb, Sr, Mo, Ba and Hg. Mean isotopic values of $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ showed distinct patterns. Fish from Spain and Portugal were more enriched in ^{13}C , ^{15}N and ^{34}S with $\delta^{13}\text{C}$ of $-18.7\text{‰} \pm 2.1\text{‰}$ and $-18.8\text{‰} \pm 1.4\text{‰}$, $\delta^{15}\text{N}$ of $11.4 \pm 1.4\text{‰}$ and $10.8 \pm 1.7\text{‰}$ and $\delta^{34}\text{S}$ of $13.5 \pm 2.8\text{‰}$ and $12.1 \pm 3.4\text{‰}$, respectively. In contrast, samples from Malta were depleted in ^{13}C , ^{15}N and ^{34}S with the average $\delta^{13}\text{C}$ value of $-22.5 \pm 0.4\text{‰}$, $\delta^{15}\text{N}$ value of $8.7 \pm 0.3\text{‰}$ and $\delta^{34}\text{S}$ value of $6.5 \pm 0.7\text{‰}$. Mean $\delta^{18}\text{O}_{\text{water}}$ values were $1.14 \pm 0.22\text{‰}$ for Malta, $-0.06 \pm 0.91\text{‰}$ for Spain and $1.13 \pm 0.16\text{‰}$ for Portugal. $\delta^{18}\text{O}_{\text{water}}$ values were significantly higher in the Malta and Portugal samples compared to the Spanish samples; however, the Maltese samples were farmed, whereas the Spanish $\delta^{18}\text{O}_{\text{water}}$ values included mostly wild samples, with only a single farmed sample. Bulk $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values showed no clear geographic patterns in the dataset. Consequently, subsequent regional comparisons focused on $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}_{\text{water}}$ and elemental profiles.

The non-parametric Mann-Whitney U test revealed significant geographical differences ($p < 0.05$) in $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values among the three countries, as well as in the elemental concentrations of Na, Mg, S, K, As, Se, Rb, and Hg. Boxplots representing isotopic values and multi-elemental compositions, which show distinct patterns among origins, are provided in the Supplementary Material 1 (Figs. S1-S10).

Wells et al. (2021) reported that mean $\delta^{15}\text{N}$ values in the European common cuttlefish (*Sepia officinalis*) are on average about 2.5‰ lower in the Mediterranean Sea than in the Northeast Atlantic, reflecting different nitrogen sources. For example, nitrogen fixation is more prevalent in the oligotrophic (nutrient-poor) Mediterranean Sea, while nitrate-based productivity dominates in the Atlantic Ocean. Within the Mediterranean, $\delta^{15}\text{N}$ values decrease toward the east, where diazotrophic bacteria contribute larger fraction of the nitrogen supply. The $\delta^{13}\text{C}$ values in *Sepia officinalis* mantle tissue showed no clear large-scale spatial trends across the Northeast Atlantic and Mediterranean, but showed local variation due to regional carbon sources. These findings are consistent with our results for farmed fish. Unfortunately, wild samples from the Mediterranean were not available in the dataset, preventing direct comparison with wild Atlantic samples.

While $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values can help discriminate wild-caught fish by geographical origin, their effectiveness may be limited by feeding habits, as fish occupying similar trophic levels or consuming comparable prey can display overlapping $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values regardless of origin (Kim et al., 2015). The $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{34}\text{S}$ values in the present study differed significantly among farmed samples from all three countries. In contrast, $\delta^{15}\text{N}$ and $\delta^{34}\text{S}$ values between wild-caught fish from Spain and Portugal did not show statistically significant differences.

Hydrogen and oxygen stable isotopes are increasingly applied in seafood traceability because they reflect the isotopic composition of ambient water and, consequently, regional climatic and hydrological conditions such as temperature, salinity, latitude, altitude, and distance

from the sea (Han et al., 2022). In fish tissue, $\delta^{18}\text{O}$ values mirror the isotopic composition of ambient water, which varies with temperature, salinity, and evaporation, while $\delta^2\text{H}$ values are influenced by precipitation and evaporation. Together, these isotopes provide complementary information on the aquatic environment and regional climate (Tulli et al., 2020). In this study, $\delta^{18}\text{O}$ and $\delta^2\text{H}$ were analysed in 10 samples per group. No clear geographical trends were observed; however, significant differences between wild and farmed seabream indicated an environmental influence. Moreover, $\delta^{18}\text{O}$ and $\delta^2\text{H}$ values differed significantly between wild and farmed seabream overall ($p < 0.001$), and within-country comparisons also showed significant differences between wild and farmed samples from Spain and Portugal. Among farmed groups, $\delta^{18}\text{O}$ values varied significantly between Maltese samples collected in July and November, as well as between Maltese samples collected in July and samples from Spain and Portugal. Boxplots are provided in the Supplementary Material 1 (Fig. S11-S15).

Previous studies have also demonstrated their effectiveness for provenance determination. For example, Camin et al. (2018) reported a strong correlation between $\delta^{18}\text{O}$ in trout muscle and culture water, while Han et al. (2021) found that both $\delta^2\text{H}$ and $\delta^{18}\text{O}$ differentiated salmonids reared in freshwater versus seawater systems, highlighting their link to water sources rather than feed. Spatial variation in $\delta^{18}\text{O}$ has also been observed in sea urchin spines (Dong et al., 2022) and octopus statoliths (Martino et al., 2022). Within Mediterranean aquaculture, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ distinguished sea bass from different farming areas (Tulli et al., 2020). Although Bell et al. (2007) and Morrison et al. (2007) included $\delta^{18}\text{O}$ in their analyses of wild and farmed seabass and seabream, most of the discrimination arose from carbon and nitrogen isotopes combined with fatty acid profiles.

In our seabream samples, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ showed only limited variability ($\delta^{18}\text{O}$ 17.0 to 18.3‰; $\delta^2\text{H}$ -85.1 to -76.3‰) with substantial overlap among countries, indicating limited stand-alone power for geographical discrimination at this spatial scale. This is consistent with previous work showing that $\delta^2\text{H}$ and $\delta^{18}\text{O}$ discriminate best across strongly different water systems (e.g., freshwater vs seawater), but vary only slightly within similar marine environments. Even so, these isotopes still provide information on the ambient water isotopic baseline, reflecting the local hydrological and oceanographic conditions experienced by the fish. For mussels, $\delta^2\text{H}$ and $\delta^{18}\text{O}$ values were clearly distinct from seabream and differed slightly between production types: farmed mussels showed $\delta^{18}\text{O} \approx 21.0\text{‰}$ and $\delta^2\text{H} \approx -99.6\text{‰}$, whereas wild mussels showed $\delta^{18}\text{O} \approx 21.7\text{‰}$ and $\delta^2\text{H} \approx -97.5\text{‰}$, indicating strong species-specific water integration; however, because mussels were available from only one origin in this study, the potential of H and O isotopes for mussel provenance could not be evaluated across countries.

Elemental fingerprints in fish muscle differ between countries because fish integrate both the trace-element composition of their environment and their diet. In gilthead seabream, muscle elemental profiles have been shown to reflect local water and sediment chemistry as well as feed composition, with diet often being the main driver of variation among origins and production systems (Mamede et al., 2022). Comparable work on mussels indicates that dissolved trace-element concentrations are shaped by regional geology, land use and pollution, producing distinct site-specific elemental signatures that allow reliable assignment of individuals to harvesting locations, even at small spatial scales (Bennion et al., 2019; Costas-Rodríguez et al., 2010). Collectively, these studies suggest that between-country differences in elemental fingerprints largely arise from contrasting environmental geochemistry and region-specific feeding regimes.

3.2.1. OPLS-DA – seabream fillet

To further evaluate the ability of stable isotope and multi-elemental data to differentiate seabream by different geographical origins, OPLS-DA models were constructed. Using only isotopic variables ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, and $\delta^{18}\text{O}_{\text{water}}$), the model achieved an overall accuracy of 77.9% when combining farmed and wild samples across the three

countries. However, separating farmed and wild fish substantially improved performance, reaching 99.1% accuracy for farmed and 79.6% for wild seabream. In both cases, the isotopes ranked by discriminant power were $\delta^{18}\text{O}_{\text{water}} > \delta^{34}\text{S} > \delta^{15}\text{N} > \delta^{13}\text{C}$.

When only elemental variables were used, the model achieved an overall accuracy of 91% for combined samples. When analysed separately, the accuracy for farmed fish was 100%, while for wild fish, it reached 98%. Nevertheless, the permutation test suggested that the two initial models, which included all measured elements, were not fully robust, suggesting potential overfitting. To address this, elements with low VIP values and weak discriminatory power were excluded. The refined model maintained similar R^2Y and Q^2 values, as well as classification accuracy (100% for farmed and 96% for wild-caught fish), while showing improved permutation validation. The final models included 13 and 10 elements, respectively. Se, Hg, Na, and As were most influential for farmed fish, while V, Fe, Cr, Co, and Se contributed most to geographical differentiation in wild-caught fish.

For geographical-origin assessment, the combination of isotopic and elemental variables improved discrimination within the investigated dataset compared with isotopic variables alone, with the combined OPLS-DA model achieving an overall classification accuracy of 95.5%. The observed performance indicates that complementary isotopic and elemental information may improve geographical differentiation of seabream; however, these results should be considered specific to the sampled origins and require validation using larger and independent sample sets before broader application. The most influential variables ($\text{VIP} > 1$) for separating samples from Malta, Portugal, and Spain were P, K, $\delta^{13}\text{C}$, $\delta^{34}\text{S}$, Mg, S, $\delta^{18}\text{O}_{\text{water}}$, $\delta^{15}\text{N}$, Zn, Hg, Rb, Se, and As. When applied separately to farmed and wild groups, classification accuracy remained high: 99.1% for farmed fish (unchanged from the isotope-only model) and 98% for wild fish (similar to the element-only model). These results suggest that isotopic composition effectively captured most geographical variation among farmed seabream, whereas elemental profiles were especially important when both farmed and wild samples were considered. A reduced OPLS-DA model built using variables with $\text{VIP} > 1$ ($\delta^{13}\text{C}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}_{\text{water}}$, K, P, S, Na, Mg, and Ca) further increased farmed fish classification accuracy to 100%. These VIP variables indicate that geographical origin discrimination among farmed seabream is driven not only by isotopic baseline and diet signals ($\delta^{13}\text{C}$, $\delta^{34}\text{S}$), but also by muscle elemental composition. The strong contribution of K, P, S, Na, Mg and Ca likely reflects differences in aquaculture feed formulation and mineral supplementation, as well as water chemistry in rearing environments, which together influence ionic balance and mineral uptake in fish tissues. Trace elements (Se, As, Rb, Hg, Zn) act as origin markers because they track regional geochemical baselines and food-web inputs (Bennion et al., 2019; Mamede et al., 2022). It is important to note that VIP scores represent multivariate contributions to class separation and do not necessarily correspond to univariate statistical significance. Some variables contribute mainly through their combined covariance structure rather than through large individual mean differences.

Despite the high classification performance observed within the investigated dataset, broader application of isotope- and element-based authentication requires representative reference datasets that capture relevant geographical, seasonal, dietary and production-related variability. Patarnello et al. (2026) identified the establishment and maintenance of such authentic reference datasets as a major challenge for seafood traceability based on stable isotope and elemental fingerprints. Accordingly, the classification performance reported here should be interpreted within the geographical and biological scope of the investigated samples, and external validation using larger and independent datasets is required before broader application.

All OPLS-DA models were statistically validated by CV-ANOVA ($p < 0.001$) and 200-permutation testing, confirming that the observed group separations were significant and not due to overfitting. Performance statistics for all OPLS-DA models, including classifications by production

type and geographical origin, are presented in Table 3.

3.2.2. DD-SIMCA seabream fillet

Following the development of an OPLS-DA model for geographical differentiation using authentic samples, a DD-SIMCA was established to verify the declared origin of commercial Portuguese samples. Unlike conventional discriminant methods, DD-SIMCA identifies objects belonging to a specific target class, in this case, authentic Portuguese samples, from all other objects and classes.

In this work, we used $k \times m$ nested cross-validation strategy to repeatedly split in different ways to account for the uncertainty arising from sampling error associated with small training sets (Mahynski et al., 2025). In each iteration, samples from the target set were randomly divided into a training set and a test set to assess model validity (sensitivity). This repeated hold-out approach provides a balance between sufficient data for model construction and reliable independent testing. Information from the nontarget samples was used to evaluate the model's performance in terms of specificity (Strojnink et al., 2022).

The DD-SIMCA model (Fig. 6) achieved a sensitivity of 93%, with four authentic samples (yellow dots) misclassified, and a specificity of 98%, with two non-Portuguese samples (blue dots) misclassified. The overall classification accuracy of the model was 96%. The most influential variables for discriminating between Portuguese and non-Portuguese fish samples were: $\delta^{13}\text{C}$, Mg, P, S, K, and Se. Both OPLS-DA and DD-SIMCA identified similar variables ($\delta^{13}\text{C}$, Mg, P, S, and K), demonstrating good agreement between the models. Selenium helped describe the typical composition of Portuguese fish; however, in the OPLS-DA, one Spanish sample clustered with the Portuguese group, suggesting that Se reflects regional similarities between neighbouring Atlantic production areas rather than serving as a clear separator.

The classification results should be interpreted within the scope of the investigated dataset. Although model performance was assessed using cross-validation, CV-ANOVA and permutation testing, the available samples do not represent the full biological and geographical variability of gilthead seabream populations. Factors such as geographical coverage, production conditions, season, diet and biological variability may influence isotopic and elemental fingerprints. Therefore, the reported classification accuracies should not be interpreted as estimates of performance for the wider seabream population, and external validation using larger, independent and geographically diverse datasets is required.

4. Conclusions

This study evaluated an integrated analytical approach combining stable isotope ratios ($\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$, $\delta^{18}\text{O}_{\text{water}}$, $\delta^{18}\text{O}$ and $\delta^2\text{H}$) and multielemental composition data for the discrimination of seafood samples according to production method and geographical origin. The results obtained for the investigated samples demonstrate the potential of this approach for seafood authenticity studies and contribute new reference data to the IsoFoodTrack database.

For production-method assessment, $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ showed clear differences between wild and farmed seabream in the investigated samples. The OPLS-DA model based on stable isotope variables achieved 100% classification accuracy within the analysed dataset, with $\delta^{34}\text{S}$, $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ among the most influential variables. These findings indicate the potential of stable isotope fingerprints for production-method discrimination, but independent validation using samples from additional geographical origins is required before the model can be considered generally applicable.

For geographical-origin assessment, combining isotopic and elemental variables improved discrimination within the investigated dataset compared with isotopic variables alone. The combined OPLS-DA model achieved an overall classification accuracy of 95.5% for the analysed samples. These results indicate the complementary value of isotopic and elemental fingerprints for geographical differentiation;

Table 3

Summary of OPLS-DA model performance for discrimination of seabream according to production type and geographical origin.

Classification	Tissue	Variables included	Number of components	R2Y	Q2 (cum)	F1 Score (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
Farmed vs Wild (F vs W)	fillet	$\delta^{18}\text{O}_{\text{water}}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$	1 + 1 + 0	0.894	0.891	100	100	100	100
		Isotopes and elements	1 + 2 + 0	0.931	0.917	100	100	100	100
Malta vs Spain vs Portugal – F & W	fillet	$\delta^{18}\text{O}_{\text{water}}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$	2 + 0 + 0	0.442	0.4	87.6	91.6	59.7	77.9
		elements	2 + 2 + 0	0.604	0.561	95.9	97.3	60.0	96.0
		Isotopes and elements	2 + 2 + 0	0.721	0.678	97.7	98.0	63.6	95.5
Malta vs Spain vs Portugal – F	fillet	$\delta^{18}\text{O}_{\text{water}}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$	2 + 1 + 0	0.867	0.86	99.5	99.1	100	99.1
		elements	2 + 2 + 0	0.93	0.914	100	100	100	100
		Isotopes and elements	2 + 3 + 0	0.957	0.943	99.5	99.1	100	99.1
Spain vs Portugal – W	fillet	$\delta^{18}\text{O}_{\text{water}}$, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, $\delta^{34}\text{S}$	1 + 1 + 0	0.482	0.48	68.6	55.0	96.6	79.6
		elements	1 + 0 + 0	0.833	0.816	95.2	90.9	100	93.1
		Isotopes and elements	1 + 0 + 0	0.846	0.83	97.6	100	96.6	98.0

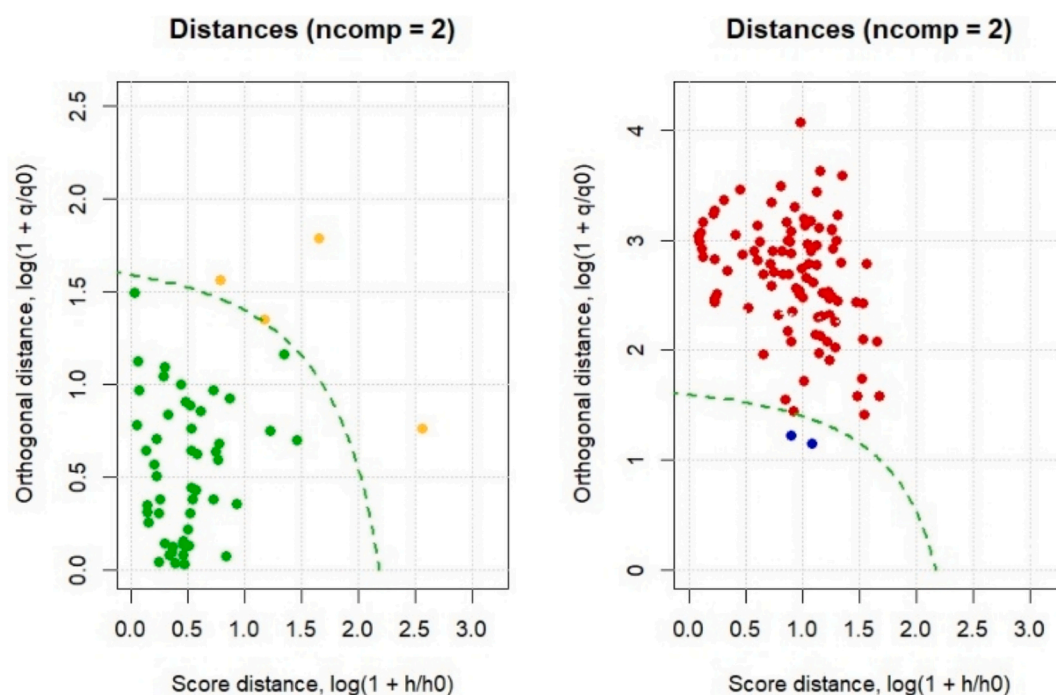


Fig. 6. DD-SIMCA classification model of a) authentic fish samples (green dots; $n = 55$) from Portugal (wild and farmed samples), b) fish samples (red dots; $n = 98$) from outside Portugal (wild and farmed samples from Spain, Malta, Slovenia, Croatia). The green line signifies the acceptance area at $\alpha = 0.05$.

however, the observed classification performance is specific to the sampled populations and geographical origins and should not be interpreted as evidence of equivalent performance for the wider seabream population. Larger, geographically diverse and independent datasets are required for external validation and generalisation of the proposed approach.

Gill samples were analysed separately as an exploratory tissue matrix in selected farmed seabream groups. Although gills exhibited tissue-specific isotopic and elemental patterns compared with fillets, they were not included in the authentication models. Further research is therefore required to evaluate their potential as an alternative matrix for seafood traceability applications.

CRediT authorship contribution statement

Brina Pavlović: Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Lidija Strojnik:** Writing – review & editing, Investigation, Data curation. **Doris Potočnik:** Methodology, Data curation. **Marta Jagodic Hudobivnik:** Methodology, Data curation. **Darja Mazej:** Data curation. **Nives Ogrinc:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

While preparing this work, the authors used ChatGPT for translation

and grammar purposes. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the published article.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Given her role as Regular Editor, Prof. Dr. Nives Ogrinc had no involvement in the peer-review of this article and has no access to information regarding its peer-review.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2026.151243>.

Data availability

Data will be made available on request.

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