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Multi-element signature of cuttlefish and its potential for the discrimination of different geographical provenances and traceability

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ABSTRACT

The measurement and analysis of fifty-two elements by quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS) and direct mercury analysis were applied to origin discrimination of Italian traditional cuttlefish (Chioggia, Venice lagoon) from Mediterranean and Atlantic samples. A total 68 specimens were analyzed in triplicates to generate 204 mass spectra profiles which were statistically processed by different chemometric techniques. Loading weights from principal component analysis as input for linear discriminant analysis (LW-LDA), stepwise-LDA (S-LDA) and variable influence of projection-partial least square discriminant analysis (VIP-PLS-DA) were used to classify samples while retaining the lowest possible number of key variables. VIP-PLS-DA was found to be the best variable selection-discriminant tool combo since the selected Na–Co–B–K–Cd–V–U–Rb–Ni–Ba–Cu–As–Sr–Mn–Mo–Li–Ca–Mg–Se–Bi–Cs–P–Y elemental pattern allowed the samples to be classified with 100% sensitivity, specificity and accuracy.

Abbreviations

Certified reference materials, CRMs, correlation analysis, CA; discriminant function, DF; high energy Helium mode, HE He; inductively coupled plasma mass spectrometry, ICP-MS; inductively coupled plasma optical emission spectrometry ICP-OES; internal standard, ISTD; kinetic energy discrimination, KED; linear discriminant analysis, LDA; leave-one-out-cross-validation, LOOCV; loading weights-based linear discriminant analysis, LW-LDA; method detection limit of the method, MDL; method limit of quantification, MLOQ; microwave digestion, MWD; partial last square-discriminant analysis, PLS-DA; principal component, PC; principal component analysis, PCA; quadrupole inductively coupled plasma mass spectrometry, Q-ICP-MS; rare earth elements, REEs; relative standard deviation, RSD; root mean square error from cross-validation, RMSECV; root mean square error of estimation, RMSEE; root mean square error of prediction, RMSEP; stepwise- linear discriminant analysis, S-LDA; variable influence on projection, VIP; variable influence of projection-partial least square discriminant analysis, VIP-PLS-DA.

Keywords: ICP-MS; element profiling; fishery products; geographical origin; chemometrics.

1. Introduction

The common cuttlefish (*Sepia officinalis*, L., 1758) is a highly valuable fishery product whose distribution extends from the Eastern Atlantic (from the Baltic and North Seas to South Africa coasts) up to the whole Mediterranean Sea. Large scale fishing operations of common cuttlefish led to an average production of 21 825 000 kg in 2018, which ensured the product's supply all over the world (especially in Italy, Spain, Japan, and South Korea), while smaller-scale fishing is particularly profitable in some Mediterranean areas where it has a high impact on local economies (FAO, 2020). This activity is particularly developed in the fishing village of Chioggia (north-western Adriatic Sea in the Eastern Mediterranean Basin), whose *S. officinalis* production reached more than 454 000 kg in 2019 (Clodia database, 2017).

Chioggia cuttlefish is well known to be of high quality thanks to the preservation of a close link with the territory and the traditional processing methods by local fish plants, which allow the products to be officially certified as Italian Traditional Agri-food Product (P.A.T.) (Ministerial Decree, G.U. n. 48, 2014). Foodstuffs with specific geographical ties (especially when a quality mark has been recognized) are in turn rated more highly in terms of quality by the consumer, mostly because of lower environmental impact and higher perceived safety of regional products.

Alongside with the compulsory labelling requirement established by European legislation concerning the provision of detailed information about the geographical origin of fish and seafood (European Parliament and Council of the European Union, 2013), the protection and the promotion of traditional foods must involve origin authenticity assessment both to ensure traceability and prevent commercial frauds (Ortea & Gallardo, 2015).

Complex multi-disciplinary and cross-disciplinary approaches are usually required to verify the geographical origin of fish and seafood since both environment and genetics can affect the final characteristics of the products (Abbas et al., 2018). Nevertheless, considering the association between the concentrations of elements in fish tissues and those in the surrounding aquatic environment, the determination of the multi-element profile of fish and seafoods can be regarded as a valid analytical strategy to guarantee fish origin authenticity. Several factors such as species, size, age, sex, and sexual maturity can directly affect the elemental composition of aquatic animals, but food resources, climate, presence of contaminants, and many water quality parameters (*p*H, dissolved oxygen, alkalinity) are those which differ to a greater extent between countries (Li, Boyd, & Sun, 2016; Smith & Watts, 2009).

Several studies reported the possibility to discriminate fishery and seafood products as croaker (Chaguri et al., 2015), sea cucumber (Kang et al., 2018), shrimps (Ortea & Gallardo, 2015), crabs (Luo et al., 2019), and bivalve mollusks such as mussels (Costas-Rodríguez, Lavilla, & Bendicho, 2010) and clams (Iguchi, Isshiki, Takashima, Yamashita, & Yamashita, 2014) using element composition. In these works, the chemical characterization of samples was based on minor and/or trace elements (Costas-Rodríguez et al., 2010; Iguchi et al., 2014), a combination of major, minor, and trace elements (Kang et al., 2018), or a combination of major and trace elements plus stable isotopes analysis (Chaguri et al., 2015; Ortea et al., 2015; Luo et al., 2019).

In the range of analytical methods applied to the determination of elements, inductively coupled plasma-mass spectrometry (ICP-MS) combined with different chemometrics techniques holds a unique position by virtue of speed, sensitivity, dynamic range, and elemental coverage (Drivelos & Georgiou, 2012; Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016). In this context, several supervised and unsupervised chemometric tools such as, principal component analysis (PCA) (Chaguri et al., 2015; Kang et al., 2018; Ortea et al., 2015), cluster analysis and *k*-means hierarchical clustering (Ortea et al., 2015), linear discriminant analysis (LDA) (Costas-Rodríguez et al., 2010; Iguchi et al., 2014; Ortea et al., 2015; Kang et al., 2018; Luo et al., 2019), support-vector machine (Luo et al., 2019), soft-independent modelling of class analogy and artificial neural networks (Costas-Rodríguez et al., 2010) were applied for authenticity and traceability purposes.

The determination of elemental composition of fish and seafood to trace their geographical provenance is particularly suitable for cephalopods mollusks. Cephalopods such as cuttlefish are in fact at high trophic levels in the aquatic food chain and the overall elements accumulated in tissues are good indicators of the surrounding habitat (Bosch, O’Neill, Sigge, Kerwath, & Hoffman, 2015). In addition, cephalopods are non-migratory animals and, therefore, their elemental composition can reasonably be expected to be constant during life (Gopi et al., 2019). Nevertheless, very few studies addressed the topic of origin discrimination of cephalopods mollusks using elemental composition, in spite of being focused on the analysis of a limited number of major and trace elements included in hard structures such as statoliths (Arbuckle & Wormuth,

2014) or ink (Bua et al., 2017). These components are in fact not always retained in the final commercial product and, therefore, not always exploitable in practical food surveillance operations to trace back the origin of cephalopods.

Based on this background, the present work aimed at outlining for the first time the *S. officinalis* multi-elemental profile of edible tissues to verify whether useful information could be extracted and specifically linked to the geographical origin of the Italian traditional cuttlefish from Chioggia (FAO subarea 37.2.1) in order to be differentiated from non-Chioggia cuttlefish caught in other fishing areas of the Mediterranean Sea (FAO 37.1/37.2) and in the French Atlantic Ocean (FAO 27.7.e). For this purpose, fifty-one elements were determined by ICP-MS and Hg was quantified via atomic absorption spectroscopy. Unsupervised and supervised pattern recognition methods were used to investigate sample characteristics, identify the key discriminant elements, and, at the same time, develop classification rules.

2. Materials and Methods

2.1. Reagents and standards

The ultrapure water (0.055 µS cm^{-1} conductivity) obtained using the Milli-Q® water purification system (Millipore, Bedford, USA) was used for the preparation of all solutions. Sub-boiled nitric acid was prepared from nitric acid (65%, w/w) of Selectipur quality (Lach-Ner, Neratovice, Czech Republic) using the distillation equipment BSB-939-IR (Berghof, Eningen, Germany). Hydrogen peroxide (Trace Select, ≥ 30%, w/w) was purchased from Fluka Chemie AG (Buchs, Switzerland).

Multi-element stock solution “A” containing 10 mg L^{-1} of Li, B, Al, V, Cr, Fe, Ni, Co, As, Se, Rb, Sr, Zr, Mo, Ru, Pd, Cd, Sn, Sb, Cs, Ba, Hf, Re, Pt, Tl, Pb, Bi, and Th was prepared from the Supelco ICP multi-element standard solution IV (Merck, Darmstadt, Germany) and single element standards of concentration 1 ± 0.002 g L^{-1} (Analytika Ltd., Prague, Czech Republic or SCP Science, Montreal, Canada). Multi-element solution “B” containing 1 mg L^{-1} of La, Ce, Pr, Nd, U (“B1”) and 0.20 mg L^{-1} of Y, Tb, Ho, Yb, Sm, Eu, Gd, Er, Lu, and Dy (“B2”) was prepared from the stock solution of rare earth elements Astasol mix “M008” (Analytika Ltd., Prague, Czech Republic). Multi-element solution “C” containing 50 mg L^{-1} of Na, Mg, P, K, Ca, Mn, Cu, and Zn was prepared from single element standards of 1 g L^{-1} obtained from Analytika Ltd. The internal standard solution (ISTD) was prepared from 1 g L^{-1} stock solution of Rh obtained from SCP Science (Montreal, Canada). Carbon reference solutions were prepared from 10 g L^{-1} of C stock solution prepared from urea of TraceSelect quality (Fluka Chemie AG, Buchs, Switzerland).

2.2. Sampling and preparation of cuttlefish

A total of 68 samples of common cuttlefish (*S. officinalis*, L.) including n = 17 samples from Adriatic Sea (Chioggia, Italy, FAO 37.2.1), n = 25 non-Chioggia samples from the Mediterranean Basin (FAO 37.1/37.2), and n = 26 samples from North-eastern Atlantic Ocean (France, FAO 27.7.e) were analyzed. Specimens were caught by fishing trawlers during the months of September and randomly collected from different batches in local fish plants located in Chioggia (Venice, Italy), by choosing homogeneous sizes and weights (mantle lengths from 10 ± 2 cm; body weight 125 ± 25 g). After collection, samples were immediately frozen and stored at −20 °C for around 3 months prior to be further processed for multi-element analysis.

2.3. Quality assurance and quality control

The following commercially supplied certified reference materials (CRMs) were analyzed: NIST SRM 1577 Bovine Liver (National Institute of Science and Technology, NIST, Gaithersburg, MD, USA); NIST SRM 1566 Oyster Tissue (NIST, Gaithersburg, MD, USA); BCR® certified reference material (CRM)184 Bovine muscle (Institute for Reference Materials and Measurements, IRMM, Geel, Belgium); BCR® 185 Bovine Liver (IRMM, Geel, Belgium); CRM NCS ZC73015 Milk Powder (National Research Centre for Certified Reference Materials, NRCRM, Beijing, China); P-WBF CRM 12-2-04 Essential and Toxic Elements in Wheat Bread Flour (pb-anal, Kosice, Slovakia); CRM12-2-03 P-Alfalfa Essential and toxic elements in Lucerne (pb-anal, Kosice, Slovakia); SMU CRM 12-02-01 Bovine liver (pb-anal, Kosice, Slovakia).

2.4. Sample preparation

2.4.1. Pre-processing of cuttlefish

Frozen samples were thawed overnight (16–18 h at +4 °C) before processing for multi-element analysis. Each specimen was then washed with deionized water and skin, cuttlebone, gills, reproductive and digestive tracts were carefully removed without causing their rupture. The head, arms and tentacles were excluded, while the mantle and the lateral fins were rinsed again with ultrapure water and minced with a ceramic knife.

2.4.2. Freeze-drying process

Pre-treated sample portions of approx. 10 g were individually transferred into 100 mL cleaned round bottom flasks wherein the material was dried and placed into a deep freezer at −80 °C for 24 hours to provide a necessary conditioning for drying. Lyophilization was conducted at −50 °C and 0.01 bar for 24 h with a LIO-5P apparatus (CinquePascal srl, Trezzano sul Naviglio, Milan, Italy). Dried samples were subsequently removed from the flasks, homogeneously ground into a powder by using ceramic mortars and pestles and then individually sealed into LDPE bags.

2.4.3. Microwave assisted digestion

Microwave digestion (MWD) of samples was carried out by using the Speedwave^{TM} MWS-3^{+} (Berghof, Eningen, Germany) microwave system with the maximum total output of the microwave generator (1450 W) and equipped with the optical sensor technology for contactless real-time recording of the sample temperature. The high-pressure resistant (up to 100 bar) PTFE vessels DAC-100S, together with the Multitube System (all from Berghof, Eningen, Germany), were used for sample digestion. This arrangement allowed the simultaneous digestion of three samples in one DAC-100S PTFE vessel by placing three PFA tubes into each vessel (Husáková et al., 2015). The maximal number of used DAC-100S vessels for one digestion cycle was eight.

A powdered cuttlefish or CRM sample aliquot of 0.1 g was weighed into a 10 mL PFA tube. Then, 4 mL of 16% HNO_3 (65%, w/w HNO_3, 1:3 diluted) and 1 mL of 30% H_2O_2 were added, leaving the vessels open until the initial reaction subsided. Three PFA tubes containing the same sample and reagents were placed into the outer 100mL PTFE digestion vessel previously filled with 25 mL of HNO_3 (16%, v/v) , by ensuring that the level of liquid in the outer PTFE vessel was higher than those in the PFA tubes. This way, the vapor pressures were compensated and the evaporation of the solution from the PFA tubes was avoided (Husáková et al., 2015). The samples were digested following a five-step program: (i) 20 min at 180 °C and 80 % power (ramp 5 min), (ii) 20 min at 220 °C and 95 % power (ramp 5 min), (iii–v) 5 min at 100 °C and 10 % power (ramp 1 min). The resulting colorless solutions were diluted to 25 mL with deionized water.

Each sample was mineralized in three replicates. Blanks, consisting of deionized water and reagents were subjected to a similar preparation procedure.

To assess the properness of the digestion process, the residual carbon content in the final cuttlefish digests was determined by a previously described inductively coupled plasma optical emission spectrometry (ICP-OES) method (Husáková et al., 2011) was 5.5 ± 0.4 % (n = 3).

2.5. ICP multi elemental analysis

ICP-MS measurements were performed by using the Agilent 7900 quadrupole mass spectrometer (Q-ICP-MS, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an octopole-based collision cell for interference removal using kinetic energy discrimination (KED).

The standard sample introduction system, consisting of a glass concentric nebulizer MicroMist (400 µL min^{-1}), the Peltier-cooled (2 °C) Scott quartz spray chamber and quartz torch with 2.5 mm internal diameter injector, was used. For precise delivery of samples and ISTD, a low-pulsation, 10-roller peristaltic pump with three separate channels was employed. The internal standard kit, including connecting tubing, connectors and the "Y" piece was used for simultaneous internal standard aspiration and its mixing with the sample. The standard sampling and skimmer nickel cones with orifices of 1 and 0.45 mm, respectively, were used. ICP-MS operating conditions were optimized during each start-up sequence by using the multi-elemental tuning solution (Agilent Technologies, Inc., Santa Clara, CA, USA) containing 1 µg L^{-1} of Ce, Co, Li, Mg, Tl and Y, in order to obtain the highest possible sensitivity for elements of low, middle and high m/z. Using the typical operating conditions summarized in Table 1, a sensitivity of 6000 counts s^{-1} per µg L^{-1} and a resolution of 0.64 amu peak width (full width at half maximum intensity) were achieved for $^{7}Li^{+}$. The same parameters were 50000 counts s^{-1} per µg L^{-1} and 0.62 for $^{89}Y^{+}$, and 30000 counts s^{-1} per µg L^{-1} and 0.60 for $^{205}Tl^{+}$.

For sample analysis the "General Purpose" plasma mode included in the ICP-MS MassHunter software was used (Agilent Technologies, Inc., Santa Clara, CA, USA). The working parameters of the cell mode "no-gas" were autotuned during the instrument start-up sequence. The working parameters of the collision cell for helium ("He") and high energy He ("HE He") modes were adjusted manually. The time required for a transition between cell modes was 5 s. Parameters related to sample introduction and plasma conditions were consistent for all modes (see Table 1).

Concentrations of a total of 51 elements were evaluated from calibration curves with coefficients of determination better than 0.999 and built up within the ranges given below.

Calibration solutions: blank, 1, 5, 10, 50, 100 µg L^{-1} of Li, B, Al, V, Cr, Fe, Ni, Co, As, Se, Rb, Sr, Zr, Mo, Ru, Pd, Cd, Sn, Sb, Cs, Ba, Hf, Re, Pt, Tl, Pb, Bi, Th; 0.1, 0.5, 1, 5, 10 µg L^{-1} La, Ce, Pr, Nd, U; 0.02, 0.1, 0.2, 1, 2 µg L^{-1} of Y, Tb, Ho, Yb, Sm, Eu, Gd, Er, Lu, and Dy; 0.5, 1, 5, 10 mg L^{-1} of Na, Mg, P, K, Ca, Mn, Cu, and Zn. The solutions were prepared daily by appropriate dilution of multi-element solutions "A" (500 µg L^{-1}), "B" (50 + 10 µg L^{-1}) and "C" (50 mg L^{-1}) in 25 mL volumetric flasks (see Section 2.1).

To compensate possible instrumental drift and matrix effects, a 200 µg L^{-1} Rh ISTD was simultaneously aspired and mixed with samples.

2.6. Mercury analysis

For the quantification of Hg content, the direct solid sampling analysis using a single-purpose atomic absorption spectrometer AMA 254 (Altec Ltd., Prague, Czech Republic), based on in situ dry ashing followed by gold amalgamation atomic absorption spectroscopy, was used. Each time, samples were weighed in a nickel boat and analyzed under the following experimental conditions: typical sample mass, 50 mg; drying time, 60 s; decomposition, ~750 °C for 150 s; waiting step, 900 °C, 45 s, necessary for quantitative release of trapped mercury from the gold amalgamator to the measuring cuvette. The peak area absorbance at 253.6 nm was monitored. The flow rate of oxygen (99.5%) carrier gas was 170 mL min^{-1}.

The AMA 254 spectrometer was regularly calibrated using blank and four working standard solutions containing Hg(II) ranging from 0.025 to 0.2 mg L^{-1}. The working standard solutions were prepared from standard aliquots of 10 mg L^{-1} prepared from a 1 g L^{-1} stock solution of Hg(II) (Analytika Ltd., Prague, Czech Republic).

2.7. Data treatment

Statistic was applied to elemental concentrations referring to cuttlefish dry matter (d.m.) and carried out using IBM-SPSS (v. 23.0, SPSS Inc., Chicago, IL, USA) SIMCA-P v.14.1 (Umetrics, Umeå, Sweden), and Statgraphics Centurion v.18.1 (StatPoint Technologies, Inc, Warrenton, VA, USA) software. The normality and homogeneity of variance of data was checked beforehand by applying the Shapiro-Wilk's and Levene's tests, respectively ($p \leq 0.05$). Since the normal distribution assumption was violated for most of the elemental data, quantitative differences between groups of cuttlefish from different origins were investigated by the nonparametric Kruskal–Wallis test with Dunn's multiple post hoc test for multiple comparison ($p \leq 0.05$). Data was presented as mean values ± margin of error (calculated at 95% confidence level), median and minimum and maximum values found.

While the nonparametric statistic was used to compensate for the lack of data normal distribution, thus preserving the original information and avoiding the loose of any intuitive meanings when describing element concentrations resulting from univariate data analysis, a Box-Cox transformation was instead applied to normalize data for subsequent bivariate and multivariate data analyses.

First, the bivariate Pearson's correlation analysis (CA) was employed to Box-Cox transformed data to explore variable distribution and look for strong positive ($r > 0.6$, $p \leq 0.01$) or strong negative ($r < -0.6$, $p \leq 0.01$) linear correlations between binary variables.

Because of the highly different magnitude of values among element concentrations, the Box-Cox data were then scaled applying a Z-score standardization and further processed by multivariate data analyses. PCA was used for further investigation of covariance patterns in elemental concentrations, data's first interpretation and multivariate outliers' removal based on Hotelling's T^2 test results (95% confidence interval). During this step, the number of variables was also reduced by selecting elements characterized by loading values (rescaled to 0–1) > 0.70.

Afterwards, supervised classifiers combined with different variable selection procedures were developed to achieve the discrimination of samples according to the geographical origin by using the lowest number and the most influential combination of variables. To that end, linear discrimination based on variable loading weights previously selected by PCA (LW-LDA), forward stepwise-LDA (S-LDA) and variable influence on projection-(VIP)-PLS-DA were independently tested and compared in terms of prediction ability. All the models were cross-validated using leave-one-out-cross-validation (LOOCV) to exclude overfitting. In addition to LOOCV, the validity of the PLS discriminant models was also checked by permutation testing (400 random permutations).

When supervised modelling was performed, about 33% of data (n = 68) was randomly sidelined from the calibration set (n = 136) and used for independent external validation. Models' overall performances in internal and external validation were evaluated in terms of accuracy (%), sensitivity (%), and specificity (%). Further details about the statistical treatments applied can be found in Supplementary material, Supplementary Experimental Section.

3. Results

3.1. Analytical performances and validation

The analytical determination of fifty-two major, trace and ultra-trace elements in mineralized cephalopod samples was carried out by Q-ICP-MS, taking advantage of its well-known sensitivity toward the targeted elements. On the other hand, analysis of Hg by thermal decomposition was performed by AMA254 without pre-treatment and/or preconcentration steps, thus overcoming the most serious problems related to ICP-MS analysis of Hg, e.g. long washout time, non-linear calibration curves, and decreasing sensitivity with time (Li et al., 2006).

The microwave-assisted pressurized digestion which involved small sample amounts (100 mg) was employed to ensure rapid sample preparation for subsequent ICP-MS analysis while attaining the high decomposition efficiency. One of the most important aspects regarding the employed MWD method is the possibility to use diluted solutions for sample preparation, thus reducing the quantity of reagents, risks of contamination and generation of residues. These are important parameters for the development of greener analytical methods (Gonzalez et al., 2009). Moreover, MWD plays an important role in decreasing some kinds of spectral and non-spectral interferences, such as those deriving from carbon containing species or different chlorides, by conversion to significantly less or non-interfering species (Husáková et al., 2011; Bizzi et al., 2017). He mode with KED or HE He mode with higher cell gas pressure and higher energy discrimination voltages, were used for the analytes that suffered from spectral effects (i.e. V, Cr, Mn, Co, Ni, Fe, Co, Cu, As, and Se) caused by polyatomic ions deriving from the plasma gas, sample solvent and other sample matrix elements such as Na, K, Ca, Mg, P, S, C, and Cl (see Table 1 and Supplementary Materials, Table S1). He and/or HE He cell modes had the additional benefit of reducing the response for low mass matrix elements like Na, K, Ca or P by an order of magnitude ($^{23}Na^+$, $^{44}Ca^+$) or more ($^{39}K^+$, $^{31}P^+$), thus effectively raising the upper linear range for these elements. Thereby elements that would have needed to be analyzed by ICP-OES, were included in the ICP-MS.

As for the ISTD, Rh was chosen for its mid-range mass and ionization potential and because of its absence in the analyzed samples. Recoveries of Rh, relative to the initial calibration blank for the entire 8-hour sequence of sample digests, are shown in Supplementary Materials, Fig. S1. ISTD behavior across the mass range was found to be very consistent overt time and downward drift was considered acceptable.

Data accuracy was evaluated by the analysis of different CRMs (NIST SRM 1577 Bovine Liver, NIST 1566 Oyster Tissue, BCR CRM 184 Bovine muscle, BCR CRM 185 Bovine Liver, CRM 12-2-01 Bovine Liver), primarily intended for the evaluation of analytical methods and instruments used for the determination of the mass fraction values of selected elements in marine tissue, foods, or similar materials. Since the levels of most lanthanides and actinides are not certified in these CRMs, three additional certified standards (NCS ZC 73015 Milk Powder, CRM 12-2-04 Wheat bread flour, and CRM 12-2-03 Essential and toxic elements in Lucerne) were analyzed to validate data for these elements. The high level of agreement between target and found values demonstrated trueness of data obtained (Supplementary Materials, Table S2 and Fig. S3).

The precision of the method was evaluated in terms of intra-day and inter-day comparison. Intra-day precision was determined by analysis of individual control materials three times during the same day. Inter-day precision was determined by analysis of the same standards on three different days over a period of one month. Within each series, every solution was analyzed in triplicate. Relative standard deviation (RSD) was calculated for both series of analyses. The RSD values of intra-day and inter-day studies were mostly found to be below 14% thus showing a good precision of the method (Supplementary Materials, Table S2).

Method detection limits (MDLs) and method quantification limits (MLOQs) - reported in Supplementary Material, Table S3 - were evaluated as a triple and tenfold standard deviation, respectively, of ten consecutive measurements of blank signal divided by the slope of the calibration curve. Detection limits were found to be low enough that selected elements could be determined at the background level. Fig. S2 and Table S3 (Supplementary Materials) also summarize relative sensitivities of Q-ICP-MS for analysis of individual elements with the use of Rh ISTD.

3.2. Concentrations of elements in cuttlefish samples

The concentrations of elements measured in the present study were presented and discussed as median values considering the non-normal distribution of the raw data. Results from descriptive statistics are listed in Table 2. The most abundant elements were found to be Na, Mg, P and K, whose concentration levels were higher than 1% of weight and quite variable among cuttlefish from different countries. According to results from Kruskal–Wallis test, Na and Mg amounts were significantly higher ($p \leq 0.05$) in samples from FAO 27.7.e (French Atlantic) compared to samples from FAO 37.1/37.2 (Mediterranean area) and FAO 37.2.1 (Chioggia). At the same time, an opposite trend was observed for P and K, for which the highest concentrations ($p \leq 0.05$) were found in Chioggia cuttlefish (Table 2).

Concentrations between 1 and 0.01% of weight (10000–100 mg kg^{-1}, d.m.) were found for two elements corresponding to Li and Ca, which showed significantly higher median concentrations in French Atlantic samples (0.51 mg kg^{-1}and 1760 mg kg^{-1}, respectively) ($p \leq 0.05$) compared to the other groups. The wide variability in both major and minor element contents found in cuttlefish specimens of different geographical origins and within samples of the same origin can be attributed to their natural variation in seawater, but the assimilation by marine animals of Na, Mg, and Ca from the organic matter in the aquatic environment also varies with the feeding status and the age of the animal (Lall, 2002). Moreover, the presence of P in fish and seafood tissues can be directly attributable to the food sources since its concentration in seawater is lower compared to the other elements (Lall, 2002).

The main trace elements, ranging between 100 and 1 mg kg^{-1}, followed the decreasing order Zn > Al > Sr > Cu > Fe > B > Rb > Se > Mn in samples from Chioggia, Zn > Cu > Sr > Fe > Al > B > Rb > Mn > Se in samples from the Mediterranean area, and Zn > Sr > Cu > B > Fe > Al > Rb > Se > Mn in samples from the French Atlantic. Among these elements, only Se, Rb, and Sr were different in median concentrations according to all three geographical regions ($p \leq 0.05$) and the concentration ranges were in line with those previously published in literature for *S. Officinalis* (Raimundo, Pereira, Vale, & Caetano, 2005; Ayas & Ozogul, 2011).

Different median contents varying between 62.3 and 75.8 mg kg^{-1} were also found for As ($p \leq 0.05$). The wide presence of arsenic in the aquatic environment is linked both to the geochemical characteristics of the region and anthropogenic activities, especially those related to agricultural practices (Neff, 2002). Due to toxic properties, some concerns regarding the dietary exposure to arsenic have arisen in recent times, but maximum levels for this metal in fishery products have not been established yet by European legislations. Fish and seafood, in particular, have been reported as the largest food contributors to overall total arsenic exposure, despite the largest proportion is represented by organic arsenic species which are known to be less or no toxic compared to relative inorganic species (European Food Safety Authority, EFSA, 2009). In addition, inorganic arsenic concentrations decrease with increasing content of total arsenic (European Food Safety Authority, EFSA, 2009).

Most of the elements analyzed in the present paper were found to be present in cuttlefish tissues at mean concentration lower than 1 mg kg^{-1}. Levels of ultra-trace elements as rare earth elements (REEs) were lower than 100 µg kg $^{-1}$ on average, with the highest median concentration for Ce (24.0 µg kg^{-1}) and the lowest one for Lu (0.047 µg kg $^{-1}$) both in in Mediterranean samples (Table 2). The natural pattern of REEs in the aquatic environment, which is associated to the mobilization of nutrients from the underlying soils, can be permanently altered by some anthropic activities and effectively used to investigate the geographical provenance of marine animals (Noack, Dzombak, & Karamalidis, 2014). In the present work, the significantly different concentrations of Tb, Dy, Ho, Er, and Yb geographically discriminated Chioggia from French specimens, while those of La, Pr, Nd, Eu, and Gd discriminated Mediterranean form French specimens ($p \leq 0.05$). In accordance with these results, the amount of both La and Ho was previously reported to be potentially useful to authenticate fish samples from different areas in the Mediterranean Sea (Varrà, Ghidini, Zanardi, Badiani, & Ianieri, 2019).

Regardless the origin, all the cuttlefish samples analyzed in the present work were in line with the maximum limits for toxic heavy metals established by European regulations No. 1881/2006/EC and subsequent amendments and additions (Commission of the European Communities, 2006). Both cadmium and lead did not exceed the threshold value of 1 mg kg^{-1} set for the edible part of cephalopods, as well as Hg was always found to be below the threshold limit of 0.5 mg kg^{-1}. The sample groups under investigation did not show significant differences in Pb and Hg concentrations ($p > 0.05$), but Cd median amount in French Atlantic specimens was found to be approximately 7 and 20 times higher than in Mediterranean and Chioggia samples, respectively (see Table 2). Furthermore, the comparison with results reported by other authors for cuttlefish from North eastern Atlantic and Adriatic Sea, showed that Hg, Pb, and Cd concentrations found in the present work fell within the same ranges (Bustamante, Lahaye, Durnez, Churlaud, & Caurant, 2006;

Storelli, Giacominelli-Stuffler, Storelli, & Marcotrigiano, 2007). Even in the case of heavy toxic metals, a close link between their concentrations in seawater and environmental pollution of specific areas exists (Carvalho, Santiago, & Nunes, 2005). This, together with Ni, Co, and Bi amounts, which also varied significantly among sample groups, makes suggestions for future insights about the effective role and contribution of these elements for the geographical origin assessment of fish and seafood.

3.3. Chemometric classification of the geographical origin of cuttlefish

Since no specific information concerning the best arrangement of elements to discriminate the geographical origin of cephalopods is available in literature, the first stages of multivariate analysis took into consideration all the fifty-two elements measured, starting with the data exploration by means of CA and PCA. Nevertheless, multicollinearity between variables as well as geographical-unrelated variability are a source of increasing noise, thus the reduction of the variables to the most predictive ones was subsequently preferred, especially in view of a future practical implementation of the methodology.

The selection of variables is strictly dependent on the sample matrix properties and dataset overall characteristics; hence its application needs to be evaluated on an individual basis. Although being the most frequently used classification technique in studies dealing with element profiling, S-LDA is coming under growing criticism because of the deceiving results deriving from the random rather than effective significance of the variables extracted, especially when the number of potential explanatory variables is high (Smith, 2018). On the other hand, one of the main problems related to the use of multivariate data analysis in food authentication studies is just the difficulty in comparing the obtained results with those already published but using a wide variety of different chemometrics techniques. Therefore, in the context of the present work, S-LDA and LW-LDA, were used for classification by variable selection, but outputs were compared with those obtained through the application of VIP-based PLS-DA. The latter, in fact, consists of a more robust and flexible algorithm, particularly suited for the classification of a large number of samples and is suggested to be a more powerful tool for reliable variable selection compared to the traditional LDA (Rashid, Hussain, Ahmad, & Abdullah, 2019).

3.3.1. Correlation and principal component analysis

Pearson's associations between all the elements were reported by plotting the correlation matrix shown in Fig. 1. The main significant patterns of covariations were found among most of the REEs and, in particular, between Ce and La ($r > 0.9$), and among Nd, Pr, Tb, Ho, Gd, Er, Eu, Dy, and Sm ($r > 0.8$). Similarly, bivariate positive correlation regarding the patterns Na–Sr–Cd–Li–Mg–Ca ($r > 0.9$), P–K ($r > 0.9$), and Cs–Rb ($r > 0.8$) were also detected. Concentrations of U were positively correlated with Ca, Mg, Pd, Li, Cd, Sr, and Na ($r > 0.8$) as well as amounts of Co with Cu, Mo, and V ($r > 0.7$). The most important and significant negative correlations were instead found among K–Rb–Cs–Mn and among Na–Sr–Cd–Li–Ca–Mg–U ($r < -0.7$) patterns.

The association between the characteristic element distributions and cuttlefish origins was further investigated by PCA. The first PCA computation took into consideration all the fifty-two elements and, as a result, the number of original variables was set by LOOCV to six final principal components (PCs) enclosing 72.6% and 57.3% of the explained and predictive data variance, respectively. The first two PCs of the model contributed for 27.5% and 20.8% of the total explained variance, with the PC2 enclosing the fraction of information related to sample provenance, as reported in Fig. 2A. The distribution of Chioggia cuttlefish along the negative axis of the PC2 was mainly dominated by the highest contribution of Rb and K, followed by Mn, Al, Cs, P, As, and, at lower levels, Bi (see loading vectors reported in Fig. 2A). On the other hand, French Atlantic cuttlefish distribution was mainly guided by contribution from Na, Sr, Cd, Li, Mg, Ca, U, and Mo, whose vectors angles nearness underlined the strong covariation previously stated by CA. Likewise, a strong contribution and a high degree of covariation was confirmed in PCA for REEs, which impacted on the negative axis of PC1.

During this preliminary stage, one sample from Chioggia was also found to be a strong outlier since it fell outside the 95% confidence interval defined by the Hotelling's T^2 range (see Fig. 2A). For this reason, it was excluded from subsequent statistics.

Since the most influential loading values were found for a total of 16 out 52 elements (Li, B, Na, Mg, Ca, Co, Cu, Zn, Sr, Mo, Ru, Pd, Cd, Hf, Pt, and U), variable filtering was performed (see Section 2.7) and PCA was thus recomputed. Loading weights (0-1 scaled) of selected elements are reported in Supplementary Materials, Table S4. The reduced PCA model extracted three PCs that explained 84.5% of the data variability. The PC1 (explained variability, 54.1%; predictive variability, 48.3%) was mainly characterized by Zn, Cd, Pd, Ca, U, Sr, while in the PC2 (explained variability, 16.3%; predictive variability, 17.1%) Pt was the most influential element (Fig. 2B). Hence, maximum variation in the original dataset was retained while reducing the number of significant features. At the same time, sample separation in the score space was significantly expressed along the PC1 using the selected variables rather than the whole set of elements.

3.3.2. Classification of cuttlefish by origin: LDA and PLS-DA approaches using selected variables

The LW-LDA classification model for cuttlefish origins, created by using variables previously selected by PCA (Li, B, Na, Mg, Ca, Co, Cu, Zn, Sr, Mo, Ru, Pd, Cd, Hf, Pt, and U) was described by two discriminant functions (DFs) explaining 99.7% of the data variability. The DF1 (explained variability, 83.1%; canonical correlation, 97.2%) and the DF 2 (explained variability, 16.6%; canonical correlation, 86.7%), whose statistical significance was confirmed by low Wilk's Lambda values (0.004 and 0.13, respectively, p = 0.000) were responsible for the clusterization of cuttlefish in the bidimensional score plot reported in Fig. 3A. Samples from French Atlantic were well discriminated from samples from Chioggia and Mediterranean Sea by the DF1 and Chioggia samples well discriminated from Mediterranean samples by the DF2. The most important loadings for the DF1 were Sr and Na, while Co was found to be the most influent element for the DF2 (Fig. 3A). Based on these results, 100% of cuttlefish were correctly recognized in CV, but, when performing the external test set validation, two samples from Mediterranean area were misclassified as samples from French Atlantic and one French Atlantic sample as of Mediterranean origin, thus resulting in an overall accuracy of the model of 97% (Table 3). Fisher's discriminant function coefficients for each geographical provenance are reported in Supplementary Material, Table S5.

In S-LDA the forward selection method based on Wilk's Lambda values was chosen to select the discriminating variables, taking 3.84 as the minimum partial F value to include a variable and 2.71 as the maximum F value to exclude a variable from the model (see Supplementary materials, Supplementary Experimental Section). Based on this, two DFs and 12 out 52 discriminating elements were finally included into the model, corresponding to Na, Co, B, K, Cd, V, U, Rb, Ni, Ba, Cu, and As (Supplementary Materials, Table S6), of which Na, Co, B, Cd, U, and Cu were the same as those selected in LW-LDA.

The DF1 and DF2 enclosed 82.5% and 17.5% of variance of original data, with a canonical correlation of 97.4% and 88.5% respectively. The significant discriminatory ability of each DF was confirmed not only by

Wilk's Lambda values of 0.03 for the DF1 and 0.114 for the DF2 ($p = 0.000$), but also by 99% accuracy in LOOCV (one Mediterranean sample erroneously classified as Atlantic sample) and 100% accuracy in external validation (Table 3). Fisher's coefficients for each provenance are reported in Supplementary Materials, Table S6.

Optimal classification results (100% sensitivity, specificity, and accuracy, Table 3) were obtained by the application of PLS-DA when 21 elements were selected on the basis of their VIP scores (VIP >1) and used as classificatory variables (see Supplementary Materials, Table S7). Among these elements, ten were found to be the same of those previously included in stepwise-LDA. Although Sr, Mn, Mo, Li, Ca, Mg, Se, Bi, Cs, P, and Y were selected in addition, some of these (Sr, Mo, Li, Ca, and Mg) were shared with LW-LDA. The highest VIP scores were 1.672 for V and 1.628 for Co, thus indicating the maximum contribution of these elements to the geographical separation between cuttlefish.

The VIP-PLS-DA model was created by using six LVs explaining 90.1% of variance ($R^2X = 0.901$), of which 92.2% was directly correlated with labels of groups ($R^2Y = 0.922$). In addition, the overall training model's predictive power of 88.8% ($Q^2 = 0.888$), resulted in 100% of samples to be correctly classified both in LOOCV and external validation (Table 3), without any possible misleading interpretation deriving from overfit or overprediction of the model as assessed by permutation test (average R^2Y-y-intercept = 0.048; average Q^2-y-intercept = -0.371). Further information about this validation is reported in Supplementary Materials, Supplementary Fig. S4.

As for the RMSECV and RMSEP values, these were found to be low (0.156 and 0.159, respectively) and very close to each other, thus remarking the outstanding ability of the simplified model in categorizing cuttlefish.

Fig. 3B shows the bidimensional score and loading graphs of the first two DFs obtained by S-LDA, while the first two LVs obtained by VIP-PLS-DA are reported in Fig. 3C. In both cases, cuttlefish were well separated from each other, with samples form Chioggia distributing in the lower right quadrant of the score plots. In addition, both the DF1 and the LV1 were mainly responsible for sample discrimination in the score plot.

By looking at the loading plots, Chioggia cuttlefish were found to be positively correlated with U, V, Ni, Ba, As, Rb, and K in S-LDA (Fig. 3B) and with Cs, Rb, K and P in PLS-DA (Fig. 3C). The highest loading scores for Mediterranean cuttlefish were instead observed for Cd in S-LDA, which in turn was diagonally opposite to the elements that characterized Chioggia samples. In PLS-DA model, the highest degree of correlation with Mediterranean cuttlefish was instead found for V, Se, Co, Cu, Mo, Ni, Bi, and Mn while for French Atlantic cuttlefish the highest contribution was exerted by Na together with Mg, U, Li, Sr, Ca and Cd. Likewise, Na showed a high loading value on the DF2 negative axis and, therefore, it allowed to distinguish French samples from the other groups in the S-LDA model.

According to the results of multi-element analysis of fish and seafood products already published in literature, some of the most discriminant elements found in the present study might be linked to anthropogenic pollution (As, V, Cd and U) or to geogenic sources (Cs and Mn) (Costas-Rodríguez et al., 2010).

Consistently to the results achieved, the major elements Na, Mg, and K were also previously identified among the most useful indicators of geographical origin of crabs (Luo et al., 2019) and prawns (Gopi et al., 2019). The variation in the amounts of Na, Mg and P in the tissues of Pacific shrimps was also correlated with specific sampling sites, whose waters were characterized by higher salinity (Li, Han, Dong, & Boyd, 2019).

The concentrations of As which, in the present work, was one of the selected discriminating elements for Chioggia cuttlefish, were also reported in mussels from Venice Lagoon (in which Chioggia is located) as being positively associated to the higher degree of salinity of this area and thus valuable for the identification of local products (Cubadda, Raggi, & Coni, 2006). Moreover, also Ni, Co, Se, Mo, and V amounts showed a strong link with anthropic sources of element contamination of the Venice Lagoon (Cubadda et al., 2006), therefore the specific pattern distribution of these trace elements in Chioggia cuttlefish tissues might be supposed to specifically reflect their origin.

Based on these results, the use of multivariate classifiers in combination with the pre-selection of the most origin-discriminant elements, was proved to be an efficient, rapid and smart analytical strategy to assure the authenticity of the provenance of cuttlefish samples. Although each of the classification techniques applied showed satisfying results, suggesting that possible users may choose the most convenient methodology to suit the specific needs, superior and unequivocal outcomes were obtained by applying VIP-PLS-DA.

4. Conclusions

Cephalopods are among some of the most consumed fishery products and are appreciated by the consumers all over the world but, to the best of our knowledge, scientific insights concerning their elemental profile and the authentication of their geographical origin are still lacking.

The results presented in this study revealed for the first time that the geographical imprint of Italian traditional cuttlefish from Chioggia can be extracted through the simultaneous quantification of more than fifty elements by ICP-MS and successfully used to discriminate the product from cuttlefish originating from different areas.

The whole elemental profile was elaborated by means of different chemometric techniques. In particular, three independent variable selection strategies merged with linear or regression pattern recognition multivariate techniques (LW-LDA, S-LDA, and VIP-PLS-DA) were tested to develop classification rules while reducing the number of variables to the lowest possible, in order to find the most parsimonious way that best described sample origins. Although the application of VIP-PLS-DA led to the extraction of the highest number of variables, analysis of performance metrics suggested the best results for this methodology, since values of sensitivity, specificity, and classification accuracy of 100% were achieved in internal and in external validation thanks to the contribution of Na, K, Ca, P, Mg, Cu, Co, Mn, Se, Ni, Mo, Li, B, Ba, Bi, Sr, As, V, Rb, Cs, Y, U, and Cd.

In summary, the elemental pattern linked to the geographical origin of cuttlefish appears to be determined by a combination of macro, trace and ultra-trace elements of both natural and anthropogenic origin, which are known to be absorbed by the animals from the surrounding environment. In particular, the contribution of anthropogenic elements here strongly emerges as a key analytical determinant for cephalopods authenticity assessment. Of note, also concentrations of some heavy toxic metals such as Cd and As, although being so low as not to represent a potential health risk, were useful for the discrimination purpose.

Considering the permanent and continuous release of all these elements in the aquatic environment deriving from modern industrial and agricultural practices, the quantification of anthropogenic contaminants in fishery products should therefore be encouraged, as well as further specific critical evaluations of their variation within seawater is recommended. In such a scenario, the promising results obtained may pave the way for practical application of the proposed methodology in the fishery sector to trace back different geographical origins, but also for the protection and ongoing promotion of traditional local fishery products for which a quality mark has been recognized.

Appendix A. Supplementary Materials

Supplementary data associated with this article can be found, in the online version, at

Declaration of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability: The dataset generated during the current study is available from the corresponding author Prof. Lenka Husáková on reasonable request.

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Figure Captions

Fig. 1. Pearson's correlation heat-map of the investigated elements.

Fig. 2. Biplot resulting from PCA applied to all the elements (A) and selected elements (B). The sample surrounded by the red dotted line in the biplot indicate an outlier according to Hotelling's T^2 test (95% confidence interval). Blue triangles: samples from Chioggia (FAO fishing area 37.2.1); red diamonds: samples from Mediterranean Sea (FAO fishing area 37.1/37.2); yellow circles: samples from French Atlantic (FAO fishing area 27.7.e).

Fig. 3. Comparison of score (left) and loading graphs (right) from LW-LDA (A), S-LDA (B) and VIP-PLS-DA (C) for cuttlefish samples from different origins. Blue triangles: samples from Chioggia (FAO fishing area 37.2.1); red diamonds: samples from Mediterranean Sea (FAO fishing area 37.1/37.2); yellow circles: samples from French Atlantic (FAO fishing area 27.7.e).

Table 1

Agilent 7900 ICP-MS operating conditions.

Parameter	Setting		
ICP			
Plasma mode	General purpose		
Rf power (27 MHz) (W)	1550		
Sampling depth (mm)	8		
Plasma gas flow (L min^{-1})	15		
Auxiliary gas flow (L min^{-1})	0.9		
Nebulizer gas flow (L min^{-1})	1		
Nebulizer pump (rps)	0.1		
Spray chamber temperature (°C)	2		
Mass spectrometer	No gas mode	He mode	HEHe mode [a]
Extract 1 (V)		0	
Extract 2 (V)		-250	
Omega bias (V)	-100	-120	-120
Omega lens (V)	9.7	7.8	9.6
Cell entrance	-30	-40	-140
Cell exit	-50	-60	-150
Deflect (V)	11.6	1	-77
Plate bias	-35	-60	-150
Helium flow (mL min^{-1})	0	5	10
OctP bias	-8	-18	-100
OctP RF		200	
Energy discrimination (V)		5	
Number of elements	39 [b]	12 [c]	5 [d]
Acquisition			
Points per peak	1		
Replicates	3		
Sweeps/replicate	100		
Total acquisition time (s)	75		

[a] HEHe mode - high energy helium mode; Monitored isotopes (integration time): b) ^{7}Li, ^{11}B, ^{24}Mg, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{95}Mo, ^{101}Ru, ^{103}Rh, ^{105}Pd, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{133}Cs, ^{138}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{153}Eu, ^{157}Gd, ^{159}Tb, ^{163}Dy,^{165}Ho, ^{166}Er, ^{172}Yb, ^{175}Lu, ^{178}Hf, ^{185}Re, ^{195}Pt, ^{205}Tl, $^{206+207+208}$Pb, ^{209}Bi, ^{232}Th, ^{238}U (all 0.1 s); c) ^{23}Na (0.3 s), ^{27}Al (0.1 s), ^{39}K, ^{44}Ca (both 0.3 s), ^{51}V (1 s), ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{103}Rh (all 0.3 s); d) ^{31}P (0.1 s), ^{75}As, ^{78}Se (both 1 s), ^{103}Rh (0.3 s).

Table2 (text with changes Marked)

Table 2 Multi-elemental composition of cuttlefish from different geographical origins. Elements are sorted according to decreasing median concentrations of cuttlefish from Chioggia.

	Chioggia (n = 17*3)				Mediterranean Sea (n = 25*3)				French Atlantic (n = 26*3)			
Element	Mean ± ME	Median	Min	Max	Mean ± ME	Median	Min	Max	Mean ± ME	Median	Min	Max
Na	11164 ± 867	11235^{a}	8659	14187	14334 ± 486	14290^{b}	12015	16338	24632 ± 1332	24730^{c}	17932	30749
K	13471 ± 1067	13768^{a}	9113	16488	10648 ± 402	10547^{b}	8898	12400	8353 ± 531	8252^{c}	6196	12039
P	10697 ± 831	10635^{a}	7459	1617	9084 ± 221	9079^{b}	7440	9883	8203 ± 308	8176^{c}	6791	10401
Mg	2297 ± 207	2274^{a}	1626	3180	2613 ± 75	2643^{a}	2164	2871	3767 ± 183	3776^{b}	2898	4795
Ca	820 ± 102	751^{a}	619	1338	1299 ± 53	1304^{b}	1019	1513	1781 ± 118	1760^{c}	1239	2493
As	79 ± 9	75.8^{a}	56.1	121.3	69 ± 4.3	67.4^{ab}	50.5	98.8	60 ± 4.7	62.3^{b}	35.5	77.1
Li	0.25 ± 0.024	0.24^{a}	0.18	0.34	0.34 ± 0.011	0.33^{b}	0.28	0.38	0.50 ± 0.025	0.51^{c}	0.36	0.65
Zn	62 ± 5.2	60.7^{a}	41.1	86.3	73 ± 1.6	72.8^{b}	63.8	80.7	74 ± 3.5	73.8^{b}	58.6	94.1
Al	16 ± 5.5	10.7^{a}	4.7	43.8	7 ± 1.9	5.69^{b}	2.97	22.7	7 ± 2.5	5.44^{b}	2.87	34.4
Cu	10 ± 1.8	8.55^{a}	5.93	20.2	24 ± 3.4	21.3^{b}	12.5	42.9	20 ± 3.4	16.3^{b}	8.29	36.2
Sr	9 ± 1.1	8.83^{a}	6.69	14.9	14.8 ± 0.44	14.9^{b}	12.0	16.8	25 ± 1.6	26.0^{c}	16.3	33.2
Fe	11 ± 4	7.85^{a}	3.62	33.0	9 ± 1.9	8.19^{a}	4.32	25.0	8 ± 2	5.59^{a}	3.41	21.7
B	6.3 ± 0.56	6.06^{ab}	4.46	8.20	5.3 ± 0.29	5.35^{a}	4.11	7.15	7.6 ± 0.85	7.88^{b}	4.15	11.8
Rb	5.3 ± 0.42	5.17^{a}	3.94	6.99	4.5 ± 0.14	4.61^{a}	3.93	5.13	3.6 ± 0.22	3.57^{b}	2.56	5.27
Se	1.3 ± 0.17	1.33^{a}	0.881	2.11	2.0 ± 0.14	1.91^{b}	1.47	2.69	1.65 ± 0.083	1.62^{c}	1.26	2.16
Mn	2.2 ± 0.87	1.28^{a}	0.93	6.86	2.5 ± 0.52	2.04^{a}	1.41	6.59	1.0 ± 0.23	0.83^{b}	0.54	3.57
Ni	0.4 ± 0.52	0.36^{a}	0.22	0.76	0.52 ± 0.056	0.50^{b}	0.27	1.98	0.28 ± 0.034	0.26^{a}	0.15	0.48
Cr	0.18 ± 0.060	0.18^{a}	0.037	0.55	0.15 ± 0.041	0.13^{a}	0.068	0.54	0.22 ± 0.082	0.13^{a}	0.081	1.07
Ba	0.3 ± 0.13	0.18^{a}	0.087	1.17	0.22 ± 0.031	0.16^{a}	0.12	0.50	0.21 ± 0.039	0.18^{a}	0.11	0.43
Pb	0.10 ± 0.024	0.093^{a}	0.050	0.23	0.10 ± 0.019	0.099^{a}	0.063	0.31	0.09 ± 0.017	0.046^{a}	0.077	0.20
V	0.07 ± 0.036	0.040^{a}	0.018	0.26	0.25 ± 0.040	0.22^{b}	0.12	0.47	0.11 ± 0.023	0.11^{a}	0.039	0.27
Zr	0.04 ± 0.018	0.027^{a}	0.0059	0.13	0.05 ± 0.015	0.036^{ab}	0.0071	0.14	0.2 ± 0.20	0.051^{b}	0.0066	2.55
Co	0.04 ± 0.019	0.025^{a}	0.016	0.15	0.21 ± 0.023	0.21^{b}	0.11	0.31	0.14 ± 0.026	0.14^{c}	0.058	0.31
Mo	0.029 ± 0.0066	0.024^{a}	0.018	0.060	0.10 ± 0.013	0.089^{b}	0.061	0.18	0.10 ± 0.019	0.084^{b}	0.044	0.23
Pd	0.016 ± 0.0013	0.016^{a}	0.012	0.020	0.024 ±0.0054	0.021^{a}	0.012	0.067	0.029 ± 0.0033	0.027^{b}	0.017	0.059
Cs	0.017 ± 0.0015	0.016^{a}	0.012	0.023	0.0146 ± 0.00085	0.014^{a}	0.012	0.023	0.012 ± 0.0014	0.012^{b}	0.0081	0.027
Y	0.02 ± 0.012	0.015^{a}	0.0057	0.11	0.008 ± 0.0012	0.0074^{b}	0.0039	0.017	0.01 ± 0.013	0.0077^{b}	0.0050	0.17
Bi	0.178 ± 0.0022	0.017^{a}	0.011	0.027	0.023 ± 0.0040	0.021^{a}	0.015	0.065	0.010 ± 0.0041	0.0066^{b}	0.0029	0.044
Sn	0.015 ± 0.0071	0.011^{a}	0.0044	0.061	0.022 ± 0.0049	0.019^{b}	0.0075	0.051	0.015 ± 0.0040	0.011^{a}	0.0052	0.053
Cd	0.02 ± 0.017	0.0099^{a}	0.0035	0.13	0.08 ± 0.013	0.071^{b}	0.035	0.15	0.25 ± 0.059	0.22^{c}	0.067	0.58
Sb	0.007 ± 0.0028	0.0053^{a}	0.0023	0.021	0.009 ± 0.0012	0.0084^{b}	0.0046	0.016	0.0075 ± 0.00080	0.0068^{b}	0.0052	0.014
U	0.0032 ± 0.00076	0.0026^{a}	0.0019	0.0074	0.0043 ± 0.00042	0.0040^{a}	0.0027	0.0069	0.010 ± 0.0015	0.0091^{b}	0.0049	0.019
Th	0.002 ± 0.0011	0.0016^{a}	0.00081	0.0082	0.005 ± 0.0036	0.0022^{a}	0.0011	0.046	0.003 ± 0.0019	0.0015^{a}	0.0011	0.024
Ce*	29 ± 10	20.6^{a}	3.56	82.0	44 ± 20	24.0^{a}	11.4	250	26 ± 8.9	18.8^{a}	8.31	91.7
La*	20 ± 11	11.9^{ab}	2.70	90.87	29 ± 12	20.3^{a}	8.03	150	16 ± 5.6	10.5^{b}	5.09	56.0
Nd*	8 ± 4.7	3.98^{a}	1.58	33.2	5 ± 1.5	4.45^{a}	1.74	20.1	4 ± 1.0	2.82^{b}	1.33	12.0
Hf*	2.5 ± 0.52	2.23^{a}	1.15	4.94	8 ± 8.4	2.89^{ab}	1.42	103	8 ± 4.9	4.19^{b}	0.92	57.3
Tl*	1.5 ± 0.11	1.42^{a}	1.07	1.94	1.4 ± 0.51	1.21^{b}	0.83	7.30	1.3 ± 0.61	1.05^{b}	0.62	8.66
Pr*	2 ± 1.0	0.96^{a}	0.38	7.43	1.2 ± 0.34	0.97^{a}	0.45	4.62	0.8 ± 0.23	0.63^{b}	0.33	2.56
Sm*	1.5 ± 0.81	0.74^{a}	0.25	5.04	0.9 ± 0.28	0.82^{a}	0.34	3.73	0.7 ± 0.18	0.55^{a}	0.33	2.32
Dy*	1.1 ± 0.44	0.74^{a}	0.34	3.22	0.8 ± 0.18	0.66^{ab}	0.27	2.21	0.7 ± 0.17	0.51^{b}	0.33	2.02
Gd*	1 ± 0.5	0.69^{ab}	0.24	3.20	0.8 ± 0.19	0.72^{a}	0.32	2.52	0.6 ± 0.15	0.50^{b}	0.31	1.97
Er*	0.7 ± 0.23	0.54^{a}	0.20	1.73	0.5 ± 0.10	0.42^{ab}	0.23	1.17	0.4 ± 0.11	0.39^{b}	0.19	1.31
Yb*	0.7 ± 0.21	0.51^{a}	0.22	1.48	0.39 ± 0.078	0.37^{ab}	0.14	0.93	0.4 ± 0.10	0.34^{b}	0.16	1.31
Eu*	0.4 ± 0.18	0.24^{ab}	0.12	1.19	0.28 ± 0.061	0.25^{a}	0.15	0.87	0.22 ± 0.044	0.19^{b}	0.12	0.62
Ho*	0.22 ± 0.078	0.17^{a}	0.062	0.60	0.15 ± 0.030	0.14^{ab}	0.047	0.39	0.14 ± 0.037	0.11^{b}	0.063	0.45
Tb*	0.20 ± 0.082	0.14^{a}	0.056	0.58	0.15 ± 0.037	0.12^{ab}	0.055	0.43	0.12 ± 0.030	0.095^{b}	0.054	0.37
Ru*	0.10 ± 0.029	0.087^{a}	0.031	0.24	0.14 ± 0.027	0.13^{a}	0.054	0.34	0.14 ± 0.023	0.13^{a}	0.044	0.29
Re*	0.10 ± 0.032	0.081^{ab}	0.016	0.24	0.12 ± 0.019	0.11^{a}	0.070	0.24	0.09 ± 0.023	0.072^{b}	0.025	0.28
Lu*	0.09 ± 0.032	0.074^{a}	0.023	0.23	0.06 ± 0.015	0.047^{a}	0.021	0.18	0.06 ± 0.017	0.053^{a}	0.021	0.22
Pt*	0.13 ± 0.034	0.10^{a}	0.060	0.25	0.2 ± 0.12	0.12^{a}	0.062	1.53	0.15 ± 0.061	0.10^{a}	0.019	0.60
Hg$^{\#}$	0.20 ± 0.030	0.18^{a}	0.15	0.34	0.20 ± 0.023	0.17^{a}	0.14	0.38	0.21 ± 0.018	0.20^{a}	0.13	0.29

Concentrations are expressed as mg kg^{-1} (d.m.) and are reported as mean values ± margin of error (ME) at 95% confidence level. Min–Max: minimum and maximum values found. *: concentrations are expressed as µg kg $^{-1}$. Hg$^{\#}$: determined by direct mercury analyzer AMA254. Values followed by different superscript letters (a–c) in the same row are significantly different ($p \leq 0.05$).

Multi-element signature of cuttlefish and its potential for the discrimination of different geographical provenances and traceability

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ABSTRACT

The measurement and analysis of fifty-two elements by quadrupole inductively coupled plasma mass spectrometry (Q-ICP-MS) and direct mercury analysis were applied to origin discrimination of Italian traditional cuttlefish (Chioggia, Venice lagoon) from Mediterranean and Atlantic samples. A total 68 specimens were analyzed in triplicates to generate 204 mass spectra profiles which were statistically processed by different chemometric techniques. Loading weights from principal component analysis as input for linear discriminant analysis (LW-LDA), stepwise-LDA (S-LDA) and variable influence of projection-partial least square discriminant analysis (VIP-PLS-DA) were used to classify samples while retaining the lowest possible number of key variables. VIP-PLS-DA was found to be the best variable selection-discriminant tool combo since the selected Na–Co–B–K–Cd–V–U–Rb–Ni–Ba–Cu–As–Sr–Mn–Mo–Li–Ca–Mg–Se–Bi–Cs–P–Y elemental pattern allowed the samples to be classified with 100% sensitivity, specificity and accuracy.

Abbreviations

Certified reference materials, CRMs, correlation analysis, CA; discriminant function, DF; high energy Helium mode, HE He; inductively coupled plasma mass spectrometry, ICP-MS; inductively coupled plasma optical emission spectrometry ICP-OES; internal standard, ISTD; kinetic energy discrimination, KED; linear discriminant analysis, LDA; leave-one-out-cross-validation, LOOCV; loading weights-based linear discriminant analysis, LW-LDA; method detection limit of the method, MDL; method limit of quantification, MLOQ; microwave digestion, MWD; partial last square-discriminant analysis, PLS-DA; principal component, PC; principal component analysis, PCA; quadrupole inductively coupled plasma mass spectrometry, Q-ICP-MS; rare earth elements, REEs; relative standard deviation, RSD; root mean square error from cross-validation, RMSECV; root mean square error of estimation, RMSEE; root mean square error of prediction, RMSEP; stepwise- linear discriminant analysis, S-LDA; variable influence on projection, VIP; variable influence of projection-partial least square discriminant analysis, VIP-PLS-DA.

Keywords: ICP-MS; element profiling; fishery products; geographical origin; chemometrics.

1. Introduction

The common cuttlefish (*Sepia officinalis*, L., 1758) is a highly valuable fishery product whose distribution extends from the Eastern Atlantic (from the Baltic and North Seas to South Africa coasts) up to the whole Mediterranean Sea. Large scale fishing operations of common cuttlefish led to an average production of 21 825 000 kg in 2018, which ensured the product's supply all over the world (especially in Italy, Spain, Japan, and South Korea), while smaller-scale fishing is particularly profitable in some Mediterranean areas where it has a high impact on local economies (FAO, 2020). This activity is particularly developed in the fishing village of Chioggia (north-western Adriatic Sea in the Eastern Mediterranean Basin), whose *S. officinalis* production reached more than 454 000 kg in 2019 (Clodia database, 2017).

Chioggia cuttlefish is well known to be of high quality thanks to the preservation of a close link with the territory and the traditional processing methods by local fish plants, which allow the products to be officially certified as Italian Traditional Agri-food Product (P.A.T.) (Ministerial Decree, G.U. n. 48, 2014). Foodstuffs with specific geographical ties (especially when a quality mark has been recognized) are in turn rated more highly in terms of quality by the consumer, mostly because of lower environmental impact and higher perceived safety of regional products.

Alongside with the compulsory labelling requirement established by European legislation concerning the provision of detailed information about the geographical origin of fish and seafood (European Parliament and Council of the European Union, 2013), the protection and the promotion of traditional foods must involve origin authenticity assessment both to ensure traceability and prevent commercial frauds (Ortea & Gallardo, 2015).

Complex multi-disciplinary and cross-disciplinary approaches are usually required to verify the geographical origin of fish and seafood since both environment and genetics can affect the final characteristics of the products (Abbas et al., 2018). Nevertheless, considering the association between the concentrations of elements in fish tissues and those in the surrounding aquatic environment, the determination of the multi-element profile of fish and seafoods can be regarded as a valid analytical strategy to guarantee fish origin authenticity. Several factors such as species, size, age, sex, and sexual maturity can directly affect the elemental composition of aquatic animals, but food resources, climate, presence of contaminants, and many water quality parameters (*p*H, dissolved oxygen, alkalinity) are those which differ to a greater extent between countries (Li, Boyd, & Sun, 2016; Smith & Watts, 2009).

Several studies reported the possibility to discriminate fishery and seafood products as croaker (Chaguri et al., 2015), sea cucumber (Kang et al., 2018), shrimps (Ortea & Gallardo, 2015), crabs (Luo et al., 2019), and bivalve mollusks such as mussels (Costas-Rodríguez, Lavilla, & Bendicho, 2010) and clams (Iguchi, Isshiki, Takashima, Yamashita, & Yamashita, 2014) using element composition. In these works, the chemical characterization of samples was based on minor and/or trace elements (Costas-Rodríguez et al., 2010; Iguchi et al., 2014), a combination of major, minor, and trace elements (Kang et al., 2018), or a combination of major and trace elements plus stable isotopes analysis (Chaguri et al., 2015; Ortea et al., 2015; Luo et al., 2019).

In the range of analytical methods applied to the determination of elements, inductively coupled plasma-mass spectrometry (ICP-MS) combined with different chemometrics techniques holds a unique position by virtue of speed, sensitivity, dynamic range, and elemental coverage (Drivelos & Georgiou, 2012; Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016). In this context, several supervised and unsupervised chemometric tools such as, principal component analysis (PCA) (Chaguri et al., 2015; Kang et al., 2018; Ortea et al., 2015), cluster analysis and *k*-means hierarchical clustering (Ortea et al., 2015), linear discriminant analysis (LDA) (Costas-Rodríguez et al., 2010; Iguchi et al., 2014; Ortea et al., 2015; Kang et al., 2018; Luo et al., 2019), support-vector machine (Luo et al., 2019), soft-independent modelling of class analogy and artificial neural networks (Costas-Rodríguez et al., 2010) were applied for authenticity and traceability purposes.

The determination of elemental composition of fish and seafood to trace their geographical provenance is particularly suitable for cephalopods mollusks. Cephalopods such as cuttlefish are in fact at high trophic levels in the aquatic food chain and the overall elements accumulated in tissues are good indicators of the surrounding habitat (Bosch, O’Neill, Sigge, Kerwath, & Hoffman, 2015). In addition, cephalopods are non-migratory animals and, therefore, their elemental composition can reasonably be expected to be constant during life (Gopi et al., 2019). Nevertheless, very few studies addressed the topic of origin discrimination of cephalopods mollusks using elemental composition, in spite of being focused on the analysis of a limited number of major and trace elements included in hard structures such as statoliths (Arbuckle & Wormuth, 2014) or ink (Bua et al., 2017). These components are in fact not always retained in the final commercial product and, therefore, not always exploitable in practical food surveillance operations to trace back the origin of cephalopods.

Based on this background, the present work aimed at outlining for the first time the *S. officinalis* multi-elemental profile of edible tissues to verify whether useful information could be extracted and specifically linked to the geographical origin of the Italian traditional cuttlefish from Chioggia (FAO subarea 37.2.1) in order to be differentiated from non-Chioggia cuttlefish caught in other fishing areas of the Mediterranean Sea (FAO 37.1/37.2) and in the French Atlantic Ocean (FAO 27.7.e). For this purpose, fifty-one elements were determined by ICP-MS and Hg was quantified via atomic absorption spectroscopy. Unsupervised and supervised pattern recognition methods were used to investigate sample characteristics, identify the key discriminant elements, and, at the same time, develop classification rules.

2. Materials and Methods

2.1. Reagents and standards

The ultrapure water (0.055 µS cm^{-1} conductivity) obtained using the Milli-Q® water purification system (Millipore, Bedford, USA) was used for the preparation of all solutions. Sub-boiled nitric acid was prepared from nitric acid (65%, w/w) of Selectipur quality (Lach-Ner, Neratovice, Czech Republic) using the distillation equipment BSB-939-IR (Berghof, Eningen, Germany). Hydrogen peroxide (Trace Select, ≥ 30%, w/w) was purchased from Fluka Chemie AG (Buchs, Switzerland).

Multi-element stock solution “A” containing 10 mg L^{-1} of Li, B, Al, V, Cr, Fe, Ni, Co, As, Se, Rb, Sr, Zr, Mo, Ru, Pd, Cd, Sn, Sb, Cs, Ba, Hf, Re, Pt, Tl, Pb, Bi, and Th was prepared from the Supelco ICP multi-element standard solution IV (Merck, Darmstadt, Germany) and single element standards of concentration 1 ± 0.002 g L^{-1} (Analytika Ltd., Prague, Czech Republic or SCP Science, Montreal, Canada). Multi-element solution “B” containing 1 mg L^{-1} of La, Ce, Pr, Nd, U (“B1”) and 0.20 mg L^{-1} of Y, Tb, Ho, Yb, Sm, Eu, Gd, Er, Lu, and Dy (“B2”) was prepared from the stock solution of rare earth elements Astasol mix “M008” (Analytika Ltd., Prague, Czech Republic). Multi-element solution “C” containing 50 mg L^{-1} of Na, Mg, P, K, Ca, Mn, Cu, and Zn was prepared from single element standards of 1 g L^{-1} obtained from Analytika Ltd. The internal standard solution (ISTD) was prepared from 1 g L^{-1} stock solution of Rh obtained from SCP Science (Montreal, Canada). Carbon reference solutions were prepared from 10 g L^{-1} of C stock solution prepared from urea of TraceSelect quality (Fluka Chemie AG, Buchs, Switzerland).

2.2. Sampling and preparation of cuttlefish

A total of 68 samples of common cuttlefish (*S. officinalis*, L.) including n = 17 samples from Adriatic Sea (Chioggia, Italy, FAO 37.2.1), n = 25 non-Chioggia samples from the Mediterranean Basin (FAO 37.1/37.2), and n = 26 samples from North-eastern Atlantic Ocean (France, FAO 27.7.e) were analyzed. Specimens were caught by fishing trawlers during the months of September and randomly collected from different batches in local fish plants located in Chioggia (Venice, Italy), by choosing homogeneous sizes and weights (mantle lengths from 10 ± 2 cm; body weight 125 ± 25 g). After collection, samples were immediately frozen and stored at −20 °C for around 3 months prior to be further processed for multi-element analysis.

2.3. Quality assurance and quality control

The following commercially supplied certified reference materials (CRMs) were analyzed: NIST SRM 1577 Bovine Liver (National Institute of Science and Technology, NIST, Gaithersburg, MD, USA); NIST SRM 1566 Oyster Tissue (NIST, Gaithersburg, MD, USA); BCR® certified reference material (CRM)184 Bovine muscle (Institute for Reference Materials and Measurements, IRMM, Geel, Belgium); BCR® 185 Bovine Liver (IRMM, Geel, Belgium); CRM NCS ZC73015 Milk Powder (National Research Centre for Certified Reference Materials, NRCRM, Beijing, China); P-WBF CRM 12-2-04 Essential and Toxic Elements in Wheat Bread Flour (pb-anal, Kosice, Slovakia); CRM12-2-03 P-Alfalfa Essential and toxic elements in Lucerne (pb-anal, Kosice, Slovakia); SMU CRM 12-02-01 Bovine liver (pb-anal, Kosice, Slovakia).

2.4. Sample preparation

2.4.1. Pre-processing of cuttlefish

Frozen samples were thawed overnight (16–18 h at +4 °C) before processing for multi-element analysis. Each specimen was then washed with deionized water and skin, cuttlebone, gills, reproductive and digestive tracts were carefully removed without causing their rupture. The head, arms and tentacles were excluded, while the mantle and the lateral fins were rinsed again with ultrapure water and minced with a ceramic knife.

2.4.2. Freeze-drying process

Pre-treated sample portions of approx. 10 g were individually transferred into 100 mL cleaned round bottom flasks wherein the material was dried and placed into a deep freezer at −80 °C for 24 hours to provide a necessary conditioning for drying. Lyophilization was conducted at −50 °C and 0.01 bar for 24 h with a LIO-5P apparatus (CinquePascal srl, Trezzano sul Naviglio, Milan, Italy). Dried samples were subsequently removed from the flasks, homogeneously ground into a powder by using ceramic mortars and pestles and then individually sealed into LDPE bags.

2.4.3. Microwave assisted digestion

Microwave digestion (MWD) of samples was carried out by using the Speedwave^{TM} MWS-3^{+} (Berghof, Eningen, Germany) microwave system with the maximum total output of the microwave generator (1450 W) and equipped with the optical sensor technology for contactless real-time recording of the sample temperature. The high-pressure resistant (up to 100 bar) PTFE vessels DAC-100S, together with the Multitube System (all from Berghof, Eningen, Germany), were used for sample digestion. This arrangement allowed the simultaneous digestion of three samples in one DAC-100S PTFE vessel by placing three PFA tubes into each vessel (Husáková et al., 2015). The maximal number of used DAC-100S vessels for one digestion cycle was eight.

A powdered cuttlefish or CRM sample aliquot of 0.1 g was weighed into a 10 mL PFA tube. Then, 4 mL of 16% HNO_3 (65%, w/w HNO_3, 1:3 diluted) and 1 mL of 30% H_2O_2 were added, leaving the vessels open until the initial reaction subsided. Three PFA tubes containing the same sample and reagents were placed into the outer 100mL PTFE digestion vessel previously filled with 25 mL of HNO_3 (16%, v/v), by ensuring that the level of liquid in the outer PTFE vessel was higher than those in the PFA tubes. This way, the vapor pressures were compensated and the evaporation of the solution from the PFA tubes was avoided (Husáková et al., 2015). The samples were digested following a five-step program: (i) 20 min at 180 °C and 80 % power (ramp 5 min), (ii) 20 min at 220 °C and 95 % power (ramp 5 min), (iii–v) 5 min at 100 °C and 10 % power (ramp 1 min). The resulting colorless solutions were diluted to 25 mL with deionized water.

Each sample was mineralized in three replicates. Blanks, consisting of deionized water and reagents were subjected to a similar preparation procedure.

To assess the properness of the digestion process, the residual carbon content in the final cuttlefish digests was determined by a previously described inductively coupled plasma optical emission spectrometry (ICP-OES) method (Husáková et al., 2011) was 5.5 ± 0.4 % (n = 3).

2.5. ICP multi elemental analysis

ICP-MS measurements were performed by using the Agilent 7900 quadrupole mass spectrometer (Q-ICP-MS, Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an octopole-based collision cell for interference removal using kinetic energy discrimination (KED).

The standard sample introduction system, consisting of a glass concentric nebulizer MicroMist (400 µL min^{-1}), the Peltier-cooled (2 °C) Scott quartz spray chamber and quartz torch with 2.5 mm internal diameter injector, was used. For precise delivery of samples and ISTD, a low-pulsation, 10-roller peristaltic pump with three separate channels was employed. The internal standard kit, including connecting tubing, connectors and the "Y" piece was used for simultaneous internal standard aspiration and its mixing with the sample. The standard sampling and skimmer nickel cones with orifices of 1 and 0.45 mm, respectively, were used. ICP-MS operating conditions were optimized during each start-up sequence by using the multi-elemental tuning solution (Agilent Technologies, Inc., Santa Clara, CA, USA) containing 1 µg L^{-1} of Ce, Co, Li, Mg, Tl and Y, in order to obtain the highest possible sensitivity for elements of low, middle and high m/z. Using the typical operating conditions summarized in Table 1, a sensitivity of 6000 counts s^{-1} per µg L^{-1} and a resolution of 0.64 amu peak width (full width at half maximum intensity) were achieved for $^{7}Li^{+}$. The same parameters were 50000 counts s^{-1} per µg L^{-1} and 0.62 for $^{89}Y^{+}$, and 30000 counts s^{-1} per µg L^{-1} and 0.60 for $^{205}Tl^{+}$.

For sample analysis the "General Purpose" plasma mode included in the ICP-MS MassHunter software was used (Agilent Technologies, Inc., Santa Clara, CA, USA). The working parameters of the cell mode "no-gas" were autotuned during the instrument start-up sequence. The working parameters of the collision cell for helium ("He") and high energy He ("HE He") modes were adjusted manually. The time required for a transition between cell modes was 5 s. Parameters related to sample introduction and plasma conditions were consistent for all modes (see Table 1).

Concentrations of a total of 51 elements were evaluated from calibration curves with coefficients of determination better than 0.999 and built up within the ranges given below.

Calibration solutions: blank, 1, 5, 10, 50, 100 µg L^{-1} of Li, B, Al, V, Cr, Fe, Ni, Co, As, Se, Rb, Sr, Zr, Mo, Ru, Pd, Cd, Sn, Sb, Cs, Ba, Hf, Re, Pt, Tl, Pb, Bi, Th; 0.1, 0.5, 1, 5, 10 µg L^{-1} La, Ce, Pr, Nd, U; 0.02, 0.1, 0.2, 1, 2 µg L^{-1} of Y, Tb, Ho, Yb, Sm, Eu, Gd, Er, Lu, and Dy; 0.5, 1, 5, 10 mg L^{-1} of Na, Mg, P, K, Ca, Mn, Cu, and Zn. The solutions were prepared daily by appropriate dilution of multi-element solutions "A" (500 µg L^{-1}), "B" (50 + 10 µg L^{-1}) and "C" (50 mg L^{-1}) in 25 mL volumetric flasks (see Section 2.1).

To compensate possible instrumental drift and matrix effects, a 200 µg L^{-1} Rh ISTD was simultaneously aspired and mixed with samples.

2.6. Mercury analysis

For the quantification of Hg content, the direct solid sampling analysis using a single-purpose atomic absorption spectrometer AMA 254 (Altec Ltd., Prague, Czech Republic), based on in situ dry ashing followed by gold amalgamation atomic absorption spectroscopy, was used. Each time, samples were weighed in a nickel boat and analyzed under the following experimental conditions: typical sample mass, 50 mg; drying time, 60 s; decomposition, ~750 °C for 150 s; waiting step, 900 °C, 45 s, necessary for quantitative release of trapped mercury from the gold amalgamator to the measuring cuvette. The peak area absorbance at 253.6 nm was monitored. The flow rate of oxygen (99.5%) carrier gas was 170 mL min^{-1}.

The AMA 254 spectrometer was regularly calibrated using blank and four working standard solutions containing Hg(II) ranging from 0.025 to 0.2 mg L^{-1}. The working standard solutions were prepared from standard aliquots of 10 mg L^{-1} prepared from a 1 g L^{-1} stock solution of Hg(II) (Analytika Ltd., Prague, Czech Republic).

2.7. Data treatment

Statistic was applied to elemental concentrations referring to cuttlefish dry matter (d.m.) and carried out using IBM-SPSS (v. 23.0, SPSS Inc., Chicago, IL, USA) SIMCA-P v.14.1 (Umetrics, Umeå, Sweden), and Statgraphics Centurion v.18.1 (StatPoint Technologies, Inc, Warrenton, VA, USA) software. The normality and homogeneity of variance of data was checked beforehand by applying the Shapiro-Wilk's and Levene's tests, respectively ($p \leq 0.05$). Since the normal distribution assumption was violated for most of the elemental data, quantitative differences between groups of cuttlefish from different origins were investigated by the nonparametric Kruskal–Wallis test with Dunn's multiple post hoc test for multiple comparison ($p \leq 0.05$). Data was presented as mean values ± margin of error (calculated at 95% confidence level), median and minimum and maximum values found.

While the nonparametric statistic was used to compensate for the lack of data normal distribution, thus preserving the original information and avoiding the loose of any intuitive meanings when describing element concentrations resulting from univariate data analysis, a Box-Cox transformation was instead applied to normalize data for subsequent bivariate and multivariate data analyses.

First, the bivariate Pearson's correlation analysis (CA) was employed to Box-Cox transformed data to explore variable distribution and look for strong positive ($r > 0.6$, $p \leq 0.01$) or strong negative ($r < -0.6$, $p \leq 0.01$) linear correlations between binary variables.

Because of the highly different magnitude of values among element concentrations, the Box-Cox data were then scaled applying a Z-score standardization and further processed by multivariate data analyses. PCA was used for further investigation of covariance patterns in elemental concentrations, data's first interpretation and multivariate outliers' removal based on Hotelling's T^2 test results (95% confidence interval). During this step, the number of variables was also reduced by selecting elements characterized by loading values (rescaled to 0–1) > 0.70.

Afterwards, supervised classifiers combined with different variable selection procedures were developed to achieve the discrimination of samples according to the geographical origin by using the lowest number and the most influential combination of variables. To that end, linear discrimination based on variable loading weights previously selected by PCA (LW-LDA), forward stepwise-LDA (S-LDA) and variable influence on projection-(VIP)-PLS-DA were independently tested and compared in terms of prediction ability. All the models were cross-validated using leave-one-out-cross-validation (LOOCV) to exclude overfitting. In addition to LOOCV, the validity of the PLS discriminant models was also checked by permutation testing (400 random permutations).

When supervised modelling was performed, about 33% of data (n = 68) was randomly sidelined from the calibration set (n = 136) and used for independent external validation. Models' overall performances in internal and external validation were evaluated in terms of accuracy (%), sensitivity (%), and specificity (%).

Further details about the statistical treatments applied can be found in Supplementary material, Supplementary Experimental Section.

3. Results

3.1. Analytical performances and validation

The analytical determination of fifty-two major, trace and ultra-trace elements in mineralized cephalopod samples was carried out by Q-ICP-MS, taking advantage of its well-known sensitivity toward the targeted elements. On the other hand, analysis of Hg by thermal decomposition was performed by AMA254 without pre-treatment and/or preconcentration steps, thus overcoming the most serious problems related to ICP-MS analysis of Hg, e.g. long washout time, non-linear calibration curves, and decreasing sensitivity with time (Li et al., 2006).

The microwave-assisted pressurized digestion which involved small sample amounts (100 mg) was employed to ensure rapid sample preparation for subsequent ICP-MS analysis while attaining the high decomposition efficiency. One of the most important aspects regarding the employed MWD method is the possibility to use diluted solutions for sample preparation, thus reducing the quantity of reagents, risks of contamination and generation of residues. These are important parameters for the development of greener analytical methods (Gonzalez et al., 2009). Moreover, MWD plays an important role in decreasing some kinds of spectral and non-spectral interferences, such as those deriving from carbon containing species or different chlorides, by conversion to significantly less or non-interfering species (Husáková et al., 2011; Bizzi et al., 2017). He mode with KED or HE He mode with higher cell gas pressure and higher energy discrimination voltages, were used for the analytes that suffered from spectral effects (i.e. V, Cr, Mn, Co, Ni, Fe, Co, Cu, As, and Se) caused by polyatomic ions deriving from the plasma gas, sample solvent and other sample matrix elements such as Na, K, Ca, Mg, P, S, C, and Cl (see Table 1 and Supplementary Materials, Table S1). He and/or HE He cell modes had the additional benefit of reducing the response for low mass matrix elements like Na, K, Ca or P by an order of magnitude ($^{23}Na^+$, $^{44}Ca^+$) or more ($^{39}K^+$, $^{31}P^+$), thus effectively raising the upper linear range for these elements. Thereby elements that would have needed to be analyzed by ICP-OES, were included in the ICP-MS.

As for the ISTD, Rh was chosen for its mid-range mass and ionization potential and because of its absence in the analyzed samples. Recoveries of Rh, relative to the initial calibration blank for the entire 8-hour sequence of sample digests, are shown in Supplementary Materials, Fig. S1. ISTD behavior across the mass range was found to be very consistent overt time and downward drift was considered acceptable.

Data accuracy was evaluated by the analysis of different CRMs (NIST SRM 1577 Bovine Liver, NIST 1566 Oyster Tissue, BCR CRM 184 Bovine muscle, BCR CRM 185 Bovine Liver, CRM 12-2-01 Bovine Liver), primarily intended for the evaluation of analytical methods and instruments used for the determination of the mass fraction values of selected elements in marine tissue, foods, or similar materials. Since the levels of most lanthanides and actinides are not certified in these CRMs, three additional certified standards (NCS ZC 73015 Milk Powder, CRM 12-2-04 Wheat bread flour, and CRM 12-2-03 Essential and toxic elements in Lucerne) were analyzed to validate data for these elements. The high level of agreement between target and found values demonstrated trueness of data obtained (Supplementary Materials, Table S2 and Fig. S3).

The precision of the method was evaluated in terms of intra-day and inter-day comparison. Intra-day precision was determined by analysis of individual control materials three times during the same day. Inter-day precision was determined by analysis of the same standards on three different days over a period of one month. Within each series, every solution was analyzed in triplicate. Relative standard deviation (RSD) was calculated for both series of analyses. The RSD values of intra-day and inter-day studies were mostly found to be below 14% thus showing a good precision of the method (Supplementary Materials, Table S2).

Method detection limits (MDLs) and method quantification limits (MLOQs) - reported in Supplementary Material, Table S3 - were evaluated as a triple and tenfold standard deviation, respectively, of ten consecutive measurements of blank signal divided by the slope of the calibration curve. Detection limits were found to be low enough that selected elements could be determined at the background level. Fig. S2 and Table S3 (Supplementary Materials) also summarize relative sensitivities of Q-ICP-MS for analysis of individual elements with the use of Rh ISTD.

3.2. Concentrations of elements in cuttlefish samples

The concentrations of elements measured in the present study were presented and discussed as median values considering the non-normal distribution of the raw data. Results from descriptive statistics are listed in Table 2. The most abundant elements were found to be Na, Mg, P and K, whose concentration levels were higher than 1% of weight and quite variable among cuttlefish from different countries. According to results from Kruskal–Wallis test, Na and Mg amounts were significantly higher ($p \leq 0.05$) in samples from FAO 27.7.e (French Atlantic) compared to samples from FAO 37.1/37.2 (Mediterranean area) and FAO 37.2.1 (Chioggia). At the same time, an opposite trend was observed for P and K, for which the highest concentrations ($p \leq 0.05$) were found in Chioggia cuttlefish (Table 2).

Concentrations between 1 and 0.01% of weight (10000–100 mg kg^{-1}, d.m.) were found for two elements corresponding to Li and Ca, which showed significantly higher median concentrations in French Atlantic samples (0.51 mg kg^{-1}and 1760 mg kg^{-1}, respectively) ($p \leq 0.05$) compared to the other groups. The wide variability in both major and minor element contents found in cuttlefish specimens of different geographical origins and within samples of the same origin can be attributed to their natural variation in seawater, but the assimilation by marine animals of Na, Mg, and Ca from the organic matter in the aquatic environment also varies with the feeding status and the age of the animal (Lall, 2002). Moreover, the presence of P in fish and seafood tissues can be directly attributable to the food sources since its concentration in seawater is lower compared to the other elements (Lall, 2002).

The main trace elements, ranging between 100 and 1 mg kg^{-1}, followed the decreasing order Zn > Al > Sr > Cu > Fe > B > Rb > Se > Mn in samples from Chioggia, Zn > Cu > Sr > Fe > Al > B > Rb > Mn > Se in samples from the Mediterranean area, and Zn > Sr > Cu > B > Fe > Al > Rb > Se > Mn in samples from the French Atlantic. Among these elements, only Se, Rb, and Sr were different in median concentrations according to all three geographical regions ($p \leq 0.05$) and the concentration ranges were in line with those previously published in literature for *S. Officinalis* (Raimundo, Pereira, Vale, & Caetano, 2005; Ayas & Ozogul, 2011).

Different median contents varying between 62.3 and 75.8 mg kg^{-1} were also found for As ($p \leq 0.05$). The wide presence of arsenic in the aquatic environment is linked both to the geochemical characteristics of the region and anthropogenic activities, especially those related to agricultural practices (Neff, 2002). Due to toxic properties, some concerns regarding the dietary exposure to arsenic have arisen in recent times, but maximum levels for this metal in fishery products have not been established yet by European legislations. Fish and seafood, in particular, have been reported as the largest food contributors to overall total arsenic exposure, despite the largest proportion is represented by organic arsenic species which are known to be less or no toxic compared to relative inorganic species (European Food Safety Authority, EFSA, 2009). In addition, inorganic arsenic concentrations decrease with increasing content of total arsenic (European Food Safety Authority, EFSA, 2009).

Most of the elements analyzed in the present paper were found to be present in cuttlefish tissues at mean concentration lower than 1 mg kg^{-1}. Levels of ultra-trace elements as rare earth elements (REEs) were lower than 100 µg kg $^{-1}$ on average, with the highest median concentration for Ce (24.0 µg kg^{-1}) and the lowest one for Lu (0.047 µg kg $^{-1}$) both in in Mediterranean samples (Table 2). The natural pattern of REEs in the aquatic environment, which is associated to the mobilization of nutrients from the underlying soils, can be permanently altered by some anthropic activities and effectively used to investigate the geographical provenance of marine animals (Noack, Dzombak, & Karamalidis, 2014). In the present work, the significantly different concentrations of Tb, Dy, Ho, Er, and Yb geographically discriminated Chioggia from French specimens, while those of La, Pr, Nd, Eu, and Gd discriminated Mediterranean form French specimens ($p \leq 0.05$). In accordance with these results, the amount of both La and Ho was previously reported to be potentially useful to authenticate fish samples from different areas in the Mediterranean Sea (Varrà, Ghidini, Zanardi, Badiani, & Ianieri, 2019).

Regardless the origin, all the cuttlefish samples analyzed in the present work were in line with the maximum limits for toxic heavy metals established by European regulations No. 1881/2006/EC and subsequent amendments and additions (Commission of the European Communities, 2006). Both cadmium and lead did not exceed the threshold value of 1 mg kg^{-1} set for the edible part of cephalopods, as well as Hg was always found to be below the threshold limit of 0.5 mg kg^{-1}. The sample groups under investigation did not show significant differences in Pb and Hg concentrations ($p > 0.05$), but Cd median amount in French Atlantic specimens was found to be approximately 7 and 20 times higher than in Mediterranean and Chioggia samples, respectively (see Table 2). Furthermore, the comparison with results reported by other authors for cuttlefish from North eastern Atlantic and Adriatic Sea, showed that Hg, Pb, and Cd concentrations found in the present work fell within the same ranges (Bustamante, Lahaye, Durnez, Churlaud, & Caurant, 2006; Storelli, Giacominelli-Stuffler, Storelli, & Marcotrigiano, 2007). Even in the case of heavy toxic metals, a close link between their concentrations in seawater and environmental pollution of specific areas exists (Carvalho, Santiago, & Nunes, 2005). This, together with Ni, Co, and Bi amounts, which also varied significantly among sample groups, makes suggestions for future insights about the effective role and contribution of these elements for the geographical origin assessment of fish and seafood.

3.3. Chemometric classification of the geographical origin of cuttlefish

Since no specific information concerning the best arrangement of elements to discriminate the geographical origin of cephalopods is available in literature, the first stages of multivariate analysis took into consideration all the fifty-two elements measured, starting with the data exploration by means of CA and PCA. Nevertheless, multicollinearity between variables as well as geographical-unrelated variability are a source of increasing noise, thus the reduction of the variables to the most predictive ones was subsequently preferred, especially in view of a future practical implementation of the methodology.

The selection of variables is strictly dependent on the sample matrix properties and dataset overall characteristics; hence its application needs to be evaluated on an individual basis. Although being the most frequently used classification technique in studies dealing with element profiling, S-LDA is coming under growing criticism because of the deceiving results deriving from the random rather than effective significance of the variables extracted, especially when the number of potential explanatory variables is high (Smith, 2018). On the other hand, one of the main problems related to the use of multivariate data analysis in food authentication studies is just the difficulty in comparing the obtained results with those already published but using a wide variety of different chemometrics techniques. Therefore, in the context of the present work, S-LDA and LW-LDA, were used for classification by variable selection, but outputs were compared with those obtained through the application of VIP-based PLS-DA. The latter, in fact, consists of a more robust and flexible algorithm, particularly suited for the classification of a large number of samples and is suggested to be a more powerful tool for reliable variable selection compared to the traditional LDA (Rashid, Hussain, Ahmad, & Abdullah, 2019).

3.3.1. Correlation and principal component analysis

Pearson's associations between all the elements were reported by plotting the correlation matrix shown in Fig. 1. The main significant patterns of covariations were found among most of the REEs and, in particular, between Ce and La ($r > 0.9$), and among Nd, Pr, Tb, Ho, Gd, Er, Eu, Dy, and Sm ($r > 0.8$). Similarly, bivariate positive correlation regarding the patterns Na–Sr–Cd–Li–Mg–Ca ($r > 0.9$), P–K ($r > 0.9$), and Cs–Rb ($r > 0.8$) were also detected. Concentrations of U were positively correlated with Ca, Mg, Pd, Li, Cd, Sr, and Na ($r > 0.8$) as well as amounts of Co with Cu, Mo, and V ($r > 0.7$). The most important and significant negative correlations were instead found among K–Rb–Cs–Mn and among Na–Sr–Cd–Li–Ca–Mg–U ($r < -0.7$) patterns. The association between the characteristic element distributions and cuttlefish origins was further investigated by PCA. The first PCA computation took into consideration all the fifty-two elements and, as a result, the number of original variables was set by LOOCV to six final principal components (PCs) enclosing 72.6% and 57.3% of the explained and predictive data variance, respectively. The first two PCs of the model contributed for 27.5% and 20.8% of the total explained variance, with the PC2 enclosing the fraction of information related to sample provenance, as reported in Fig. 2A. The distribution of Chioggia cuttlefish along the negative axis of the PC2 was mainly dominated by the highest contribution of Rb and K, followed by Mn, Al, Cs, P, As, and, at lower levels, Bi (see loading vectors reported in Fig. 2A). On the other hand, French Atlantic cuttlefish distribution was mainly guided by contribution from Na, Sr, Cd, Li, Mg, Ca, U, and Mo, whose vectors angles nearness underlined the strong covariation previously stated by CA. Likewise, a strong contribution and a high degree of covariation was confirmed in PCA for REEs, which impacted on the negative axis of PC1.

During this preliminary stage, one sample from Chioggia was also found to be a strong outlier since it fell outside the 95% confidence interval defined by the Hotelling's T^2 range (see Fig. 2A). For this reason, it was excluded from subsequent statistics.

Since the most influential loading values were found for a total of 16 out 52 elements (Li, B, Na, Mg, Ca, Co, Cu, Zn, Sr, Mo, Ru, Pd, Cd, Hf, Pt, and U), variable filtering was performed (see Section 2.7) and PCA was thus recomputed. Loading weights (0-1 scaled) of selected elements are reported in Supplementary Materials, Table S4. The reduced PCA model extracted three PCs that explained 84.5% of the data variability. The PC1 (explained variability, 54.1%; predictive variability, 48.3%) was mainly characterized by Zn, Cd, Pd, Ca, U, Sr, while in the PC2 (explained variability, 16.3%; predictive variability, 17.1%) Pt was the most influential element (Fig. 2B). Hence, maximum variation in the original dataset was retained while reducing the number of significant features. At the same time, sample separation in the score space was significantly expressed along the PC1 using the selected variables rather than the whole set of elements.

3.3.2. Classification of cuttlefish by origin: LDA and PLS-DA approaches using selected variables

The LW-LDA classification model for cuttlefish origins, created by using variables previously selected by PCA (Li, B, Na, Mg, Ca, Co, Cu, Zn, Sr, Mo, Ru, Pd, Cd, Hf, Pt, and U) was described by two discriminant functions (DFs) explaining 99.7% of the data variability. The DF1 (explained variability, 83.1%; canonical correlation, 97.2%) and the DF 2 (explained variability, 16.6%; canonical correlation, 86.7%), whose statistical significance was confirmed by low Wilk's Lambda values (0.004 and 0.13, respectively, p = 0.000) were responsible for the clusterization of cuttlefish in the bidimensional score plot reported in Fig. 3A. Samples from French Atlantic were well discriminated from samples from Chioggia and Mediterranean Sea by the DF1 and Chioggia samples well discriminated from Mediterranean samples by the DF2. The most important loadings for the DF1 were Sr and Na, while Co was found to be the most influent element for the DF2 (Fig. 3A). Based on these results, 100% of cuttlefish were correctly recognized in CV, but, when performing the external test set validation, two samples from Mediterranean area were misclassified as samples from French Atlantic and one French Atlantic sample as of Mediterranean origin, thus resulting in an overall accuracy of the model of 97% (Table 3). Fisher's discriminant function coefficients for each geographical provenance are reported in Supplementary Material, Table S5.

In S-LDA the forward selection method based on Wilk's Lambda values was chosen to select the discriminating variables, taking 3.84 as the minimum partial *F* value to include a variable and 2.71 as the maximum *F* value to exclude a variable from the model (see Supplementary materials, Supplementary Experimental Section). Based on this, two DFs and 12 out 52 discriminating elements were finally included into the model, corresponding to Na, Co, B, K, Cd, V, U, Rb, Ni, Ba, Cu, and As (Supplementary Materials, Table S6), of which Na, Co, B, Cd, U, and Cu were the same as those selected in LW-LDA.

The DF1 and DF2 enclosed 82.5% and 17.5% of variance of original data, with a canonical correlation of 97.4% and 88.5% respectively. The significant discriminatory ability of each DF was confirmed not only by Wilk's Lambda values of 0.03 for the DF1 and 0.114 for the DF2 (p = 0.000), but also by 99% accuracy in LOOCV (one Mediterranean sample erroneously classified as Atlantic sample) and 100% accuracy in external validation (Table 3). Fisher's coefficients for each provenance are reported in Supplementary Materials, Table S6.

Optimal classification results (100% sensitivity, specificity, and accuracy, Table 3) were obtained by the application of PLS-DA when 21 elements were selected on the basis of their VIP scores (VIP >1) and used as classificatory variables (see Supplementary Materials, Table S7). Among these elements, ten were found to be the same of those previously included in stepwise-LDA. Although Sr, Mn, Mo, Li, Ca, Mg, Se, Bi, Cs, P, and Y were selected in addition, some of these (Sr, Mo, Li, Ca, and Mg) were shared with LW-LDA. The highest VIP scores were 1.672 for V and 1.628 for Co, thus indicating the maximum contribution of these elements to the geographical separation between cuttlefish.

The VIP-PLS-DA model was created by using six LVs explaining 90.1% of variance ($R^2X = 0.901$), of which 92.2% was directly correlated with labels of groups ($R^2Y = 0.922$). In addition, the overall training model's predictive power of 88.8% ($Q^2 = 0.888$), resulted in 100% of samples to be correctly classified both in LOOCV and external validation (Table 3), without any possible misleading interpretation deriving from overfit or overprediction of the model as assessed by permutation test (average R^2Y-y-intercept = 0.048; average Q^2-y-intercept = -0.371). Further information about this validation is reported in Supplementary Materials, Supplementary Fig. S4.

As for the RMSECV and RMSEP values, these were found to be low (0.156 and 0.159, respectively) and very close to each other, thus remarking the outstanding ability of the simplified model in categorizing cuttlefish.

Fig. 3B shows the bidimensional score and loading graphs of the first two DFs obtained by S-LDA, while the first two LVs obtained by VIP-PLS-DA are reported in Fig. 3C. In both cases, cuttlefish were well separated from each other, with samples form Chioggia distributing in the lower right quadrant of the score plots. In addition, both the DF1 and the LV1 were mainly responsible for sample discrimination in the score plot.

By looking at the loading plots, Chioggia cuttlefish were found to be positively correlated with U, V, Ni, Ba, As, Rb, and K in S-LDA (Fig. 3B) and with Cs, Rb, K and P in PLS-DA (Fig. 3C). The highest loading scores for Mediterranean cuttlefish were instead observed for Cd in S-LDA, which in turn was diagonally opposite to the elements that characterized Chioggia samples. In PLS-DA model, the highest degree of correlation with Mediterranean cuttlefish was instead found for V, Se, Co, Cu, Mo, Ni, Bi, and Mn while for French Atlantic cuttlefish the highest contribution was exerted by Na together with Mg, U, Li, Sr, Ca and Cd. Likewise, Na showed a high loading value on the DF2 negative axis and, therefore, it allowed to distinguish French samples from the other groups in the S-LDA model.

According to the results of multi-element analysis of fish and seafood products already published in literature, some of the most discriminant elements found in the present study might be linked to anthropogenic pollution (As, V, Cd and U) or to geogenic sources (Cs and Mn) (Costas-Rodríguez et al., 2010).

Consistently to the results achieved, the major elements Na, Mg, and K were also previously identified among the most useful indicators of geographical origin of crabs (Luo et al., 2019) and prawns (Gopi et al., 2019). The variation in the amounts of Na, Mg and P in the tissues of Pacific shrimps was also correlated with specific sampling sites, whose waters were characterized by higher salinity (Li, Han, Dong, & Boyd, 2019).

The concentrations of As which, in the present work, was one of the selected discriminating elements for Chioggia cuttlefish, were also reported in mussels from Venice Lagoon (in which Chioggia is located) as being positively associated to the higher degree of salinity of this area and thus valuable for the identification of local products (Cubadda, Raggi, & Coni, 2006). Moreover, also Ni, Co, Se, Mo, and V amounts showed a strong link with anthropic sources of element contamination of the Venice Lagoon (Cubadda et al., 2006), therefore the specific pattern distribution of these trace elements in Chioggia cuttlefish tissues might be supposed to specifically reflect their origin.

Based on these results, the use of multivariate classifiers in combination with the pre-selection of the most origin-discriminant elements, was proved to be an efficient, rapid and smart analytical strategy to assure the authenticity of the provenance of cuttlefish samples. Although each of the classification techniques applied showed satisfying results, suggesting that possible users may choose the most convenient methodology to suit the specific needs, superior and unequivocal outcomes were obtained by applying VIP-PLS-DA.

4. Conclusions

Cephalopods are among some of the most consumed fishery products and are appreciated by the consumers all over the world but, to the best of our knowledge, scientific insights concerning their elemental profile and the authentication of their geographical origin are still lacking.

The results presented in this study revealed for the first time that the geographical imprint of Italian traditional cuttlefish from Chioggia can be extracted through the simultaneous quantification of more than fifty elements by ICP-MS and successfully used to discriminate the product from cuttlefish originating from different areas.

The whole elemental profile was elaborated by means of different chemometric techniques. In particular, three independent variable selection strategies merged with linear or regression pattern recognition multivariate techniques (LW-LDA, S-LDA, and VIP-PLS-DA) were tested to develop classification rules while reducing the number of variables to the lowest possible, in order to find the most parsimonious way that best described sample origins. Although the application of VIP-PLS-DA led to the extraction of the highest number of variables, analysis of performance metrics suggested the best results for this methodology, since values of sensitivity, specificity, and classification accuracy of 100% were achieved in internal and in external validation thanks to the contribution of Na, K, Ca, P, Mg, Cu, Co, Mn, Se, Ni, Mo, Li, B, Ba, Bi, Sr, As, V, Rb, Cs, Y, U, and Cd.

In summary, the elemental pattern linked to the geographical origin of cuttlefish appears to be determined by a combination of macro, trace and ultra-trace elements of both natural and anthropogenic origin, which are known to be absorbed by the animals from the surrounding environment. In particular, the contribution of anthropogenic elements here strongly emerges as a key analytical determinant for cephalopods authenticity assessment. Of note, also concentrations of some heavy toxic metals such as Cd and As, although being so low as not to represent a potential health risk, were useful for the discrimination purpose.

Considering the permanent and continuous release of all these elements in the aquatic environment deriving from modern industrial and agricultural practices, the quantification of anthropogenic contaminants in fishery products should therefore be encouraged, as well as further specific critical evaluations of their variation within seawater is recommended. In such a scenario, the promising results obtained may pave the way for practical application of the proposed methodology in the fishery sector to trace back different geographical origins, but also for the protection and ongoing promotion of traditional local fishery products for which a quality mark has been recognized.

Appendix A. Supplementary Materials

Supplementary data associated with this article can be found, in the online version, at

Declaration of interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability: The dataset generated during the current study is available from the corresponding author Prof. Lenka Husáková on reasonable request.

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Figure Captions

Fig. 1. Pearson's correlation heat-map of the investigated elements.

Fig. 2. Biplot resulting from PCA applied to all the elements (A) and selected elements (B). The sample surrounded by the red dotted line in the biplot indicate an outlier according to Hotelling's T^2 test (95% confidence interval). Blue triangles: samples from Chioggia (FAO fishing area 37.2.1); red diamonds: samples from Mediterranean Sea (FAO fishing area 37.1/37.2); yellow circles: samples from French Atlantic (FAO fishing area 27.7.e).

Fig. 3. Comparison of score (left) and loading graphs (right) from LW-LDA (A), S-LDA (B) and VIP-PLS-DA (C) for cuttlefish samples from different origins. Blue triangles: samples from Chioggia (FAO fishing area 37.2.1); red diamonds: samples from Mediterranean Sea (FAO fishing area 37.1/37.2); yellow circles: samples from French Atlantic (FAO fishing area 27.7.e).

Table1 (text UNmarked)

Table 1

Agilent 7900 ICP-MS operating conditions.

Parameter	Setting		
ICP			
Plasma mode	General purpose		
Rf power (27 MHz) (W)	1550		
Sampling depth (mm)	8		
Plasma gas flow (L min^{-1})	15		
Auxiliary gas flow (L min^{-1})	0.9		
Nebulizer gas flow (L min^{-1})	1		
Nebulizer pump (rps)	0.1		
Spray chamber temperature (°C)	2		
Mass spectrometer	No gas mode	He mode	HEHe mode [a]
Extract 1 (V)		0	
Extract 2 (V)		-250	
Omega bias (V)	-100	-120	-120
Omega lens (V)	9.7	7.8	9.6
Cell entrance	-30	-40	-140
Cell exit	-50	-60	-150
Deflect (V)	11.6	1	-77
Plate bias	-35	-60	-150
Helium flow (mL min^{-1})	0	5	10
OctP bias	-8	-18	-100
OctP RF		200	
Energy discrimination (V)		5	
Number of elements	39 [b]	12 [c]	5 [d]
Acquisition			
Points per peak	1		
Replicates	3		
Sweeps/replicate	100		
Total acquisition time (s)	75		

[a] HEHe mode - high energy helium mode; Monitored isotopes (integration time): b) ^{7}Li, ^{11}B, ^{24}Mg, ^{66}Zn, ^{85}Rb, ^{88}Sr, ^{89}Y, ^{90}Zr, ^{95}Mo, ^{101}Ru, ^{103}Rh, ^{105}Pd, ^{111}Cd, ^{118}Sn, ^{121}Sb, ^{133}Cs, ^{138}Ba, ^{139}La, ^{140}Ce, ^{141}Pr, ^{146}Nd, ^{147}Sm, ^{153}Eu, ^{157}Gd, ^{159}Tb, ^{163}Dy,^{165}Ho, ^{166}Er, ^{172}Yb, ^{175}Lu, ^{178}Hf, ^{185}Re, ^{195}Pt, ^{205}Tl, $^{206+207+208}$Pb, ^{209}Bi, ^{232}Th, ^{238}U (all 0.1 s); c) ^{23}Na (0.3 s), ^{27}Al (0.1 s), ^{39}K, ^{44}Ca (both 0.3 s), ^{51}V (1 s), ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{103}Rh (all 0.3 s); d) ^{31}P (0.1 s), ^{75}As, ^{78}Se (both 1 s), ^{103}Rh (0.3 s).

Table2 (text UNmarked)

Table 2 Multi-elemental composition of cuttlefish from different geographical origins. Elements are sorted according to decreasing median concentrations of cuttlefish from Chioggia.

	Chioggia (n = 17*3)				Mediterranean Sea (n = 25*3)				French Atlantic (n = 26*3)			
Element	Mean ± ME	Median	Min	Max	Mean ± ME	Median	Min	Max	Mean ± ME	Median	Min	Max
Na	11164 ± 867	11235^{a}	8659	14187	14334 ± 486	14290^{b}	12015	16338	24632 ± 1332	24730^{c}	17932	30749
K	13471 ± 1067	13768^{a}	9113	16488	10648 ± 402	10547^{b}	8898	12400	8353 ± 531	8252^{c}	6196	12039
P	10697 ± 831	10635^{a}	7459	1617	9084 ± 221	9079^{b}	7440	9883	8203 ± 308	8176^{c}	6791	10401
Mg	2297 ± 207	2274^{a}	1626	3180	2613 ± 75	2643^{a}	2164	2871	3767 ± 183	3776^{b}	2898	4795
Ca	820 ± 102	751^{a}	619	1338	1299 ± 53	1304^{b}	1019	1513	1781 ± 118	1760^{c}	1239	2493
As	79 ± 9	75.8^{a}	56.1	121.3	69 ± 4.3	67.4^{ab}	50.5	98.8	60 ± 4.7	62.3^{b}	35.5	77.1
Li	0.25 ± 0.024	0.24^{a}	0.18	0.34	0.34 ± 0.011	0.33^{b}	0.28	0.38	0.50 ± 0.025	0.51^{c}	0.36	0.65
Zn	62 ± 5.2	60.7^{a}	41.1	86.3	73 ± 1.6	72.8^{b}	63.8	80.7	74 ± 3.5	73.8^{b}	58.6	94.1
Al	16 ± 5.5	10.7^{a}	4.7	43.8	7 ± 1.9	5.69^{b}	2.97	22.7	7 ± 2.5	5.44^{b}	2.87	34.4
Cu	10 ± 1.8	8.55^{a}	5.93	20.2	24 ± 3.4	21.3^{b}	12.5	42.9	20 ± 3.4	16.3^{b}	8.29	36.2
Sr	9 ± 1.1	8.83^{a}	6.69	14.9	14.8 ± 0.44	14.9^{b}	12.0	16.8	25 ± 1.6	26.0^{c}	16.3	33.2
Fe	11 ± 4	7.85^{a}	3.62	33.0	9 ± 1.9	8.19^{a}	4.32	25.0	8 ± 2	5.59^{a}	3.41	21.7
B	6.3 ± 0.56	6.06^{ab}	4.46	8.20	5.3 ± 0.29	5.35^{a}	4.11	7.15	7.6 ± 0.85	7.88^{b}	4.15	11.8
Rb	5.3 ± 0.42	5.17^{a}	3.94	6.99	4.5 ± 0.14	4.61^{a}	3.93	5.13	3.6 ± 0.22	3.57^{b}	2.56	5.27
Se	1.3 ± 0.17	1.33^{a}	0.881	2.11	2.0 ± 0.14	1.91^{b}	1.47	2.69	1.65 ± 0.083	1.62^{c}	1.26	2.16
Mn	2.2 ± 0.87	1.28^{a}	0.93	6.86	2.5 ± 0.52	2.04^{a}	1.41	6.59	1.0 ± 0.23	0.83^{b}	0.54	3.57
Ni	0.4 ± 0.52	0.36^{a}	0.22	0.76	0.52 ± 0.056	0.50^{b}	0.27	1.98	0.28 ± 0.034	0.26^{a}	0.15	0.48
Cr	0.18 ± 0.060	0.18^{a}	0.037	0.55	0.15 ± 0.041	0.13^{a}	0.068	0.54	0.22 ± 0.082	0.13^{a}	0.081	1.07
Ba	0.3 ± 0.13	0.18^{a}	0.087	1.17	0.22 ± 0.031	0.16^{a}	0.12	0.50	0.21 ± 0.039	0.18^{a}	0.11	0.43
Pb	0.10 ± 0.024	0.093^{a}	0.050	0.23	0.10 ± 0.019	0.099^{a}	0.063	0.31	0.09 ± 0.017	0.046^{a}	0.077	0.20
V	0.07 ± 0.036	0.040^{a}	0.018	0.26	0.25 ± 0.040	0.22^{b}	0.12	0.47	0.11 ± 0.023	0.11^{a}	0.039	0.27
Zr	0.04 ± 0.018	0.027^{a}	0.0059	0.13	0.05 ± 0.015	0.036^{ab}	0.0071	0.14	0.2 ± 0.20	0.051^{b}	0.0066	2.55
Co	0.04 ± 0.019	0.025^{a}	0.016	0.15	0.21 ± 0.023	0.21^{b}	0.11	0.31	0.14 ± 0.026	0.14^{c}	0.058	0.31
Mo	0.029 ± 0.0066	0.024^{a}	0.018	0.060	0.10 ± 0.013	0.089^{b}	0.061	0.18	0.10 ± 0.019	0.084^{b}	0.044	0.23
Pd	0.016 ± 0.0013	0.016^{a}	0.012	0.020	0.024 ±0.0054	0.021^{a}	0.012	0.067	0.029 ± 0.0033	0.027^{b}	0.017	0.059
Cs	0.017 ± 0.0015	0.016^{a}	0.012	0.023	0.0146 ± 0.00085	0.014^{a}	0.012	0.023	0.012 ± 0.0014	0.012^{b}	0.0081	0.027
Y	0.02 ± 0.012	0.015^{a}	0.0057	0.11	0.008 ± 0.0012	0.0074^{b}	0.0039	0.017	0.01 ± 0.013	0.0077^{b}	0.0050	0.17
Bi	0.178 ± 0.0022	0.017^{a}	0.011	0.027	0.023 ± 0.0040	0.021^{a}	0.015	0.065	0.010 ± 0.0041	0.0066^{b}	0.0029	0.044
Sn	0.015 ± 0.0071	0.011^{a}	0.0044	0.061	0.022 ± 0.0049	0.019^{b}	0.0075	0.051	0.015 ± 0.0040	0.011^{a}	0.0052	0.053
Cd	0.02 ± 0.017	0.0099^{a}	0.0035	0.13	0.08 ± 0.013	0.071^{b}	0.035	0.15	0.25 ± 0.059	0.22^{c}	0.067	0.58
Sb	0.007 ± 0.0028	0.0053^{a}	0.0023	0.021	0.009 ± 0.0012	0.0084^{b}	0.0046	0.016	0.0075 ± 0.00080	0.0068^{b}	0.0052	0.014
U	0.0032 ± 0.00076	0.0026^{a}	0.0019	0.0074	0.0043 ± 0.00042	0.0040^{a}	0.0027	0.0069	0.010 ± 0.0015	0.0091^{b}	0.0049	0.019
Th	0.002 ± 0.0011	0.0016^{a}	0.00081	0.0082	0.005 ± 0.0036	0.0022^{a}	0.0011	0.046	0.003 ± 0.0019	0.0015^{a}	0.0011	0.024
Ce*	29 ± 10	20.6^{a}	3.56	82.0	44 ± 20	24.0^{a}	11.4	250	26 ± 8.9	18.8^{a}	8.31	91.7
La*	20 ± 11	11.9^{ab}	2.70	90.87	29 ± 12	20.3^{a}	8.03	150	16 ± 5.6	10.5^{b}	5.09	56.0
Nd*	8 ± 4.7	3.98^{a}	1.58	33.2	5 ± 1.5	4.45^{a}	1.74	20.1	4 ± 1.0	2.82^{b}	1.33	12.0
Hf*	2.5 ± 0.52	2.23^{a}	1.15	4.94	8 ± 8.4	2.89^{ab}	1.42	103	8 ± 4.9	4.19^{b}	0.92	57.3
Tl*	1.5 ± 0.11	1.42^{a}	1.07	1.94	1.4 ± 0.51	1.21^{b}	0.83	7.30	1.3 ± 0.61	1.05^{b}	0.62	8.66
Pr*	2 ± 1.0	0.96^{a}	0.38	7.43	1.2 ± 0.34	0.97^{a}	0.45	4.62	0.8 ± 0.23	0.63^{b}	0.33	2.56
Sm*	1.5 ± 0.81	0.74^{a}	0.25	5.04	0.9 ± 0.28	0.82^{a}	0.34	3.73	0.7 ± 0.18	0.55^{a}	0.33	2.32
Dy*	1.1 ± 0.44	0.74^{a}	0.34	3.22	0.8 ± 0.18	0.66^{ab}	0.27	2.21	0.7 ± 0.17	0.51^{b}	0.33	2.02
Gd*	1 ± 0.5	0.69^{ab}	0.24	3.20	0.8 ± 0.19	0.72^{a}	0.32	2.52	0.6 ± 0.15	0.50^{b}	0.31	1.97
Er*	0.7 ± 0.23	0.54^{a}	0.20	1.73	0.5 ± 0.10	0.42^{ab}	0.23	1.17	0.4 ± 0.11	0.39^{b}	0.19	1.31
Yb*	0.7 ± 0.21	0.51^{a}	0.22	1.48	0.39 ± 0.078	0.37^{ab}	0.14	0.93	0.4 ± 0.10	0.34^{b}	0.16	1.31
Eu*	0.4 ± 0.18	0.24^{ab}	0.12	1.19	0.28 ± 0.061	0.25^{a}	0.15	0.87	0.22 ± 0.044	0.19^{b}	0.12	0.62
Ho*	0.22 ± 0.078	0.17^{a}	0.062	0.60	0.15 ± 0.030	0.14^{ab}	0.047	0.39	0.14 ± 0.037	0.11^{b}	0.063	0.45
Tb*	0.20 ± 0.082	0.14^{a}	0.056	0.58	0.15 ± 0.037	0.12^{ab}	0.055	0.43	0.12 ± 0.030	0.095^{b}	0.054	0.37
Ru*	0.10 ± 0.029	0.087^{a}	0.031	0.24	0.14 ± 0.027	0.13^{a}	0.054	0.34	0.14 ± 0.023	0.13^{a}	0.044	0.29
Re*	0.10 ± 0.032	0.081^{ab}	0.016	0.24	0.12 ± 0.019	0.11^{a}	0.070	0.24	0.09 ± 0.023	0.072^{b}	0.025	0.28
Lu*	0.09 ± 0.032	0.074^{a}	0.023	0.23	0.06 ± 0.015	0.047^{a}	0.021	0.18	0.06 ± 0.017	0.053^{a}	0.021	0.22
Pt*	0.13 ± 0.034	0.10^{a}	0.060	0.25	0.2 ± 0.12	0.12^{a}	0.062	1.53	0.15 ± 0.061	0.10^{a}	0.019	0.60
Hg$^{\#}$	0.20 ± 0.030	0.18^{a}	0.15	0.34	0.20 ± 0.023	0.17^{a}	0.14	0.38	0.21 ± 0.018	0.20^{a}	0.13	0.29

Concentrations are expressed as mg kg^{-1} (d.m.) and are reported as mean values ± margin of error (ME) at 95% confidence level. Min–Max: minimum and maximum values found. *: concentrations are expressed as µg kg $^{-1}$. Hg$^{\#}$: determined by direct mercury analyzer AMA254. Values followed by different superscript letters (a–c) in the same row are significantly different ($p \leq 0.05$).

Table 3 Supervised classification model performances in cross-validation (CV) and in external test set validation.

Model	Validation	Overall classification rate (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)
LW-LDA	CV	100	100	100	100
	External	96	96	98	97
S-LDA	CV	99	99	99	99
	External	100	100	100	100
VIP-PLS-DA	CV	100	100	100	100
	External	100	100	100	100

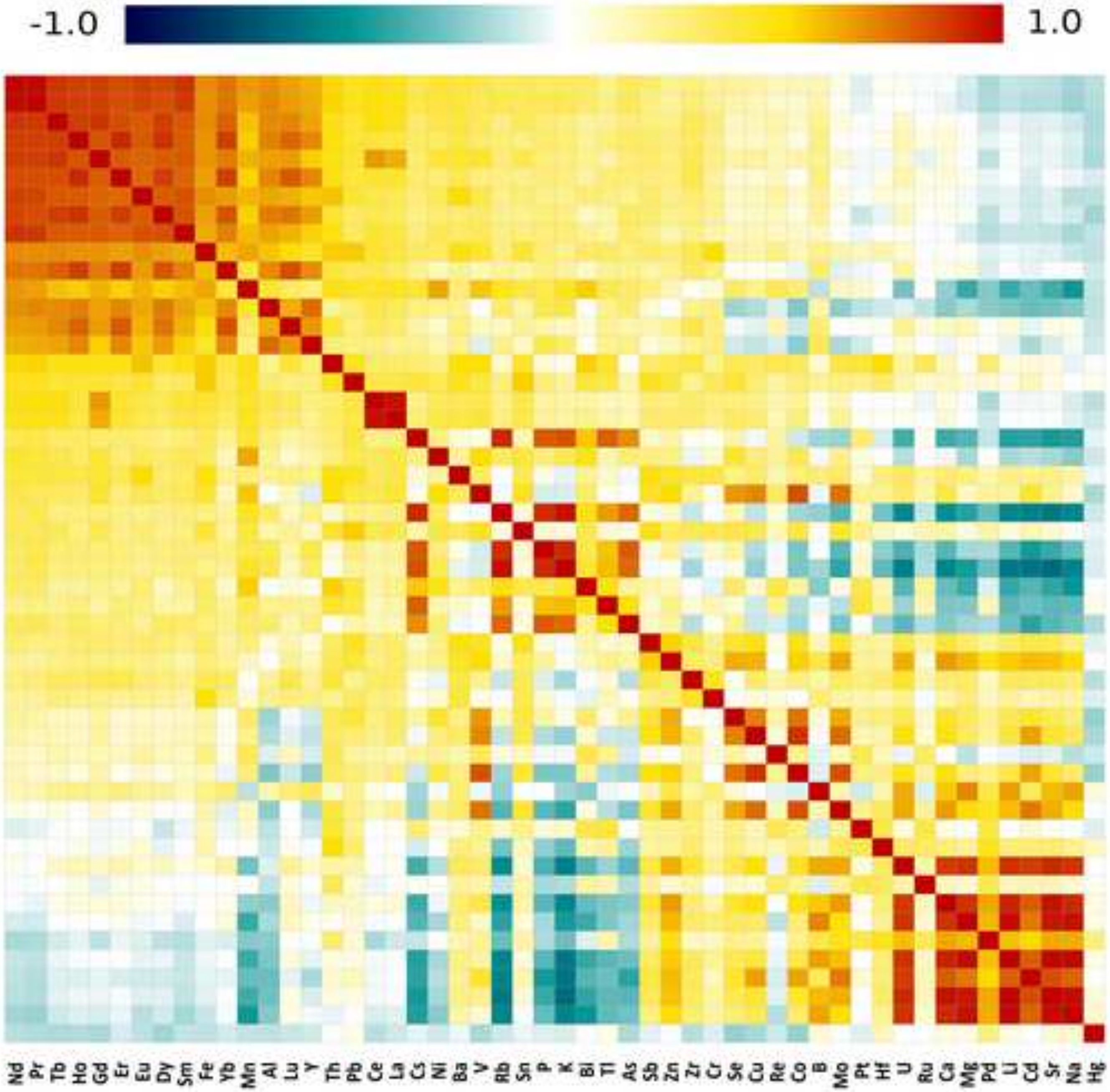

-1.0
1.0
Nd
Pr
Tb
Ho
Gd
Er
Eu
Dy
Sm
Fe
Yb
Mn
Al
Lu
Y
Th
Pb
Ce
La
Cs
Ni
Ba
V
Rb
Sn
P
K
Bi
Tl
As
Sb
Zn
Zr
Cr
Se
Cu
Re
Co
B
Mo
Pt
Hf
U
Ru
Ca
Mg
Pd
Li
Cd
Sr
Na
Hg

Figure 2

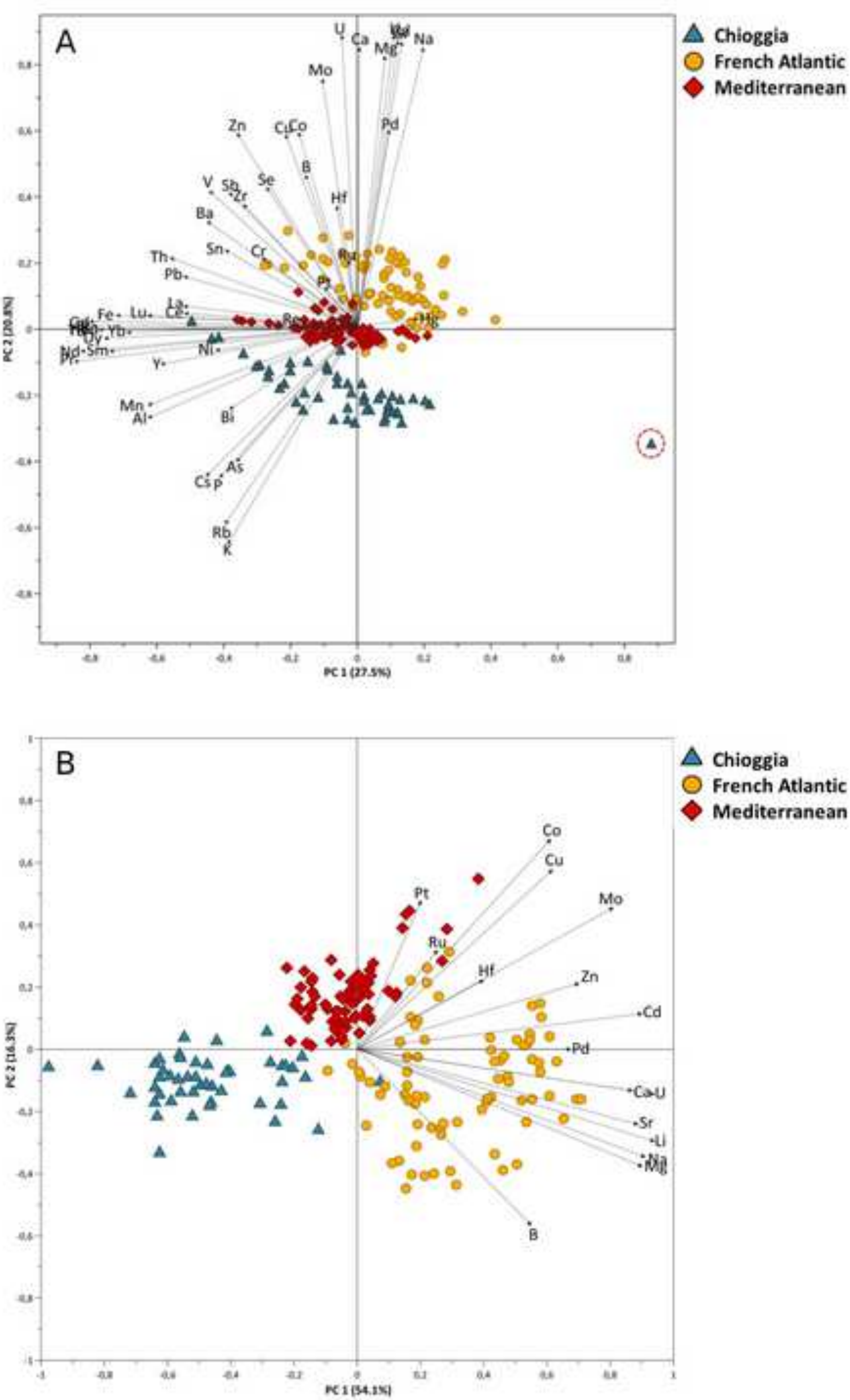
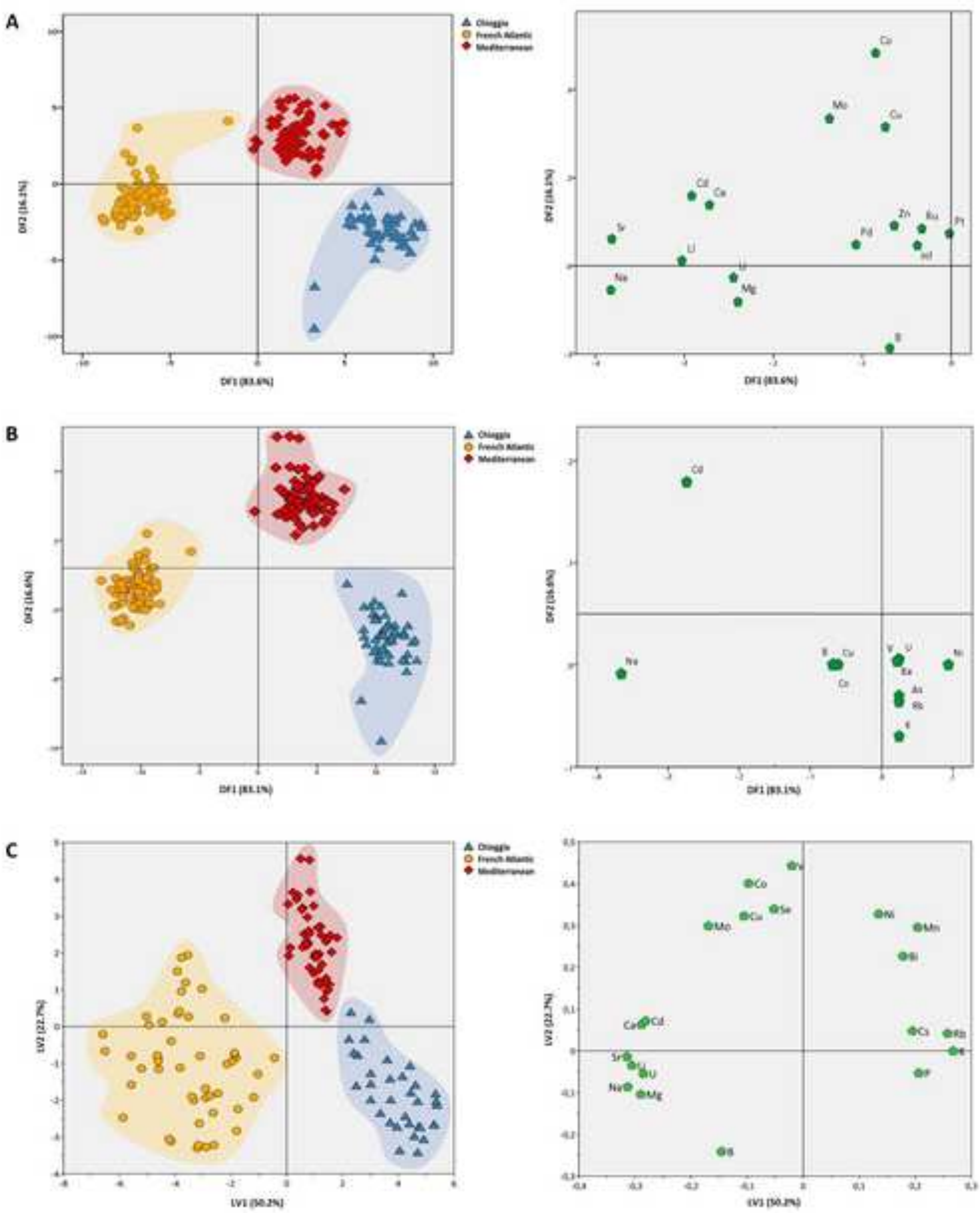

A
Chioggia
French Atlantic
Mediterranean
U
Ca
Mg
Na
Mo
Zn
Cu
Co
Pd
B
Se
V
Sb
Zr
Hf
Ba
Sn
Cr
Ru
Th
Pb
Pt
La
Ce
Fe
Lu
Yb
Dy
Nd
Sm
Pr
Y
Ni
Mn
Al
Bi
As
Cs
P
Rb
K
PC 2 (20.8%)
PC 1 (27.5%)
B
Chioggia
French Atlantic
Mediterranean
Co
Cu
Mo
Pt
Ru
Hf
Zn
Cd
Pd
Ca U
Sr
Li
Na
Mg
B
PC 2 (16.3%)
PC 1 (54.1%)

Figure 3


A
Chioggia
French Atlantic
Mediterranean
DF1 (83.6%)
DF2 (16.1%)
Co
Mo
Cu
Cd
Ca
Sr
Li
Zn
Ba
Pt
Pd
Na
U
Mg
B
B
Chioggia
French Atlantic
Mediterranean
DF1 (83.1%)
DF2 (16.6%)
Cd
Na
B
Cu
Co
V
U
Ni
Ba
As
Rb
C
Chioggia
French Atlantic
Mediterranean
LV1 (50.2%)
LV2 (22.7%)
V
Co
Se
Cu
Mo
Ni
Mn
Bi
Ca
Cd
Cs
Rb
Sr
Li
U
Na
Mg
P
B

Supplementary Materials

Multi-element signature of cuttlefish and its potential for the discrimination of different geographical provenances and traceability

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Supplementary Experimental Section

Essential elements such as calcium, iron, copper, and zinc are frequently normally distributed in fish tissues since concentrations are metabolically regulated by the organisms. Contrarily, non-essential trace elements' concentrations are completely dependent on the exposure to the specific environment and, therefore, frequently characterized by a positive skewed lognormal distribution (Phillips & Russo, 1978). As expected, both data homoscedasticity and normality assumptions tested by the Shapiro-Wilk's and Levene's tests were violated for the largest portion of the elements in the analyzed cuttlefish samples ($p \leq 0.05$), thus nonparametric statistics was used to investigate and describe elemental concentrations, taking into consideration that, alongside with this, the main advantage of nonparametric statistical tests is the low sensitivity to small samples sizes and to the potential bias of outlying data (Helsel & Hisrsch, 1992). The comparison among groups of cuttlefish was therefore reported after having performed the nonparametric Kruskal–Wallis analysis of variance followed Dunn's post hoc (Sawilowsky & Fahoome 2014) test for multiple comparison ($p \leq 0.05$).

The first chemometric technique applied to analyze the huge amount of ICP-MS data obtained from cuttlefish sample was principal component analysis (PCA). The PCA is an unsupervised projection method aimed at dimensionality reduction of the original set of correlated variables into new fewer uncorrelated latent variables extracting as much systematic variation as possible of the original data. PCA is mostly used as a preliminary explorative tool to study any intrinsic correlation among observations, variables as well as the bidirectional relation between variables and observation and it is a powerful technique for the rapid detection of strong multivariate outliers (Jolliffe & Cadima, 2016). In the present work, the application of PCA to elemental data was mainly aimed at obtaining the first global description of dataset, after having corrected the non-normal data distribution by Box-Cox transformation (Sakia, 1992) and the wide range of magnitude of values by Z-score standardization (Shiffler, 1988). In combination with Pearson's correlation analysis applied to Box-Cox transformed data, PCA was also useful for data collinearity reduction necessary for the subsequent step in supervised classification.

To define the optimal number of principal components to retain and avoid data overfitting and overoptimistic results, leave-one-out cross validation (LOOCV) following the approach of Krzanowski was employed (Eastment & Krzanowski, 1982). According to this, one observation per time was retained and predicted by the model which, in turn, was trained on the remaining observations. This sequence was repeated until all observations were kept out one by one. Thus, considering the sample size, LOOCV statistics resulted from a number of 68*3 trained models. The selected internal validation method was chosen taking into consideration the effective number of independent samples in the dataset and because since it is faster to be computed compared to other CV methods.

In study dealing with elemental fingerprints, linear discriminant analysis (LDA) is the most frequently used supervised classification method. By using Euclidean distance, LDA is based on defining new linear combinations of the original variables able to maximize the separation between class of samples while minimizing intra-class variability. The maximum likelihood ratio criterion, referred to as Wilks' lambda ($p \leq 0.05$) was applied to verify the statistical significance of each discriminant function, where smaller values suggested greater discriminatory power associated to the function (Todorov, 2007). Since LDA may be prone to failing in classification, especially when the samples size for each class is greatly unbalanced, equal priors in estimations were not set, but probabilities were calculated based on unequal sample sizes (Sanchez, 1974; Xue & Titterington, 2008).

With the aim to define a classification model by using the minimum number of variables that increased between-group variability and, at the same time, decreased within-class variability, forward stepwise-LDA (S-LDA) was applied to Box-Cox transformed and Z-score standardized data. An *F*-statistic was used to define the statistical significance of each variable to discrimination, where *F*-to-remove value (indicating the cut off value for a variable to be excluded) was set to 2.71 and *F*-to-enter value (indicating the cut-off value for a variable to be included) was set to 3.84.

Standard LDA and S-LDA were evaluate in terms of recognition ability in LOOCV applied to 66% of original observations (training set), i.e. the percentage of samples correctly assigned to the proper class. Considering that the internal cross-validation is often insufficient to accurately assess the predictability of the model, an external validation of the training model was also performed using the excluded 33% of data which were randomly but consistently selected from the whole dataset. External test observations which do not uniformly cover the range of training set distribution, may led to misleading results (Consonni, Ballabio & Todeschini, 2010).

Partial least square-discriminant analysis (PLS-DA) is a regression-based supervised technique aimed at finding interrelation between original variables and categorical variables by building new (latent) variables for the maximum separation between the different groups of samples. In particular, the categorical variable matrix is transformed into a dummy variable matrix, which boundaries of classification of samples into one class are defined using Bayes theorem. In the present work, the quality and of discriminant models based on PLS-DA of Box-Cox transformed and Z-score standardized data was assessed by applying LOOCV on the 66% of observation of the training set (Eastment & Krzanowski, 1982) and by evaluating the resulting estimating of fitting (R^2X and R^2Y) and predictive ability (Q^2) and the root-mean square error of cross-validation (RMSECV) (Bellabio & Consonni, 2013). Here too, the external validation of the regression model was performed using the independent 33% of observation left out during the calibration step (test set) and the number of samples correctly classified was evaluated. The reliability, in terms overoptimistic fitting and predictability results, was further checked by permutation tests (400 random permutation). The significance of the model was thus confirmed if the y-intercept values of the R^2Y were ≤ 0.4 and y-intercept values of Q^2 were ≤ 0.05 (Van der Voet, 1994). When building classification regression models, the discrimination among informative, redundant, and noisy variables is often an important step. The variable-influence on projection (VIP) index was used to identify and select the most discriminant variables in PLS-DA (Andersen & Bro, 2010), which were afterward employed to build a new simplified model. The VIP index summarizes the cumulative importance of each variable and represents the weighted sum of squares of the PLS weights, considering the whole explained variability related to responses (sample groups) in all extracted components (Andersen & Bro, 2010). VIP indexes higher than one are considered to be the most significant for explaining the correlation of the observation to all the responses (Andersen & Bro, 2010). The overall quality and robustness of all the supervised models was assessed by taking into consideration the experimental percentages of true positive samples (sensitivity %), true negative samples (specificity %) and prediction accuracy (%). These metrics were calculated as follows, following what has been previously reported by Fawcett (2006):

$$\text{Sensitivity (\%)} = \frac{\text{TP}}{\text{TP} + \text{FN}} * 100$$

$$\text{Specificity (\%)} = \frac{\text{TN}}{\text{TN} + \text{FP}} * 100$$

$$\text{Accuracy (\%)} = \frac{\text{TN} + \text{TP}}{\text{TN} + \text{TP} + \text{FN} + \text{FP}} * 100$$

Whereas: TP = true positive samples

TN = true negative samples

FP = false positive samples

FN = false negative samples

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Figure S1. Internal standard (ISTD) stability for sequence including 111 samples of cuttlefish (sample name is displayed on the x-axis) measured during the 8-hour run. Due to limited space, not all sample names are shown in the X-axis labels. The ISTD recoveries are displayed relative to the calibration blank.

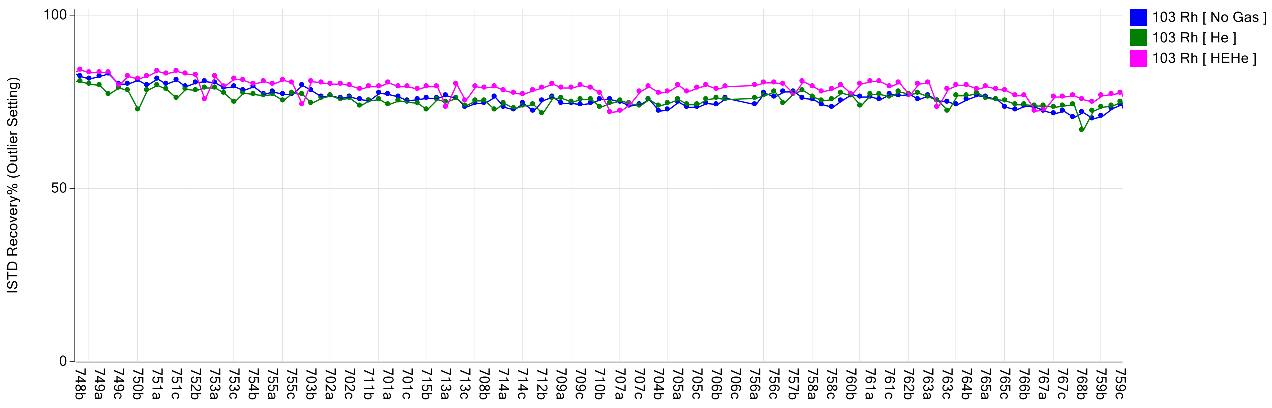


Table S1. Principal spectral interferences encountered on the Agilent 7900 from the major components of food matrices, plasma gas and sample solvent onto the analysis of selected elements.

Interfered isotope	Interfering ion
$^{27}\mathrm{Al}^{+}$	$^{12}\mathrm{C}^{15}\mathrm{N}^{+}$, $^{12}\mathrm{C}^{14}\mathrm{NH}^{+}$
$^{31}\mathrm{P}^{+}$	$^{14}\mathrm{N}^{16}\mathrm{OH}^{+}$, $^{15}\mathrm{N}^{16}\mathrm{O}^{+}$,
$^{39}\mathrm{K}^{+}$	$^{23}\mathrm{Na}^{16}\mathrm{O}^{+}$, $^{38}\mathrm{ArH}^{+}$
$^{44}\mathrm{Ca}^{+}$	$^{12}\mathrm{C}^{16}\mathrm{O}^{16}\mathrm{O}^{+}$
$^{48}\mathrm{Ca}^{+}$	$^{31}\mathrm{P}^{17}\mathrm{O}^{+}$, $^{31}\mathrm{P}^{16}\mathrm{O}^{1}\mathrm{H}^{+}$
$^{51}\mathrm{V}^{+}$	$^{35}\mathrm{Cl}^{16}\mathrm{O}^{+,}$ $^{37}\mathrm{Cl}^{14}\mathrm{N}^{+}$
$^{52}\mathrm{Cr}^{+}$	$^{40}\mathrm{Ar}^{12}\mathrm{C}^{+}$, $^{35}\mathrm{Cl}^{16}\mathrm{OH}^{+}$, $^{37}\mathrm{Cl}^{14}\mathrm{NH}^{+}$
$^{53}\mathrm{Cr}^{+}$	$^{40}\mathrm{Ar}^{13}\mathrm{C}^{+}$, $^{35}\mathrm{Cl}^{18}\mathrm{O}^{+}$, $^{37}\mathrm{Cl}^{16}\mathrm{O}^{+}$, $^{36}\mathrm{Ar}^{16}\mathrm{OH}^{+}$
$^{55}\mathrm{Mn}^{+}$	$^{39}\mathrm{K}^{16}\mathrm{O}^{+}$, $^{37}\mathrm{Cl}^{18}\mathrm{O}^{+}$, $^{23}\mathrm{Na}^{32}\mathrm{S}^{+}$
$^{54}\mathrm{Fe}^{+}$	$^{37}\mathrm{Cl}^{17}\mathrm{O}^{+}$, $^{37}\mathrm{Cl}^{16}\mathrm{O}^{1}\mathrm{H}^{+}$
$^{56}\mathrm{Fe}^{+}$	$^{40}\mathrm{Ar}^{16}\mathrm{O}^{+}$, $^{40}\mathrm{Ca}^{16}\mathrm{O}^{+}$
$^{57}\mathrm{Fe}^{+}$	$^{40}\mathrm{Ar}^{16}\mathrm{OH}^{+}$, $^{40}\mathrm{Ca}^{16}\mathrm{OH}^{+}$
$^{58}\mathrm{Ni}^{+}$	$^{40}\mathrm{Ca}^{18}\mathrm{O}^{+}$, $^{40}\mathrm{Ar}^{18}\mathrm{O}^{+}$, $^{23}\mathrm{Na}^{35}\mathrm{Cl}^{+}$
$^{59}\mathrm{Co}^{+}$	$^{43}\mathrm{Ca}^{16}\mathrm{O}^{+}$, $^{42}\mathrm{Ca}^{16}\mathrm{O}^{1}\mathrm{H}^{+}$, $^{40}\mathrm{Ar}^{18}\mathrm{OH}^{+}$
$^{60}\mathrm{Ni}^{+}$	$^{44}\mathrm{Ca}^{16}\mathrm{O}^{1+}$, $^{43}\mathrm{Ca}^{16}\mathrm{O}^{1}\mathrm{H}^{+}$, $^{23}\mathrm{Na}^{37}\mathrm{Cl}^{+}$
$^{61}\mathrm{Ni}^{+}$	$^{44}\mathrm{Ca}^{16}\mathrm{O}^{1}\mathrm{H}^{+}$, $^{36}\mathrm{Ar}^{25}\mathrm{Mg}^{+}$, $^{38}\mathrm{Ar}^{23}\mathrm{Na}^{+}$, $^{23}\mathrm{Na}^{37}\mathrm{ClH}^{+}$
$^{62}\mathrm{Ni}^{+}$	$^{23}\mathrm{Na}^{23}\mathrm{Na}^{16}\mathrm{O}^{+}$, $^{44}\mathrm{Ca}^{18}\mathrm{O}^{+}$, $^{36}\mathrm{Ar}^{26}\mathrm{Mg}^{+}$
$^{63}\mathrm{Cu}^{+}$	$^{40}\mathrm{Ar}^{23}\mathrm{Na}^{+}$, $^{31}\mathrm{P}^{16}\mathrm{O}^{16}\mathrm{O}^{+}$, $^{12}\mathrm{C}^{16}\mathrm{O}^{35}\mathrm{Cl}^{+}$, $^{12}\mathrm{C}^{14}\mathrm{N}^{37}\mathrm{Cl}^{+}$
$^{64}\mathrm{Zn}^{+}$	$^{31}\mathrm{P}^{16}\mathrm{O}^{17}\mathrm{O}^{+}$, $^{32}\mathrm{S}^{16}\mathrm{O}_2^{+}$, $^{32}\mathrm{S}_2^{+}$, $^{36}\mathrm{Ar}^{12}\mathrm{C}^{16}\mathrm{O}^{+}$, $^{38}\mathrm{Ar}^{12}\mathrm{C}^{14}\mathrm{N}^{+}$, $^{48}\mathrm{Ca}^{16}\mathrm{O}^{+}$
$^{65}\mathrm{Cu}^{+}$	$^{31}\mathrm{P}^{16}\mathrm{O}^{18}\mathrm{O}^{+}$, $^{32}\mathrm{S}^{33}\mathrm{S}^{+}$, $^{33}\mathrm{S}^{16}\mathrm{O}_2^{+}$, $^{32}\mathrm{S}^{16}\mathrm{O}_2{}^{1}\mathrm{H}^{+}$, $^{32}\mathrm{S}_2\mathrm{H}^{+}$, $^{14}\mathrm{N}^{16}\mathrm{O}^{35}\mathrm{Cl}^{+}$, $^{48}\mathrm{Ca}^{16}\mathrm{O}^{+}$
$^{66}\mathrm{Zn}^{+}$	$^{34}\mathrm{S}^{16}\mathrm{O}_2^{+}$, $^{32}\mathrm{S}^{16}\mathrm{O}^{18}\mathrm{O}^{+}$, $^{32}\mathrm{S}^{17}\mathrm{O}_2^{+}$, $^{33}\mathrm{S}^{16}\mathrm{O}^{17}\mathrm{O}^{+}$, $^{32}\mathrm{S}^{34}\mathrm{S}^{+}$, $^{33}\mathrm{S}_2^{+}$, $^{48}\mathrm{Ca}^{18}\mathrm{O}^{+}$
$^{67}\mathrm{Zn}^{+}$	$^{33}\mathrm{S}^{34}\mathrm{S}^{+}$, $^{34}\mathrm{S}^{16}\mathrm{O}^{17}\mathrm{O}^{+}$, $^{33}\mathrm{S}^{16}\mathrm{O}^{18}\mathrm{O}^{+}$, $^{32}\mathrm{S}^{17}\mathrm{O}^{18}\mathrm{O}^{+}$, $^{33}\mathrm{S}^{17}\mathrm{O}_2^{+}$, $^{33}\mathrm{S}_2\mathrm{H}^{+}$, $^{48}\mathrm{Ca}^{18}\mathrm{OH}^{+}$, $^{14}\mathrm{N}^{16}\mathrm{O}^{37}\mathrm{Cl}^{+}$, $^{16}\mathrm{O}_2{}^{35}\mathrm{Cl}^{+}$
$^{68}\mathrm{Zn}^{+}$	$^{34}\mathrm{S}_2^{+}$, $^{32}\mathrm{S}^{18}\mathrm{O}_2^{+}$
$^{69}\mathrm{Ga}^{+}$	$^{34}\mathrm{S}_2\mathrm{H}^{+}$, $^{32}\mathrm{S}^{18}\mathrm{O}_2\mathrm{H}^{+}$, $^{16}\mathrm{O}_2{}^{37}\mathrm{Cl}^{+}$
$^{71}\mathrm{Ga}^{+}$	$^{34}\mathrm{S}^{18}\mathrm{O}_2\mathrm{H}^{+}$
$^{72}\mathrm{Ge}^{+}$	$^{40}\mathrm{Ar}^{32}\mathrm{S}^{+}$, $^{35}\mathrm{Cl}^{37}\mathrm{Cl}^{+}$, $^{40}\mathrm{Ar}^{16}\mathrm{O}_2^{+}$
$^{73}\mathrm{Ge}^{+}$	$^{40}\mathrm{Ar}^{33}\mathrm{S}^{+}$, $^{35}\mathrm{Cl}^{37}\mathrm{ClH}^{+,}$ $^{40}\mathrm{Ar}^{16}\mathrm{O}_2\mathrm{H}^{+}$
$^{74}\mathrm{Ge}^{+}$	$^{40}\mathrm{Ar}^{33}\mathrm{S}^{+}$, $^{37}\mathrm{Cl}_2^{+}$, $^{39}\mathrm{K}^{35}\mathrm{Cl}^{+}$
$^{75}\mathrm{As}^{+}$	$^{43}\mathrm{Ca}^{16}\mathrm{O}_2^{+}$, $^{40}\mathrm{Ca}^{35}\mathrm{Cl}^{+}$, $^{40}\mathrm{Ar}^{34}\mathrm{SH}^{+}$
$^{77}\mathrm{Se}^{+}$	$^{40}\mathrm{Ca}^{37}\mathrm{Cl}^{+}$, $^{40}\mathrm{Ar}^{37}\mathrm{Cl}^{+}$
$^{78}\mathrm{Se}^{+}$	$^{41}\mathrm{K}^{37}\mathrm{Cl}^{+}$, $^{38}\mathrm{Ar}^{40}\mathrm{Ca}^{+}$
$^{80}\mathrm{Se}^{+}$	$^{40}\mathrm{Ar}_2^{+}$, $^{40}\mathrm{Ca}_2^{+}$, $^{40}\mathrm{Ar}^{40}\mathrm{Ca}^{+}$
$^{82}\mathrm{Se}^{+}$	$^{32}\mathrm{S}^{17}\mathrm{O}_2{}^{16}\mathrm{O}^{+}$, $^{33}\mathrm{S}^{16}\mathrm{O}_2{}^{17}\mathrm{O}^{+}$

Table S2. Comparison of measured and certified concentrations in selected control standards, recoveries (R), and intra-day and inter-day relative standard deviation (RSD).

Analyte	Reference sample	Declared (mg kg^{-1})	Found[a] (mg kg^{-1})	R[b] (%)	RSD (%)	
					Intra	Inter
^{7}Li	NCS ZC73015 Milk Powder	0.040	0.0414 ± 0.0032	104	3.86	6.70
	CRM 12-2-04 Wheat bread flour	0.020	0.0195 ± 0.0021	98	5.45	6.07
^{11}B	NCS ZC73015 Milk Powder	1.56 ± 0.22	1.53 ± 0.08	98	2.75	4.85
	CRM 12-2-04 Wheat bread flour	< 0.5	0.49 ± 0.04	[d]	3.96	7.08
	CRM12-2-03 Lucerne	30	30.2 ± 2.0	101	3.35	9.20
^{23}Na	BCR 184 Bovine muscle	2000	2080 ± 56	104	1.35	2.07
	BCR-CRM 185 Bovine Liver	2100	2101 ± 72	102.80	1.71	3.32
	NIST 1577 Bovine Liver	2033 ± 64	1918 ± 88	94	2.31	3.78
	NIST 1566 Oyster Tissue	5100 ± 3000	5013 ± 250	98	2.48	3.02
	NCS ZC 73015 Milk Powder	4700 ± 300	4400 ± 752	94	8.57	6.79
	CRM12-2-03 Lucerne	474 ± 23	474 ± 24	100	2.51	3.53
^{24}Mg	CRM 12-2-01 Bovine Liver	650 ± 112	645 ± 60	99	4.68	5.54
	BCR 184 Bovine muscle	1020	1026 ± 30	101	1.39	6.38
	NIST 1577 Bovine Liver	620 ± 42	594 ± 16	96	1.30	1.40
	NIST 1566 Oyster Tissue	1280 ± 90	1316 ± 78	103	2.95	8.28
	NCS ZC 73015 Milk Powder	960 ± 70	880 ± 38	92	2.24	5.77
	CRM 12-2-04 Wheat bread flour	556 ± 29	554 ± 7.2	99	0.65	7.85
	CRM12-2-03 Lucerne	3520 ± 125	3475 ± 336	99	4.82	6.44
^{27}Al	CRM 12-2-04 Wheat bread flour	3	3.4 ± 0.2	113	1.39	3.16
	CRM12-2-03 Lucerne	330	330 ± 5	100	0.78	9.20
^{31}P	BCR 184 Bovine muscle	8300	8351 ± 328	101	1.97	2.62
	BCR-CRM 185 Bovine Liver	11700	11618 ± 452	99	1.94	11.2
	NIST 1566 Oyster Tissue	8100	8131 ± 324	101	1.99	4.91
	NCS ZC 73015 Milk Powder	7600 ± 300	7300 ± 448	96	3.17	5.30
	CRM 12-2-04 Wheat bread flour	2280 ± 85	2289 ± 95	100	2.07	2.24
	CRM12-2-03 Lucerne	3030 ± 90	2982 ± 102	98	1.71	4.60
^{39}K	CRM 12-2-04 Wheat bread flour	2550 ± 100	2596 ± 83	102	1.60	3.73
	BCR 184 Bovine muscle	16600	16354 ± 200	99	0.61	2.57
	BCR-CRM 185 Bovine Liver	11200	10807 ± 350	97	1.62	3.60
	NIST 1577 Bovine Liver	10230 ± 640	10270 ± 512	101	2.49	4.01
	NIST 1566 Oyster Tissue	9690 ± 50	9610 ± 514	99	2.67	3.83
	NCS ZC 73015 Milk Powder	12500 ± 500	11910 ± 312	95	1.33	2.74
	CRM12-2-03 Lucerne	18700 ± 650	18897 ± 638	101	1.69	1.66
^{44}Ca	BCR 184 Bovine muscle	150	151 ± 4	101	1.17	3.54
	BCR-CRM 185R Bovine Liver	131	133 ± 5	102	1.87	3.73
	NIST 1566 Oyster Tissue	1500 ± 200	1400 ± 14	93	0.49	2.28
	NCS ZC 73015 Milk Powder	9400 ± 300	8630 ± 740	92	4.29	4.75
	CRM12-2-03 Lucerne	17500 ± 750	17348 ± 229	99	0.65	1.30

Table S2. Continued

Analyte	Reference sample	Declared (mg kg^{-1})	Found[a] (mg kg^{-1})	R[b] (%)	RSD (%) Intra	RSD (%) Inter
^{51}V	NIST 1566 Oyster Tissue	2.3 ± 0.1	2.1 ± 0.1	91	1.43	8.27
	CRM 12-2-01 Bovine Liver	0.26 ± 0.05	0.27 ± 0.01	104	1.73	3.14
	NCS ZC 73015 Milk Powder	0.06	0.050 ± 0.002	83	1.93	3.03
^{52}Cr	BCR 184 Bovine muscle	0.076	0.075 ± 0.003	99	2.13	7.28
	CRM 12-2-01 Bovine Liver	0.044	0.048 ± 0.002	109	2.25	9.74
	NCS ZC 73015 Milk Powder	0.39 ± 0.04	0.40 ± 0.02	103	2.22	1.98
	CRM12-2-03 Lucerne	0.900	0.74 ± 0.03	82	2.02	7.00
^{55}Mn	CRM 12-2-04 Wheat bread flour	22.6 ± 1.1	22.4 ± 0.9	99	1.93	8.13
	BCR 184 Bovine muscle	0.334 ± 0.028	0.320 ± 0.017	96	2.68	4.36
	BCR-CRM 185R Bovine Liver	9.3 ± 0.3	9.4 ± 0.2	101	0.90	10.9
	NIST 1577 Bovine Liver	10.46 ± 0.47	10.2 ± 0.4	98	2.15	2.53
	NIST 1566 Oyster Tissue	17.5 ± 1.2	16.4 ± 2.3	94	7.02	6.73
	CRM 12-2-01 Bovine Liver	7.6 ± 0.5	7.46 ± 0.09	98	0.58	4.20
	NCS ZC 73015 Milk Powder	0.51 ± 0.17	0.50 ± 0.08	98	2.85	9.56
	CRM12-2-03 Lucerne	34.2 ± 1.15	34.7 ± 0.3	101	0.44	1.86
^{56}Fe	BCR 184 Bovine muscle	79 ± 2	78 ± 3	99	1.80	2.72
	BCR-CRM 185R Bovine Liver	214 ± 5	217 ± 6	101	1.40	13.8
	NIST 1577 Bovine Liver	495 ± 32.5	501 ± 24	101	2.39	3.15
	NIST 1566 Oyster Tissue	195 ± 34	183 ± 18	94	4.81	3.62
	CRM 12-2-01 Bovine Liver	495 ± 28	501 ± 24	101	2.39	3.15
	NCS ZC 73015 Milk Powder	7.8 ± 1.3	6.8 ± 0.4	87	2.83	3.06
	CRM 12-2-04 Wheat bread flour	23.8 ± 1.5	22.7 ± 1.3	95	2.97	3.00
	CRM12-2-03 Lucerne	355 ± 18	350 ± 36	99	5.08	1.68
^{59}Co	NIST 1577 Bovine Liver	0.300 ± 0.018	0.303 ± 0.003	101	0.48	1.44
	NIST 1566 Oyster Tissue	0.400	0.320 ± 0.028	80	4.65	7.39
	CRM 12-2-01 Bovine Liver	0.37 ± 0.03	0.36 ± 0.02	97	2.71	1.37
	CRM12-2-03 Lucerne	0.193 ± 0.0185	0.175 ± 0.003	91	0.97	4.79
^{60}Ni	BCR 184 Bovine muscle	0.270	0.274 ± 0.018	102	3.28	9.96
	NIST 1566 Oyster Tissue	1.03 ± 0.19	0.94 ± 0.09	91	4.91	6.41
	CRM 12-2-04 Wheat bread flour	0.3	0.29 ± 0.05	97	3.22	5.70
	CRM12-2-03 Lucerne	2.54 ± 0.18	2.9 ± 0.2	114	2.60	5.89
^{63}Cu	BCR 184 Bovine muscle	2.36 ± 0.06[c]	2.30 ± 0.10	98	2.31	4.33
	BCR-CRM 185R Bovine Liver	189 ± 4	189 ± 3	100	0.85	9.91
	NIST 1577 Bovine Liver	275.2 ± 4.6	271 ± 8	98	1.42	1.74
	NIST 1566 Oyster Tissue	63.0 ± 3.5	62 ± 2	98	1.68	0.17
	CRM 12-2-01 Bovine Liver	26.3 ± 1.6	25.7 ± 1.4	98	2.69	4.80
	NCS ZC 73015 Milk Powder	0.51 ± 0.13	0.49 ± 0.01	96	1.33	7.66
	CRM 12-2-04 Wheat bread flour	2.77 ± 0.03	2.74 ± 0.02	99	0.36	3.49
	CRM12-2-03 Lucerne	11.7 ± 0.75	11.3 ± 0.3	97	1.11	4.87

Table S2. Continued

Analyte	Reference sample	Declared (mg kg^{-1})	Found[a] (mg kg^{-1})	R[b] (%)	RSD (%)	
					Intra	Inter
^{66}Zn	BCR 184 Bovine muscle	166 ± 3	168 ± 4	101	1.34	1.45
	BCR-CRM 185 Bovine Liver	142 ± 3	140 ± 35	99	0.58	10.6
	NIST 1577 Bovine Liver	181.1 ± 1.0	180 ± 5	99	1.27	2.95
	NIST 1566 Oyster Tissue	852 ± 14	851 ± 2	99	1.18	0.93
	CRM 12-2-01 Bovine Liver	162 ± 6	162 ± 10	100	3.10	2.44
	NCS ZC 73015 Milk Powder	34 ± 2	34 ± 4	100	5.95	2.49
^{75}As	BCR-CRM 185 Bovine Liver	0.024 ± 0.003	0.025 ± 0.002	104	3.45	6.77
	NIST 1566 Oyster Tissue	13.4 ± 1.9	12.9 ± 0.4	96	1.46	1.58
	CRM 12-2-01 Bovine Liver	0.110 ± 0.016	0.107 ± 0.005	97	2.42	6.20
	CRM 12-2-04 Wheat bread flour	0.017 ± 0.0046	0.0178 ± 0.001	105	2.78	0.45
	CRM12-2-03 Lucerne	0.262 ± 0.020	0.264 ± 0.017	101	3.17	2.38
^{78}Se	BCR 184 Bovine muscle	0.183 ± 0.012	0.187 ± 0.014	102	3.79	3.52
	BCR-CRM 185R Bovine Liver	0.446 ± 0.013	0.444 ± 0.040	99	4.50	5.42
	NIST 1577 Bovine Liver	2.031 ± 0.045	2.03 ± 0.06	100	1.39	5.20
	NIST 1566 Oyster Tissue	2.1 ± 0.5	2.2 ± 0.5	105	2.55	9.27
	CRM 12-2-01 Bovine Liver	0.325 ± 0.014	0.33 ± 0.03	102	3.88	10.2
	CRM 12-2-04 Wheat bread flour	0.040	0.039 ± 0.005	98	6.83	5.69
	NCS ZC 73015 Milk Powder	0.11 ± 0.03	0.104 ± 0.006	95	2.75	7.36
	CRM12-2-03 Lucerne	0.050	0.051 ± 0.002	102	0.23	2.83
^{85}Rb	NIST 1566 Oyster Tissue	4.45 ± 0.09	4.3 ± 0.3	97	2.85	3.81
	CRM 12-2-01 Bovine Liver	16.0 ± 2.7	16.1 ± 1.0	101	3.19	3.00
	NCS ZC 73015 Milk Powder	11.6 ± 0.7	11 ± 1	95	5.33	6.71
	CRM 12-2-04 Wheat bread flour	1.5	1.48 ± 0.08	99	2.61	2.04
	CRM12-2-03 Lucerne	16.1 ± 2.2	15.5 ± 0.8	96	2.49	5.11
^{88}Sr	NIST 1566 Oyster Tissue	10.36 ± 0.56	9.91 ± 0.05	96	0.27	2.90
	NCS ZC 73015 Milk Powder	5.3 ± 0.6	4.7 ± 0.1	89	1.15	1.65
	CRM 12-2-04 Wheat bread flour	1.53 ± 0.16	1.56 ± 0.11	102	2.04	1.14
^{89}Y	NCS ZC 73015 Milk Powder	8 ± 3[c]	8.9 ± 0.4[c]	111	1.93	3.03
^{95}Mo	NIST 1577 Bovine Liver	3.30 ± 0.13	3.40 ± 0.15	103	2.18	2.46
	NIST 1566 Oyster Tissue	0.2	0.20 ± 0.02	100	4.00	5.04
	CRM 12-2-01 Bovine Liver	3.5 ± 0.6	3.8 ± 0.3	109	3.35	5.96
	NCS ZC 73015 Milk Powder	0.28 ± 0.03	0.27 ± 0.03	96	5.43	4.16
	CRM 12-2-04 Wheat bread flour	0.2	0.203 ± 0.011	102	2.80	5.16
	CRM12-2-03 Lucerne	0.200	0.191 ± 0.011	96	2.94	3.50
^{111}Cd	BCR 184 Bovine muscle	0.013 ± 0.002	0.0132 ± 0.002	102	7.81	9.32
	BCR-CRM 185 Bovine Liver	0.298 ± 0.025	0.278 ± 0.008	93	1.38	1.34
	NIST 1577 Bovine Liver	0.097 ± 0.0014	0.096 ± 0.005	99	2.46	2.52
	NIST 1566 Oyster Tissue	3.5 ± 0.4	3.24 ± 0.09	93	1.42	2.00
	CRM 12-2-01 Bovine Liver	0.48 ± 0.03	0.47 ± 0.03	98	3.13	3.68
	CRM 12-2-04 Wheat bread flour	0.0415 ± 0.0032	0.039 ± 0.0003	94	0.35	1.41
	CRM12-2-03 Lucerne	0.136 ± 0.0065	0.136 ± 0.005	100	2.00	2.27

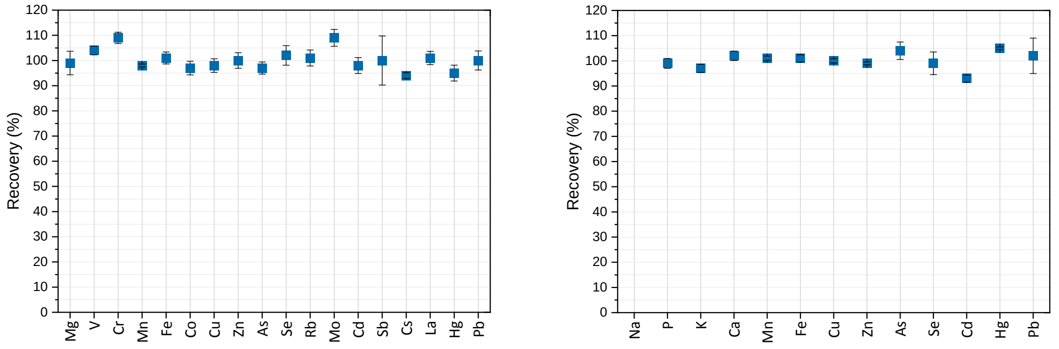
Table S2. Continued

Analyte	Reference sample	Declared (mg kg^{-1})	Found[a] (mg kg^{-1})	R[b] (%)	RSD (%)	
					Intra	Inter
^{118}Sn	CRM 12-2-04 Wheat bread flour	< 3	0.31 ± 0.04	[d]	5.78	4.47
^{121}Sb	CRM 12-2-01 Bovine liver	0.033	0.033 ± 0.006	100	9.76	6.99
	NCS ZC 73015 Milk Powder	6[c]	5.98 ± 0.26[c]	99	2.23	11.6
	CRM 12-2-03 Lucerne	0.080	0.075 ± 0.008	94	5.12	4.00
^{133}Cs	NIST 1577 Bovine Liver	21.7 ± 1.4[c]	22 ± 3[c]	101	6.91	12.1
	CRM 12-02-01 Bovine liver	0.047	0.044 ± 0.001	94	1.12	3.88
	NCS ZC 73015 Milk Powder	0.034 ± 0.005	0.031 ± 0.005	91	7.54	10.1
	CRM 12-2-04 Wheat bread flour	5[c]	4.9 ± 0.8[c]	98	8.59	14.6
	CRM 12-2-03 Lucerne	0.090	0.086 ± 0.002	96	1.37	1.75
^{138}Ba	NCS ZC 73015 Milk Powder	1.0 ± 0.3	0.86 ± 0.03	86	1.77	8.89
	CRM 12-2-04 Wheat bread flour	1.5	1.58 ± 0.22	105	7.26	0.15
	CRM 12-2-03 Lucerne	23.4 ± 2.1	23.6 ± 0.7	101	1.57	4.06
^{139}La	CRM 12-2-01 Bovine Liver	0.070	0.071 ± 0.004	101	2.66	7.36
	CRM12-2-03 Lucerne	0.940	0.95 ± 0.03	101	1.73	4.00
^{140}Ce	CRM12-2-03 Lucerne	1.0	1.04 ± 0.06	104	3.01	4.06
^{141}Pr	NCS ZC 73015 Milk Powder	0.7[c]	0.59 ± 0.02[c]	85	1.35	12.0
^{146}Nd	NCS ZC 73015 Milk Powder	2[c]	2.2 ± 0.4[c]	110	8.82	12.7
^{147}Sm	NCS ZC 73015 Milk Powder	0.5[c]	0.46 ± 0.09[c]	92	11.4	13.6
	CRM12-2-03 Lucerne	0.140	0.141 ± 0.006	101	2.16	3.27
^{153}Eu	NCS ZC 73015 Milk Powder	0.4[c]	0.45 ± 0.09[c]	112	2.28	8.98
	CRM12-2-03 Lucerne	0.030	0.031 ± 0.001	103	1.53	2.39
^{159}Tb	NCS ZC 73015 Milk Powder	0.7[c]	0.61 ± 0.07[c]	87	5.70	12.1
	CRM12-2-03 Lucerne	0.020	0.018 ± 0.001	90	2.93	3.63
^{163}Dy	NCS ZC 73015 Milk Powder	0.45[c]	0.40 ± 0.03[c]	89	4.49	10.9
	CRM12-2-03 Lucerne	0.090	0.087 ± 0.007	97	3.89	4.96
^{165}Ho	NCS ZC 73015 Milk Powder	0.07[c]	0.073 ± 0.004[c]	104	2.46	11.8
^{166}Er	NCS ZC 73015 Milk Powder	0.16[c]	0.19 ± 0.004[c]	118	0.84	1.10
^{175}Lu	CRM12-2-03 Lucerne	5[c]	4 ± 0.2[c]	80	3.22	6.83
^{178}Hf	CRM12-2-03 Lucerne	0.100	0.082 ± 0.002	82	1.77	5.83
^{202}Hg	BCR 185 Bovine Liver	0.044 ± 0.003	0.0460 ± 0.0005	105	0.54	[d]
	NIST 1577c Bovine Liver	5.36 ± 0.17[c]	5.0 ± 0.4[c]	93	3.80	7.06
	NIST 1566 Oyster Tissue	0.057 ± 0.015	0.0528 ± 0.0004	93	0.38	[d]
	CRM 12-2-01 Bovine Liver	0.37 ± 0.02	0.35 ± 0.02	95	3.14	5.92
	NCS ZC 73015 Milk Powder	2.2[c]	2.0 ± 0.2	91	4.50	7.73
^{205}Tl	NIST 1566 Oyster Tissue	5[c]	4.92 ± 0.05[c]	98	1.04	10.9
	NCS ZC 73015 Milk Powder	0.9[c]	0.89 ± 0.03[c]	99	1.66	6.40

Table S2. Continued

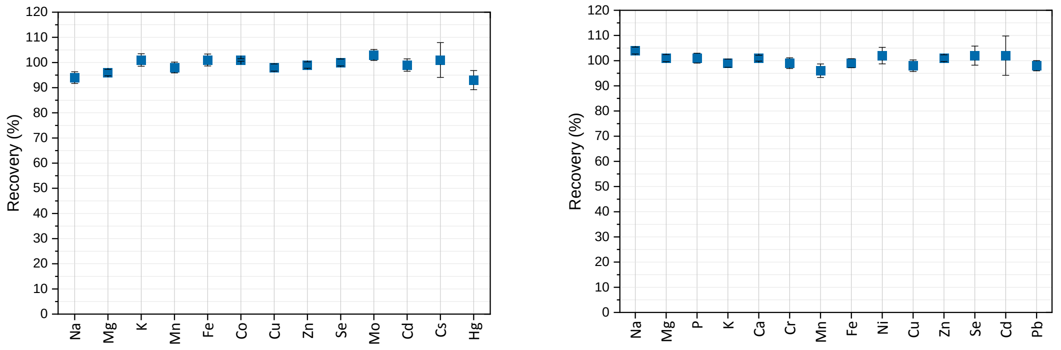
Analyte	Reference sample	Declared (mg kg^{-1})	Found[a] (mg kg^{-1})	R[b] (%)	RSD (%) Intra	RSD (%) Inter
Pb[e]	CRM 12-2-04 Wheat bread flour	0.041 ± 0.0078	0.043 ± 0.0076	104	9.97	8.64
	BCR 184 Bovine muscle	0.239 ± 0.011	0.2343 ± 0.0096	98	2.04	2.35
	BCR-CRM 185 Bovine Liver	0.501 ± 0.027	0.512 ± 0.072[c]	102	7.03	5.92
	NIST 1566 Oyster Tissue	0.480 ± 0.040	0.475 ± 0.040	99	4.20	4.95
	CRM 12-2-01 Bovine Liver	0.71 ± 0.08	0.71 ± 0.05	100	3.79	4.54
	NCS ZC 73015 Milk Powder	0.07 ± 0.02	0.071 ± 0.003	101	1.77	6.55
	CRM12-2-03 Lucerne	1.84 ± 0.17	1.89 ± 0.07	103	1.74	4.86
^{209}Bi	NCS ZC 73015 Milk Powder	1.2[c]	1.27 ± 0.22[c]	106	8.89	13.8
^{232}Th	NCS ZC 73015 Milk Powder	2.8[c]	2.64 ± 0.05[c]	99	1.00	10.5
	CRM12-2-03 Lucerne	0.110	0.109 ± 0.006	99	2.78	3.68
^{238}U	NIST 1566 Oyster Tissue	0.116 ± 0.006	0.112 ± 0.006	97	2.79	7.58
	NCS ZC 73015 Milk Powder	3[c]	3.12 ± 0.14[c]	104	2.18	5.18

[a] Mean ± 2 S.D. (n = 3).
[b] Recovery (%) = (Found value/Declared value)×100.
[c] μg kg^{-1}
[d] Not determined.
[e] Pb was measured as the sum of the three most abundant isotopes, ^{206}Pb^{+}, ^{207}Pb^{+} and ^{208}Pb^{+}.



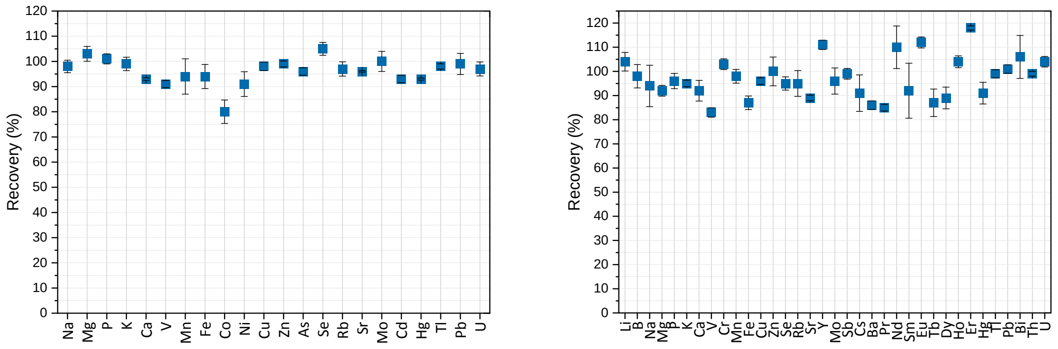
CRM 12-2-01 Bovine Liver

BCR-CRM 185R Bovine Liver



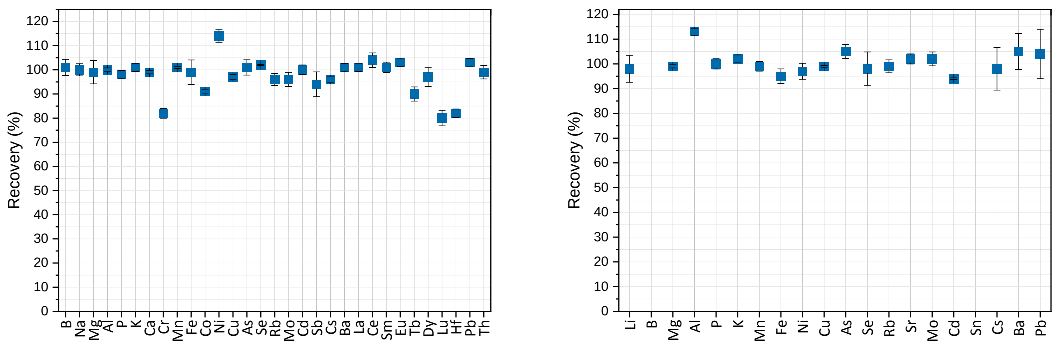
NIST 1577 Bovine Liver

BCR 184 Bovine muscle



NIST 1566 Oyster Tissue

NCS ZC 73015 Milk Powder



CRM12-2-03 Lucerne

CRM 12-2-04 Wheat bread flour

Figure S2. Graphical summary of the recovery values of the eight different certified reference materials (CRMs) used to validate the analytical methods for the quantification of different elements in cuttlefish samples. B and Sn were not determined in CRM 12-02-04 wheat bread flour, therefore missing values are present in the relative plot.

Table S3. Method Detection Limits (MDL)[a] and Method Limits of Quantification (MLOQ)[a] ($\mu g\ kg^{-1}$) and normalized calibration slopes (NCS) ($1/\mu g\ L^{-1}$) of Agilent 7900 Q-ICP-MS for analysis of different elements in cuttlefish samples with the use of Rh as internal standard.

Analyte	Cell mode	NCS	MDL	MLOQ	Analyte	NCS	Cell mode	MDL	MLOQ
$^{7}Li^{+}$	No gas	5.2×10^{-3}	0.59	1.98	$^{118}Sn^{+}$	1.73×10^{-2}	No gas	0.49	1.6
$^{11}B^{+}$	No gas	1.4×10^{-3}	5.7	19	$^{121}Sb^{+}$	2.3×10^{-2}	No gas	0.15	0.49
$^{23}Na^{+}$	He	1.2×10^{-3}	3500	11667	$^{133}Cs^{+}$	6.4×10^{-2}	No gas	0.015	0.051
$^{24}Mg^{+}$	No gas	1.3×10^{-2}	15	51	$^{138}Ba^{+}$	5.6×10^{-2}	No gas	0.55	1.83
$^{27}Al^{+}$	He	1.1×10^{-4}	8.6	29	$^{139}La^{+}$	6.4×10^{-2}	No gas	0.013	0.043
$^{31}P^{+}$	HE He	3.4×10^{-5}	161	536	$^{140}Ce^{+}$	6.4×10^{-2}	No gas	1.50	5.0
$^{39}K^{+}$	He	4.0×10^{-4}	735	2448	$^{141}Pr^{+}$	7.8×10^{-2}	No gas	0.010	0.033
$^{44}Ca^{+}$	He	1.9×10^{-5}	1291	4304	$^{146}Nd^{+}$	1.2×10^{-2}	No gas	0.07	0.22
$^{51}V^{+}$	He	8.1×10^{-3}	0.06	0.21	$^{147}Sm^{+}$	1.1×10^{-2}	No gas	0.27	0.88
$^{52}Cr^{+}$	He	1.1×10^{-2}	0.97	3.2	$^{153}Eu^{+}$	4.0×10^{-2}	No gas	0.019	0.063
$^{55}Mn^{+}$	He	4.3×10^{-3}	2.4	8.1	$^{157}Gd^{+}$	1.8×10^{-2}	No gas	0.046	0.153
$^{56}Fe^{+}$	He	7.7×10^{-3}	6.5	22	$^{159}Tb^{+}$	8.1×10^{-2}	No gas	0.0095	0.032
$^{59}Co^{+}$	He	2.1×10^{-2}	0.12	0.41	$^{163}Dy^{+}$	1.9×10^{-2}	No gas	0.070	0.23
$^{60}Ni^{+}$	He	5.7×10^{-3}	6.3	21	$^{165}Ho^{+}$	7.7×10^{-2}	No gas	0.054	0.18
$^{63}Cu^{+}$	He	1.7×10^{-2}	2.0	6.7	$^{166}Er^{+}$	2.6×10^{-2}	No gas	0.031	0.102
$^{66}Zn^{+}$	No gas	5.8×10^{-3}	159	528	$^{172}Yb^{+}$	1.7×10^{-2}	No gas	0.047	0.157
$^{75}As^{+}$	HE He	2.5×10^{-3}	0.49	1.6	$^{175}Lu^{+}$	7.2×10^{-3}	No gas	0.011	0.035
$^{78}Se^{+}$	HE He	3.7×10^{-4}	1.6	5.5	$^{178}Hf^{+}$	2.1×10^{-3}	No gas	0.0023	0.0075
$^{85}Rb^{+}$	No gas	4.3×10^{-2}	0.09	0.31	$^{185}Re^{+}$	2.3×10^{-3}	No gas	0.12	0.39
$^{88}Sr^{+}$	No gas	5.6×10^{-2}	0.19	0.62	$^{195}Pt^{+}$	1.6×10^{-3}	No gas	0.18	0.59
$^{89}Y^{+}$	No gas	6.8×10^{-2}	0.03	0.10	$^{205}Tl^{+}$	4.1×10^{-2}	No gas	0.027	0.088
$^{90}Zr^{+}$	No gas	3.5×10^{-2}	0.11	0.35	Pb [b]	5.3×10^{-2}	No gas	0.12	0.40
$^{95}Mo^{+}$	No gas	1.0×10^{-2}	0.58	1.93	$^{209}Bi^{+}$	4.4×10^{-2}	No gas	0.033	0.11
$^{101}Ru^{+}$	No gas	1.2×10^{-2}	0.07	0.22	$^{232}Th^{+}$	4.7×10^{-2}	No gas	0.033	0.11
$^{105}Pd^{+}$	No gas	1.34×10^{-2}	0.14	0.45	$^{238}U^{+}$	4.8×10^{-2}	No gas	0.0005	0.0017
$^{111}Cd^{+}$	No gas	6.0×10^{-3}	0.0038	0.013	Hg [c]	2.8×10^{-2}		0.2	0.7

[a] Values were calculated assuming a sample mass of 0.100 g.

[b] Pb is measured as the sum of the three most abundant isotopes, $^{206}Pb^{+}$, $^{207}Pb^{+}$ and $^{208}Pb^{+}$.

[c] Values were evaluated for direct analysis of Hg by single purpose atomic absorption spectrometer AMA 254.

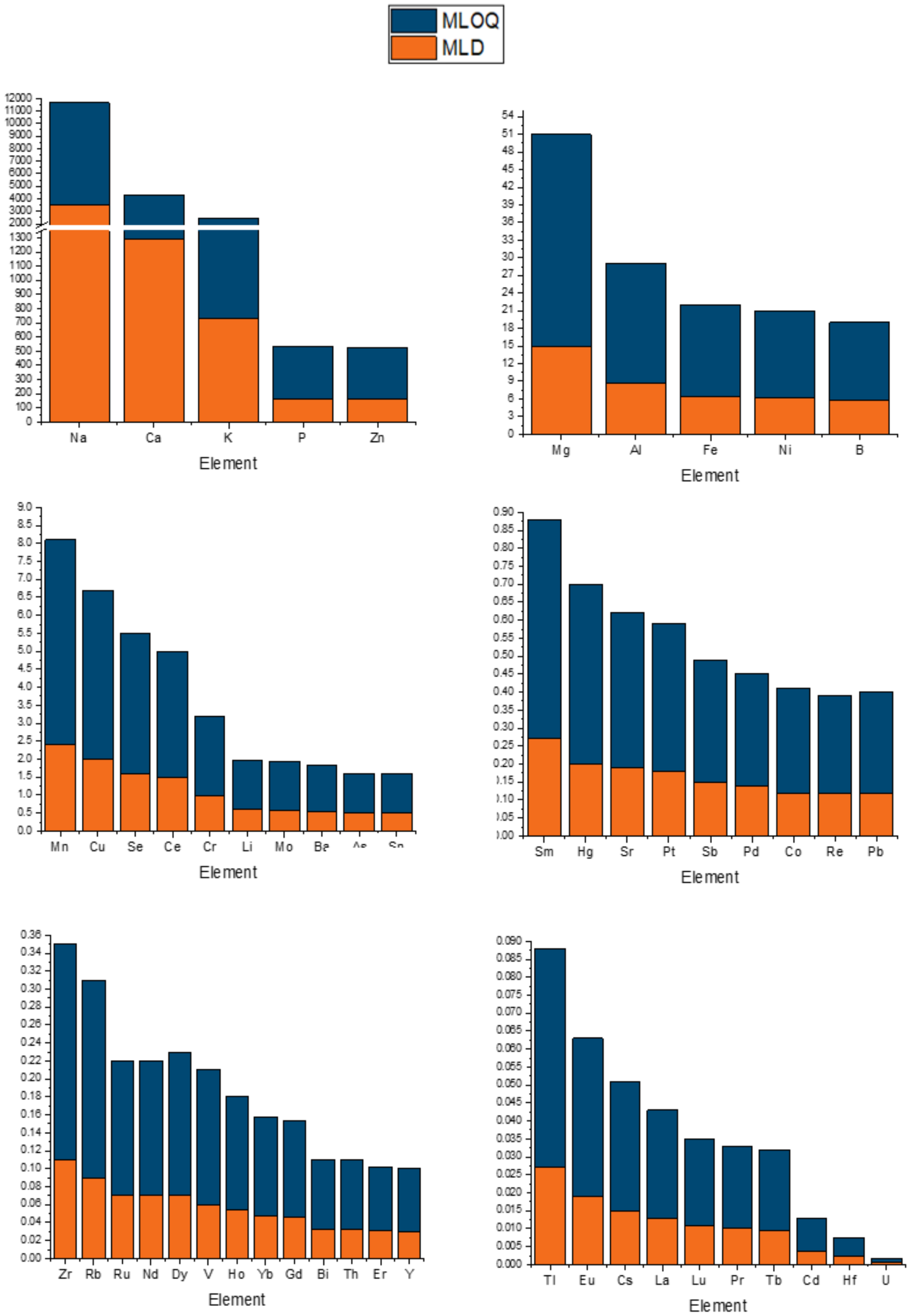


Figure S3. Graphical summary of Method Detection Limits (MDL) (µg kg^{-1}) and Method Limits of Quantification (MLOQ) (µg kg^{-1}) for the analysis of different elements in cuttlefish samples.

Table S4. Loading weights (0-1 scaled) on the first two principal components from PCA performed using all the elements. Variables with values higher than 0.7 were selected and used for the creation of LW-LDA model.

Variable	Loading Weight		Variable	Loading Weight	
	PC 1	PC 2		PC 1	PC 2
Li	0.9161	0.9828	Sn	0.4052	0.5677
B	0.6713	0.7207	Sb	0.4487	0.6867
Na	0.9835	0.9617	Cs	0.3746	0.1279
Mg	0.8714	0.9447	Ba	0.3775	0.6344
Al	0.2004	0.2489	La	0.3005	0.4644
P	0.4261	0.1216	Ce	0.2928	0.4470
K	0.4533	0.0000	Pr	0.0005	0.3591
Ca	0.8132	0.9881	Nd	0.0000	0.3737
V	0.3980	0.6934	Sm	0.0758	0.3760
Cr	0.5316	0.5639	Eu	0.0588	0.4170
Fe	0.1041	0.4436	Gd	0.0242	0.4294
Mn	0.1649	0.2719	Tb	0.0233	0.4141
Ni	0.3848	0.3796	Dy	0.0654	0.3997
Co	0.6354	0.8007	Ho	0.0248	0.4180
Cu	0.5961	0.7946	Er	0.0443	0.4210
Zn	0.4682	0.8007	Yb	0.1320	0.4139
As	0.4623	0.1563	Lu	0.1959	0.4488
Se	0.5489	0.6970	Hf	0.7509	0.6634
Rb	0.4229	0.0328	Re	0.6194	0.4359
Sr	0.9352	1.0000	Pt	0.7033	0.5132
Y	0.2576	0.3511	Tl	0.4505	0.2269
Zr	0.4817	0.6681	Pb	0.2908	0.5203
Mo	0.6908	0.9030	Bi	0.4470	0.2509
Ru	0.7650	0.5617	Th	0.2504	0.5523
Pd	0.8914	0.8030	U	0.7600	0.9832
Cd	0.9222	0.9777	Hg	0.6800	0.4359

Table S5. Fisher's classification coefficients for the LW-LDA model.

	Fisher's classification coefficients		
Variable selected	CH	MED	AT
Li	–13.496	3.080	5.994
B	14.378	0.615	–10.118
Na	–24.484	–6.308	23.663
Mg	30.145	1.805	–21.952
Ca	19.229	11.045	–24.197
Co	1.275	9.925	–9.745
Cu	3.603	2.396	–4.396
Zn	–0.484	–0.790	1.294
Sr	–49.228	–14.310	45.787
Mo	–3.046	1.769	0.438
Ru	0.305	–0.666	0.142
Pd	–0.615	1.774	–0.946
Cd	–6.960	–11.474	13.922
Hf	1.132	–0.357	–0.287
Pt	0.882	–0.344	–0.211
U	–2.835	–3.589	5.752
(Constant)	–31.893	–8.675	–24.855

CH = Cuttlefish from Chioggia (FAO 37.2.1). MED = cuttlefish from Mediterranean Sea (FAO 37.1/37.2) AT = cuttlefish from French Atlantic Ocean (FAO 27.7.e).

Table S6. Variables selected according to Wilk's Lambda values used for the creation of stepwise-LDA models and relative Fisher's classification coefficients for each group.

Variable selected	Lambda value	*T*	*F* to remove	*p*	Fisher's classification coefficients		
					CH	MED	AT
Na	0.011	0.199	153.030	0.000	–38.122	–1.622	26.301
Co	0.004	0.219	12.568	0.000	0.879	7.071	–7.012
B	0.006	0.207	65.093	0.000	15.871	0.960	–11.211
K	0.005	0.110	34.483	0.000	24.739	2.006	–17.469
Cd	0.004	0.171	24.886	0.000	–6.304	–12.504	14.739
V	0.005	0.319	29.741	0.000	–3.456	6.218	–3.797
U	0.004	0.194	14.543	0.000	1.876	–7.844	6.759
Rb	0.003	0.138	5.703	0.000	–9.350	0.011	5.943
Ni	0.003	0.740	7.558	0.000	1.404	2.285	–2.878
Ba	0.003	0.700	4.316	0.000	2.614	–0.112	–1.444
Cu	0.003	0.274	5.639	0.000	1.087	3.725	–3.948
As	0.003	0.428	4.393	0.000	–2.660	–1.828	3.321
(Constant)					–33.155	–10.750	–27.163

T = tolerance. *F* to remove = maximum *F* set to 2.71. *p* = significant level of 0.05. CH = Cuttlefish from Chioggia (FAO 37.2.1). MED = cuttlefish from Mediterranean Sea (FAO 37.1/37.2) AT = cuttlefish from French Atlantic Ocean (FAO 27.7.e)

Table S7. VIP values used for the creation of VIP-PLS-DA model and relative regression coefficients for each group.

Variable selected	VIP value	Regression coefficients		
		CH	MED	AT
V	1.672	–0.2584	0.4376	–0.2115
Co	1.628	–0.1722	0.2502	–0.0995
Na	1.477	–0.1700	0.0678	0.0809
Sr	1.447	–0.2113	0.1065	0.0783
Mn	1.420	0.1365	0.0292	–0.1487
Mo	1.416	–0.0867	0.0393	0.0365
Cd	1.411	0.1963	–0.4848	0.3131
Li	1.391	–0.2127	0.7827	0.0257
U	1.386	0.2916	–0.5152	0.2601
Cu	1.375	–0.0285	0.0930	–0.0681
Ni	1.370	0.0056	0.0572	–0.0620
Ca	1.368	–0.2666	0.2128	0.0204
Mg	1.361	–0.0263	–0.1046	0.1276
B	1.342	0.3117	–0.1665	–0.1062
Se	1.273	–0.1543	0.1802	–0.0451
K	1.242	0.1371	–0.0803	–0.0397
Bi	1.227	0.3117	–0.1665	–0.1062
Rb	1.174	–0.0167	0.1343	–0.1197
Cs	1.060	0.0269	–0.0675	0.0440
P	1.046	0.1007	–0.1280	0.0398
Y	1.003	0.0509	0.1523	0.0212
(Constant)		0.5546	0.7827	0.7827

CH = Cuttlefish from Chioggia (FAO 37.2.1). MED = cuttlefish from Mediterranean Sea (FAO 37.1/37.2) AT = cuttlefish from French Atlantic Ocean (FAO 27.7.e).

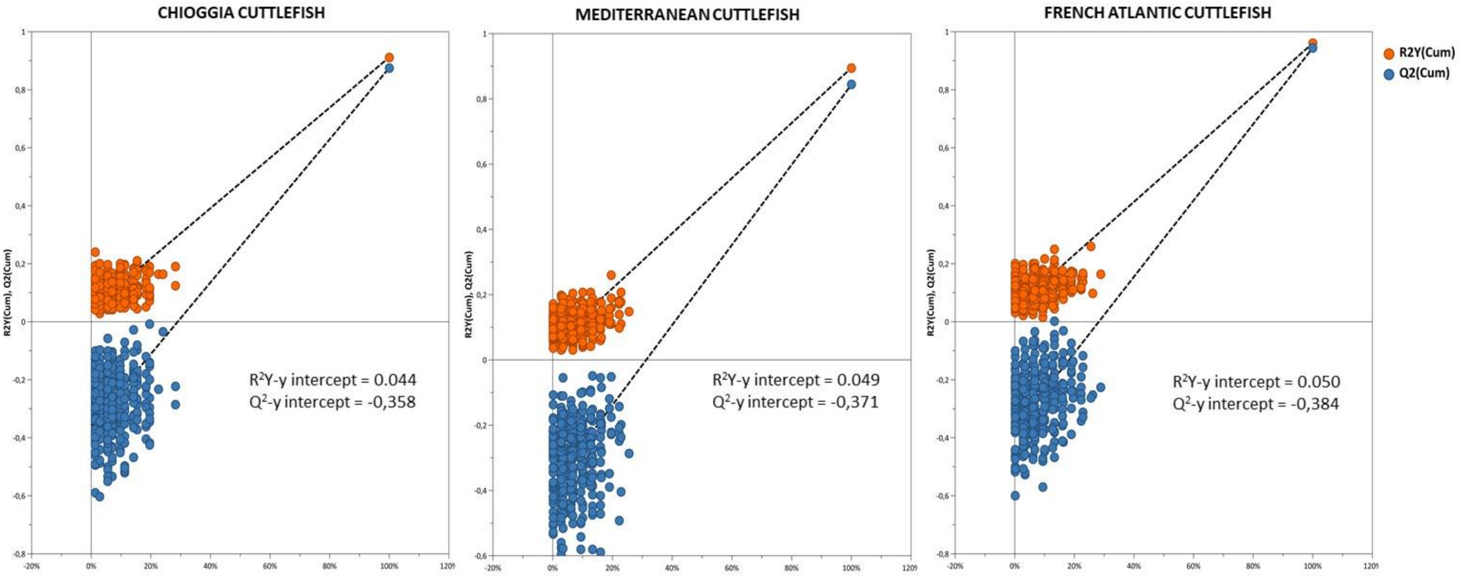


Figure S4. Permutation plot (400 random permutations) assessing the validity of VIP-PLS-DA training model. The intercept to the y-axis of the regression line, correlating the R2Y and Q2 values between the original and the permuted y-variables (displayed on the X-axis) and the cumulative value of R2Y and Q2 values (displayed on the X-axis), outline the degree of model's overfitting.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Maria Olga Varra: Writing – original draft, Investigation, Data curation, Formal analysis, Validation, Resources. **Lenka Husáková:** Writing - review & editing, Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Supervision, Funding acquisition. **Jan Patočka:** Methodology, Investigation, Validation, Formal analysis. **Emanuela Zanardi:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition, Supervision. **Sergio Ghidini:** Resources, Conceptualization.