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Elemental composition in soft tissues as a model for identifying batches of juvenile Atlantic bluefin tuna (*Thunnus thynnus*)

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ABSTRACT

Integral Atlantic bluefin tuna (*Thunnus thynnus*) aquaculture will become a reality in the coming years and so tuna batches will have to be clearly identifiable to avoid commercial fraud and ensure this species' conservation. Consequently, the objective of this study was to analyse the components of juvenile bluefin tissue to be able to discriminate between three tuna batches: specimens born in captivity and raised in inland facilities (onshore tanks), fish born in captivity and raised in the sea (sea cages), and wild tuna. Ten macro and trace elements (Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn) were selected, and their concentrations were analysed in four soft tissues: liver, kidney, brain and muscle. Only one of the elements (Cu) showed statistically significant differences for fish batch in all tissues, so multivariate tests (Principal Component Analysis, PCA and Canonical Discriminant Analysis, DCA) were performed. In the PCA, there were partial batches separation in kidney and muscle. In DCA, the percentage of cases correctly classified using this validation were 60.8 % (liver), 88.6 % (kidney), 79.5 % (muscle) and 82.2 % (brain). Globally, muscle appear to be the best tissue for discriminating the batch of tunas, and wild specimens are the most readily identifiable.

1. Introduction

The Atlantic bluefin tuna (Thunnus thynnus, ABFT) is a species of great commercial importance and as such its capture is subject to rigorous controls to ensure its quality and compliance with international laws. In general, the aquaculture of tuna species is a relatively recent activity (Mylonas et al., 2010; Benetti et al., 2015) and capture-based aquaculture is a type of intensive production that in ABFT has only been practiced since the late 1990s (De la Gándara et al., 2016). However, production techniques have changed since the biological cycle of the ABFT was first fully disentangled in 2016 (Ortega and De la Gándara, 2017), thereby enabling the development of integral aquaculture for this species. In future years, juvenile specimens of ABFT will be bred in aquaculture facilities and, once established, new tools will be required to discriminate batches of these specimens and ensure correct adherence to sanitary regulations.

In recent years, studies using marking methods have been developed in aquaculture for various fish species (Canonico et al., 2005; Krkošek et al., 2006; Brooks and Jones, 2008; Glover et al., 2013). To identify captivity-born fish, some marking techniques can be applied, like external labels, intramuscular microchips, otolith marking, stable isotopes and genetic markers (Greene et al., 2009; Huelga-Suárez et al., 2012; Thorrold et al., 2001). However, techniques to mass tagging are difficult to implement and sometimes cause complications in growth and mortality due to handling remain (Gilderhus and Marking, 1987; Mohler, 2003). Thus, non-invasive methods that can guarantee the traceability of products of different batches are required. In fact, some techniques to differ fish stocks based on their origin, mainly by otoliths composition or by genetic markers are used. However, other techniques about the composition of different tissues of fish are tools rarely documented, even though it is known that, in the growth and development of both terrestrial and aquatic animals, growing conditions play a key role in tissue configuration (Jara & Chodyniecki, 1999; Brucka-Jastrzębska et al., 2009).

Recently, certain trace elements have been proposed in the Turkish Mediterranean as non-invasive and natural tools for determining the origin of ABFT specimens weighing over 50 kg (Sogut et al., 2011). As well, the use of this technique to identify and analyse biological

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variables is beginning to receive greater attention in stock identification (Kusznierz et al., 2008; Bektas and Belduz, 2009; Specziar et al., 2009). Multi-element studies are valuable tools for performing standardised chemical composition profiles and are potentially of great interest in food authentication and possibly for application in fisheries (Cubadda et al., 2006). However, several biotic and abiotic factors such as the age and weight of fish, place of capture, the tissues studied, and the statistical model employed could distort results.

In this study, differences in the main trace elements (Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn) in four soft tissues (liver, kidney, brain and muscle) of juvenile ABFT (less than 1-year old) from different batches (wild, raised in onshore tanks and in sea cages) were investigated. Two multivariant statistical models were used to classify the origin of the fish: principal component analysis (PCA) and discriminant canonical analysis (DCA).

2. Material and methods

2.1. Sample collection

Samples of ABFT weighing less than 1000 g were taken in 2018. The number of the samples was (for batch 1, 2 and 3): 24–22–28 (liver), 15–13–9 (kidney), 24–22–27 (muscle), and 24–15–29 (brain). The fish of batch 1 and 2 consisted of ABFT hatched from eggs from naturally spawning captive adults in sea cages and raised in the facilities of the Spanish Institute of Oceanography (Mazarrón, Spain). The larval culture was fed on rotifer and copepod in a 40-m^3 tank; weaned fish were fed an artificial diet (Magokoro S-3, Marubeni Nissin Feed Co., Ltd., Tokyo, Japan) and maintained at 24.9 °C at a salinity of 32.0 g L^{-1} in a 20-m^3 tank. At 41 days post-hatching (individuals with suffisent body mass to be transported to the tanks and sea cages), the specimens were split into two groups: fish of batch 1 was transferred to a 900-m^3 overflow system tank in the Infraestructura de Control de Reproducción del Atún Rojo (Cartagena, Spain) where they were fed with herring *Clupea spp.*, Brazilian menhaden *Brevoortia aurea* and Atlantic mackerel *Scomber scombrus*; fish of batch 2 was placed in floating cages in the sea at Cartagena (37°34'39.2"N, 0°52'35.9"O). All fish dying due to traumatic events were collected soon after death and sampled. The batch 3 (wild tunas) were caught by trolling in October 2018 in Mazarrón Bay (Murcia, Spain) and sampled immediately after capture. In accordance with European legislation (Directive 2010/63/EU), the procedures employed did not require ethical permissions.

Tissue samples taken from liver, kidney, muscle and brain were frozen immediately and stored at − 20 °C until analysis. Muscle samples were taken from the front of the head, liver samples from the ventral and cranial regions, and kidney samples from the cranial region; the brain was removed whole (or as intact as possible).

2.2. Sample preparation and elemental analysis

To determine the concentrations of Ca, Fe, K, Mg, Na, P, S, Cu, Mn and Zn, samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, *ICAP 6500 Duo, Thermo Scientific,* Waltham, USA). Samples (0.1–0.2 g) were treated with 4 mL of trace mineral grade HNO_3 (69 % Suprapure, *Merck*, Darmstadt, Germany) and 1 mL of H_2O_2 (33 % Suprapure, *Merck*, Darmstadt, Germany) in special Teflon reaction tubes and heated at 220 ºC in a microwave digestion system (UltraClave-Microwave Milestone®, Sorisole, Italy) for 20 min, and then diluted with double deionised water to 10 mL. The detection limit (DL) was 10 μg g^{-1} for major constituents (Ca, K, Mg, Na, P and S) and 0.001 μg g^{-1} for the remaining elements. For every sample, two readings were made, the mean of which was used as the concentration value. To check for possible metal contaminants, one blank sample for every 11 samples was also analysed.

Multi-element calibration standards (SCP Science, in 4% HNO^3, Québec, Canada) were prepared with specific concentrations for each element, taking as a reference UNE-EN ISO 11885 for the determination of elements by ICP-OES. Furthermore, intermediate patterns of all elements were prepared. The recovery percentages of standar reference material (1577b -National Institute of Standards & Technology- Dicoex, Bilbao, Spain) were 91.62 (Ca), 97.99 (Cu), 106.86 (Fe), 98.33 (K), 103.79 (Mg), 111.21 (Mn), 98.06 (Na), 97.48 (P), 99.48 (S) and 96.34 (Zn)".

2.3. Statistical analysis

The results obtained were subjected to statistical analysis using the SPSS software (*Statistical Package for the Social Sciences*, *IBM 24.0*, New York, USA). For the elemental concentrations, means and standard deviations were obtained. An ANOVA (Tukey and Games-Howell post hoc tests) test was used as a statistical method to study differences between specimens of different batches, while Levene's test was used to assess the homogeneity of variance. The significance levels for all tests were set at 0.05.

In order to classify the batch of the fish using the chemical data, two multivariate techniques were used: PCA and DCA. For the PCA, a threshold factor loading of 0.32 corresponding to an explained average variance of 56.6 % was considered (Peterson, 2000). In addition, to evaluate the validity of the method, the Kaiser Meyer Olkin (KMO) index, a p-value lower than 0.05 (Bartlett's Test of Sphericity) and the eigenvalue criterion (greater than 1) were employed. For the DCA, Wilk's Lambda was used to test the significance of the discrimination ($p < 0.05$). Two functions were created, and a split-sample validation (cross-validation testing procedure) was performed to assess the capacity of the selected variables to predict different batches for the tested fish. In this validation, one individual is removed from the original matrix. The DCA is then performed using the remaining observations to classify the omitted individual; the number of misclassified individuals indicate the degree of intermingling, while the proportion of individuals correctly reallocated is taken as an integrity measurement for a group (Poulet et al., 2005; Yakubu and Osenbor, 2011). The formulas from the case classification were obtained to classify the new specimens of unknown batch. In these formulas, the constant and function coefficients were obtained for each of the tissues, batches and elements:

$$F(x) = a + (b * [X])$$

where a = a constant for the combination of a tissue and a batch; b = a coefficient of classification function for the combination of an element and batch; and X = the concentration of an element for a given tissue and batch (in a particular specimen). Once the formula has been applied, the result with the highest value indicates the possible batch of the fish.

3. Results

The concentrations of the trace elements detected in ABFT tissues are shown in Table 1. Copper was the only element with statistical differences between groups for all tissues; no differences between batches for any tissue were found for Ca, Na and Zn. Of all the tissues, the liver had the fewest elements with significant differences.

In the PCA, the integration of all 10 elements was represented by four (liver and muscle) and three (kidney and brain) principal components that explained 71–79 % of the total variance in the original data set. The KMO index was low in all cases (liver = 0.462, kidney = 0.521, muscle;= 0.529 and brain = 0.563). For kidney, the fishes from the batch 1 (onshore tank) were separated from the other groups in PC1 (Cu, Fe, Na and K; Fig. 1a); for muscle, fishes of batch 3 (wild tunas) were separated based on component 2 (Cu, K and Mg; Fig. 1b); and for liver and brain, no differentiation between groups was observed (Figs. 1c and 1d).

With the DCA, three elements (Ca, Na and Zn) were not considered in any tissues. Data from the canonical discriminant functions (CDF) are

Table 1
Concentration of trace elements in tissues of ABFT. Data: mean ± standard deviation, μg g^{-1}, ww. For each element and tissue, the same superscript letter shows statistical differences between batches (1- tanks, 2- sea cages, 3- wild); superscripts in parentheses means marginally significant (p = 0.05–0.1).

	Batch	n	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Liver	1	24	161 ± 109	2.45 ± 0.8^{b}	116 ± 53	2647 ± 292^{a,b}	260 ± 88.6	3.25 ± 1.2	1861 ± 912	2433 ± 423$^{(b)}$	2953 ± 466$^{(b)}$	25 ± 6
	2	22	174 ± 129	3.37 ± 2.3	103 ± 46	3124 ± 328^{a,C}	256 ± 109	3.31 ± 1	1774 ± 832	2620 ± 390	2952 ± 476$^{(c)}$	27.5 ± 4
	3	28	170 ± 85.9	4 ± 2^{b}	94.7 ± 40.9	3627 ± 770^{b,c}	306 ± 45.5	3.36 ± 0.7	2155 ± 1104	2777 ± 678$^{(b)}$	3272 ± 560$^{(b)(c)}$	26.8 ± 5.2
Kidney	1	15	244 ± 125	12.7 ± 10.9^{a,b}	182 ± 62^{a,b}	3059 ± 555^{a,b}	213 ± 39.4$^{(b)}$	2.23 ± 0.8	2002 ± 884$^{(b)}$	3087 ± 576	2820 ± 482	31.4 ± 7.2
	2	13	185 ± 160	2.07 ± 1.3^{a}	87.4 ± 32.3^{a}	4142 ± 510^{a}	272 ± 100	2.49 ± 1.02	1383 ± 935	2974 ± 660$^{(c)}$	3259 ± 727^{c}	28.2 ± 6.8
	3	9	243 ± 56.4	1.53 ± 0.4^{b}	66 ± 21^{b}	4291 ± 443^{b}	296 ± 82.2$^{(b)}$	2.39 ± 0.9	1181 ± 465$^{(b)}$	3554 ± 480$^{(c)}$	2407 ± 377^{c}	27.1 ± 8.4
Muscle	1	24	84.4 ± 67.9	0.281 ± 0.1^{b}	3.05 ± 0.8^{a,b}	3657 ± 494^{b}	278 ± 52.1^{b}	2.34 ± 0.5	807 ± 564	2800 ± 833	2415 ± 423	5.28 ± 2.2
	2	22	124 ± 135	0.314 ± 0.05^{c}	4.31 ± 1.8^{a}	3733 ± 479^{c}	285 ± 44.8^{c}	2.59 ± 0.4	998 ± 657	2964 ± 396	2464 ± 534	5.09 ± 1.1
	3	27	112 ± 173	0.515 ± 0.08^{b,c}	4.53 ± 1.4^{b}	4365 ± 734^{b,c}	346 ± 56.9^{b,c}	2.32 ± 0.7	677 ± 410	3018 ± 845	2613 ± 478	4.56 ± 1.3
Brain	1	24	142 ± 35.8	0.98 ± 0.2^{b}	32.9 ± 16.3	2284 ± 375	93.4 ± 30.3^{b}	0.69 ± 0.2^{a,b}	2462 ± 713	2401 ± 337	1768 ± 243	11.2 ± 7.8
	2	15	183 ± 66.2	1.12 ± 0.2^{c}	38.7 ± 25.4	2338 ± 485	121 ± 34	1.34 ± 0.4$^{a,(c)}$	2791 ± 537	2737 ± 679^{c}	1624 ± 256	8.87 ± 1.3
	3	29	185 ± 145	1.508 ± 0.4^{b,c}	28.8 ± 13.7	2388 ± 523	129 ± 55.5^{b}	1.06 ± 0.5$^{b,(c)}$	2906 ± 1190	2201 ± 470^{c}	1687 ± 221	8.23 ± 2.2

shown in Table 2. Of the three different fish batches, two CDFs were created for kidney, muscle and brain. Membership of the predicted groups in terms of cross-validation are shown in Table 3, while the formulas for the case classifications are given in Table 4. The percentage of cases correctly classified using this validation were 60.8 % (liver), 88.6 % (kidney), 79.5 % (muscle) and 82.2 % (brain). Differences between groups are shown in Fig. 2 (the histogram for liver only shows one element, K) and Fig. 3 (dispersion plot of CDF for kidney, muscle and brain).

4. Discussion

Elemental composition in tuna tissues is commonly used to determine the concentration of pollutants – mainly Hg – in commercial-size specimens for reasons of food safety (i.e., Annibaldi et al., 2019). However, trace element composition has attracted interest in recent decades in the food industry (Percin et al., 2011) and some studies have been performed on ABFT (i.e., Sogut and Percin, 2011; Sogut et al., 2011; Ugarte et al., 2012; Belmonte et al., 2021) to detect elements such as Cu, Mn, Ni, Zn, Fe, Mg and Se in tissues including muscle, liver, kidney, heart, brain, bone, gill and the first dorsal spine. However, to date these studies have only provided data for wild and fattened tuna weighing over 50 kg, and not for juvenile fish.

4.1. Trace element concentrations

In our study, only one of the elements (Cu) showed statistically significant differences for fish batch in all tissues (Table 1). This essential trace element is required for cellular functioning (Lall and Kaushik, 2021) and previous studies of ABFT have reported similar concentrations to those we found in muscle (Di Bella et al., 2015; Vizzini et al., 2010; Milatou et al., 2015; Ugarte et al., 2012). In this tissue, Percin et al. (2011) reported significantly higher concentrations in wild than in farmed tuna, which agrees with our results. In kidney, other authors have reported statistical differences between wild and farmed tunas, with greater concentrations in wild fish (Sogut and Percin, 2011), which contradicts our results, probably due to the high coefficient of variation detected in kidney from tuna from the onshore tanks (batch 1). In liver and brain, a similar pattern was found for Cu concentrations, with statistical differences observed between wild (batch 3) and onshore tank tunas. Vizzini et al. (2010) report similar Cu concentrations in the liver of wild and farmed tunas. To the best of our knowledge, no data regarding significant differences in Cu in brain of this fish have ever been reported.

The remaining elements (K, Mg, Fe, S, Mn and P) are essential for fish health (National Research Council, 1993, 2011). Potassium is an important cation involved in the acid:base balance and osmoregulation (Lall, 2002), and we found statistical differences for P between the batches of fish in three tissues (liver, kidney and muscle, Table 1). No data on P in tunas could be found in the literature. Magnesium is an important macroelement present in soft tissues such as muscle (Knox et al., 1981). Our data reveal greater concentrations ($p < 0.05$) in wild specimens, similar to those reported by Ugarte et al. (2012). In addition, there were statistical differences for this element between brain tissue from tuna from the onshore tank and the wild tuna, although, once again, no previous references in the literature to Mg in brain could be found. Iron is an essential trace element for vertebrates (Lall and Kaushik, 2021) and is used mainly in the production and functioning of enzymes including haemoglobin, myoglobin and cytochromes. Iron concentrations were also higher ($p < 0.05$) in muscle in wild tuna than in onshore tanks tuna (4.53 vs. 3.05 μg g^{-1}, respectively). Even though the statistical differences between these two batches agree with those reported by other authors (Percin et al., 2011), the levels we detected were lower than those reported by these and other authors (Di Bella et al., 2015; Milatou et al., 2015; Ugarte et al., 2012; Girolametti et al., 2021); in general, tunas are deemed a good source of Fe (HealthLinkBC, 2020). In the studied batches, the Fe differences could be related to the distinct feeding conditions, a fact also observed in Percın et al. (2011). For consumers, Fe intake would be slightly lower in the case of farmed tunas' muscle, but the edible part of both groups would be considered good for the intake of this element. In kidney, a tissue that eliminates Fe (Bury et al., 2012), the inverse situation was found (lower concentrations in wild tuna), which contrasts with the results of Sogut and Percin (2011). These authors also reported lower concentrations than those found in this study in kidney (10.4–14.02 vs. 66–182 μg g^{-1}, respectively), which could be due to the different weights of the tunas studied (54–57 kg vs. 0.3–1.0 kg). Sulphur, Mn and P are all relevant elements in biochemical processes and are constituents of amino acids or nucleotides (Lall and Kaushik, 2021; Aschner and Aschner, 2005; Leach et al., 1997; National Research Council, 2011). We only found statistical differences

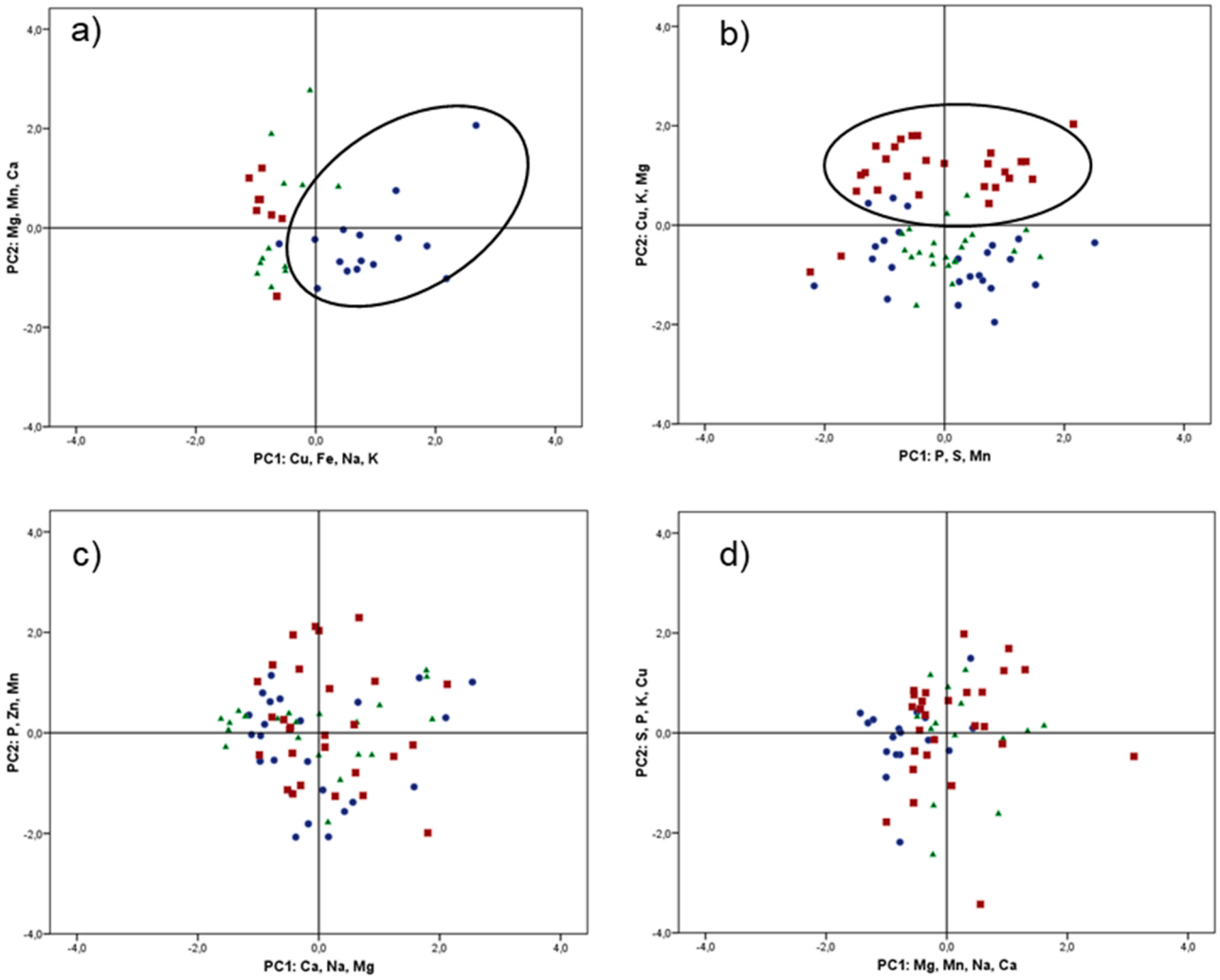

Fig. 1. PCA analyses carried out with 10 trace elements: Ca, Fe, K, Mg, Na, P, S, Cu, Mn, Zn. (a) Kidney; (b) Muscle; (c) Liver; (d) Brain. Two components (axis 1 and axis 2) explaining 45.3 (liver), 53.0 (kidney), 46.8 (muscle) and 53.8% (brain) of the total variance. The circle shows the exemplars grouped due to their similar characteristics. ● Batch 1; ▲ Batch 2; ■ Batch 3.

Table 2
Canonical discriminant functions and statistic data (DCA) from ABFT soft tissues.

	Function	Eigenvalue	% variance	Canonic correlation	Lambda of Wilks	Canonical discriminant function coefficients (Standarized)
Liver	1	0.615	100.0	0.617	0.619, $p < 0.001$	K (1.0)
Kidney	1	4.02	78.4	0.895	0.095, $p < 0.001$	Fe (0.69), K (−1.06), P (0.151), S (0.475)
	2	1.11	21.6	0.725	0.475, $p < 0.001$	Fe (−0.004), K (0.223), P (−1.09), S (1.07)
Muscle	1	5.05	95.9	0.914	0.136, $p < 0.001$	Cu (1.23), Fe (0.041), Mn (−0.517), Zn (−0.4)
	2	0.215	4.1	0.421	0.823, $p < 0.05$	Cu (−0.217), Fe (0.914), Mn (0.74), Zn (−0.738)
Brain	1	2.68	75.4	0.853	0.145, $p < 0.001$	Cu (1.03), Mg (1.61), Mn (−1.86), P (−0.851), S (0.164)
	2	0.873	24.6	0.683	0.534, $p < 0.001$	Cu (0.629), Mg (−0.196), Mn (0.791), P (0.204), S (−0.792)

between onshore tanks and wild tunas in Mn in brain (Table 1), a result that differs from the results reported in muscle by Percin et al. (2011) and kidney by Sogut and Percin (2011) in tunas weighing approximately 50 kg. Interestingly, we found high levels of Mn in all tissues, higher than those reported in muscle, liver and kidney by other authors (Percin et al., 2011; Sogut and Percin, 2011; Di Bella et al., 2015; Licata et al., 2005; Ugarte et al., 2012).

Another noteworthy finding was that most wild tuna had greater ($p < 0.05$) concentrations of elements than tuna reared in onshore tanks (Table 1). According to Percin et al. (2011), differences in tissue accumulation might be related to factors such as weight, feeding profile or habitat. In our study, although fish were chosen specifically with similar weights, there were small differences between the specimens kept in onshore tanks and wild tunas. Only three correlations with weight were detected: S in kidney in tuna from the sea cage (batch 2) and Mg in muscle and brain in tuna from the onshore tanks (batch 1). Therefore, elements such as Cu, K, Fe, Mn and P could be used to study the batches and origin of fish.

In relation with food safety, none of the analysed elements in this study is described in the Regulation 1881/2006 (and posterior modifications), which control the maximum allowed concentrations for some metals in the UE (Pb, Cd, Hg, As and inorganic Sn). Nevertheless, the studied elements could be considered as toxic if found at high levels in the edible parts of tuna (i.e., Cu and Fe; Tietz et al., 1990; Watanabe

Table 3
Pronosticated belonging groups: DCA classification accuracy (*) or missclasification (remaining) by batch and tissue from the cross-validation test. Data=percentage.

	Batch	1	2	3
Liver	**1**	75.0*	25.0	0.0
	2	31.8	36.4*	31.8
	3	14.3	17.9	67.9*
Kidney	**1**	86.7*	13.3	0.0
	2	0.0	91.7*	8.3
	3	0.0	12.5	87.5*
Muscle	**1**	70.8*	29.2	0.0
	2	31.8	68.2*	0.0
	3	0.0	3.7	96.3*
Brain	**1**	83.3*	5.6	11.1
	2	0.0	93.3*	6.7
	3	10.7	7.1	82.1*

Table 4
Classification case formulas; [element]= element concentration for the case to be classified.

	Batch	Formula
Liver	1	-13.4 + (92.8 *[K])
	2	-18.3 + (110 *[K])
	3	-24 + (127 *[K])
Kidney	1	-25.1 + (0.061 *[Fe])+ (66.3 *[K])+ (22.8 *[P])+ (35.7 *[S])
	2	-34.6 + (0.012 *[Fe])+ (140 *[K])+ (−14.1 *[P])+ (37 *[S])
	3	-37.1 + (−0.005 *[Fe])+ (153 *[K])+ (32 *[P])+ (−23.6 *[S])
Muscle	1	-13.6 + (42.7 *[Cu])+ (0.418 *[Fe])+ (4.54 *[Mn])+ (0.207 *[Zn])
	2	-17.5 + (48.2 *[Cu])+ (1.18 *[Fe])+ (5.64 *[Mn])+ (−0.432 *[Zn])
	3	-35.4 + (133 *[Cu])+ (0.862 *[Fe])+ (0.631 *[Mn])+ (−1.18 *[Zn])
Brain	1	-30.3 + (1.27 *[Cu])+ (−578 *[Mg])+ (4.32 *[Mn])+ (−35.3 *[P])+ (384 *[S])
	2	-30 + (−2.23 *[Cu])+ (−1495 *[Mg])+ (19.6 *[Mn])+ (12.3 *[P])+ (297 *[S])
	3	-30.9 + (11.5 *[Cu])+ (−48.9 *[Mg])+ (0.12 *[Mn])+ (−56.4 *[P])+ (331 *[S])

et al., 1997; Olsson, 1998; Percin and Konyalioglu, 2008; Vizzini et al., 2010). In addition, the specific legislation to food safety is complex and perpetually evolving (Bondoc, 2016) and the levels of trace element have attracted interest in recent decades in the food industry, developing more strict regulations (Percin et al., 2011). Specifically, ABFT has a wide food spectrum and a long-life span (Santamaria et al., 2009) with a life cycle of 20 years (Chase, 2002). Therefore, the (bio)accumulation of trace elements in soft tissues is quite important (Licata et al., 2005; Storelli et al., 2005; Kojadinavic et al., 2007; Tuzen and Soylak, 2007; Yildirim et al., 2009; Vizzini et al., 2010; Cammilleri et al., 2018). However, the standards of trace elements from fish muscle that we could consider (USEPA, 1989; MAFF, 2000; USDA, 2009) offer differing ranges of concentration (i.e. for Cu: 20.0 μg g^{-1} in MAFF, 120 μg g^{-1} in USEPA, and 0.86 μg g^{-1} in USDA; see Percin et al., 2011 for detailed information) and it could not signal unequivocable if the concentrations found are or not above these limits.

4.2. Principal component analysis

In order to obtain an overall picture of elemental composition in tuna of different batches, all the trace elements were integrated into a PCA for each tissue (Fig. 1). Bartlett's Test of Sphericity, the eigenvalue criterion and explained variance were appropriate in all tissues, although the KMO indices were low (0.462–0.563). According to Shrestha (2021), if the KMO is below 0.5, the results are not suitable for data analysis and so the PCA for liver was not taken into account. For kidney, while batch 1 tunas were clearly different, batch 2 and 3 specimens were not (Fig. 1a). In fish, the kidney has both exocrine and endocrine functions, i.e. hormone production and haematopoietic functions (Hyttel et al. 2009; Zapata & Amemiya, 2000; Press & Evensen, 1999), and play a vital role in osmoregulation and homoeostasis (Davidson, 2014). Therefore, differences in the characteristics of the water (batch 1, onshore tank tunas) could have affected the results. In addition, Cu and K (two elements with statistical differences for their origins) were part of the principal component that separated the groups (PC1). Finally, a clear separation between the batches was found for muscle (Fig. 1b). In this case, specimens from the batch 1 and 2 were mixed in both components (PC1 and PC2); meanwhile Cu, K and Mg (forming the PC2) separated the batch 3 from the other groups (Cu, K and Mg were elements with statistical differences between batch 3 and the other two batches). Several authors have reported that diet is the main source of Cu and Mg (Lall, 2002; Cowey et al., 1977; Bury et al., 2003; Kamunde et al., 2002). For these elements, the wild specimens (batch 3) had higher concentrations than those found in remaining groups, being the tendance wild $>$ sea cages $\geq$ tanks. Tank and sea cage individuals (batch 1 and 2, respectively) were fed on defrosted bait ad libitum, but wild juveniles have an opportunistic diet with the presence of shrimps, cephalopods and crustaceans (Uotani et al., 1990; Sarà and Sarà, 2007; Sinopoli et al., 2004), especially in the Mediterranean Sea (Karakulak et al., 2009; Van Beveren et al., 2016). In cephalopods and crustaceans, microelements play essential roles in biological functions (Rjeibi et al., 2015, cephalopods; Jacobo et al., 2016, crustaceans). This diet could explain the higher concentration in wild's muscle of Cu and K. However, as stated above, data for Mg should be viewed with caution due to the correlation found in muscles in fish of batch 1 (onshore tank). In summary, this statistical model does not allow us to discriminate these groups for muscle.

4.3. Discriminant canonical analysis

The DCA enables differences between populations to be maximised or made more evident (Balzarini et al., 2015). In this test, for each tissue a pair of functions describing the differences between the three batches was created. These functions are composed of some of the elements selected by the analytical software as the most discriminant (Yakubu and Okunsebor, 2011). Wilk's lambda was used to test the significance of the discrimination, which were significant for all functions (Table 2).

Calcium and Na are key elements involved in functions such as the development and maintenance of the skeletal system, the osmotic balance, and the acid:base equilibrium (Lall, 2002; Lall and Kaushik, 2021; Zimmer et al., 2019). Nevertheless, these elements showed non-validity for this method of analysis (Table 2) and so no data for Ca and Na were taken from the tuna tissue.

Only one function was found for liver (K), which had the lowest classification value (60.8 %); the remaining elements were used to build two functions for kidney, muscle and brain (Table 2). Again, good separation in kidney was found (88.6 %, cross-validation) with no confusion between specimens from the batches 1 and 3 (Table 3). In terms of functions, there were two elements (Fe and K) in kidney that also showed differences between groups in the ANOVA test and PCA, so a priori these tissues probably could be used to ascertain the batch of fish. However, the batch 2 (sea-cage fish) was the best identified group (91.7 %), in contrast to the PCA results (batch 1, onshore tanks).

Muscle was the third-best tissue for identifying the batch of fish (79.5 %), although a number of factors should be borne in mind. First, specimens of batch 3 were discriminated in 96.3 % of cases and there was no confusion with tunas of batch 1, and only in 3.7 % of cases was there confusion with specimens from batches 2 and 3 (Table 3). This is a very interesting result that could be used to differentiate different batches as wild and reared tuna. Second, the batch 3 was a separate group in the PCA for muscle, which adds to the potential of this tissue for discriminate the origin of fish. Finally, Cu was conclusive in all three statistical analyses. According to a number of authors (Bury et al., 2003; Kamunde et al., 2002), diet is the major source of the Cu required for physiological

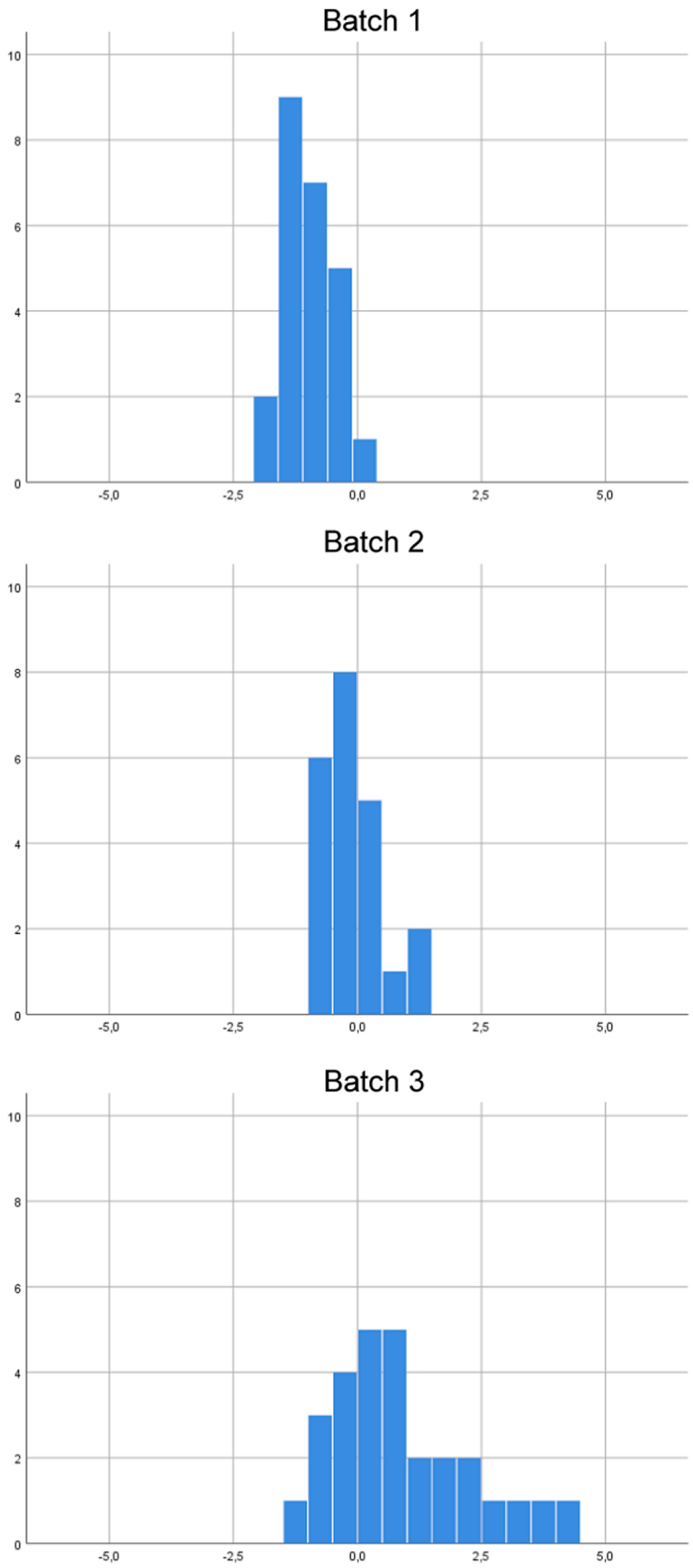

Fig. 2. Canonical Discriminant Funcions of the liver from the three different batches (for the element K).

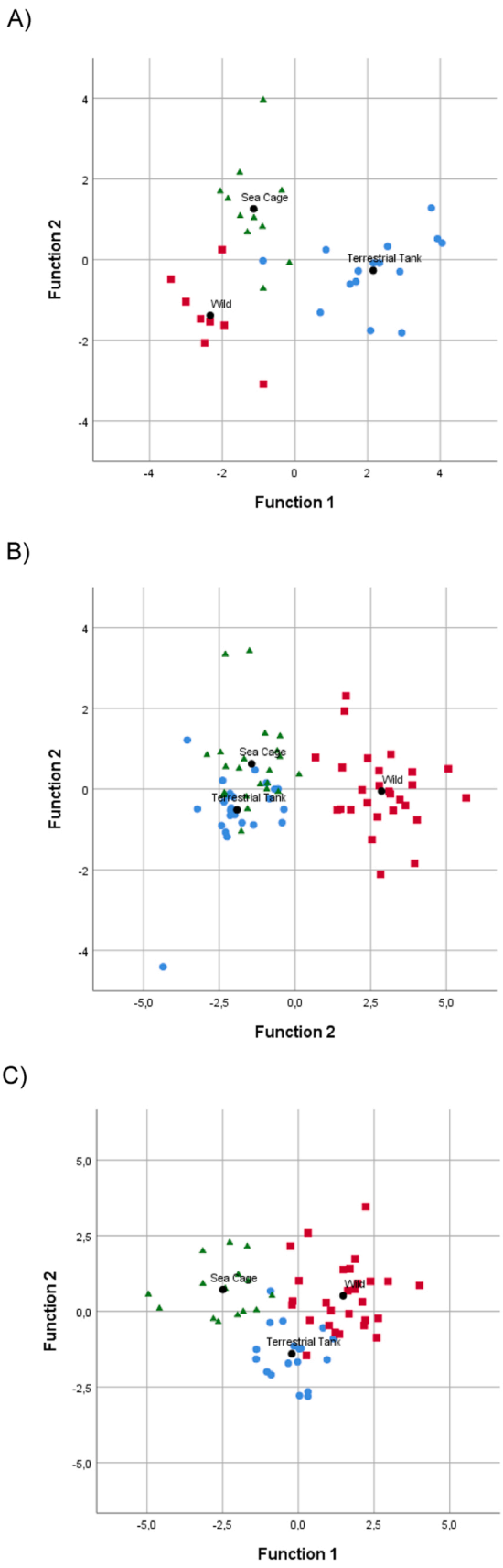

Fig. 3. Batch spatial distribution based in functions outcoming from the DCA analysis and group separation by tissue. A= kidney; B= muscle; C= brain. The small point in the middle shows the group centroid. fx1 Batch 1; fx2 Batch 2; fx3 Batch 3.

functions, growth and fish development. Myoglobin (an abundant protein in muscle) is one of the most important compounds containing Fe (Lall and Kaushik, 2021). Percin et al. (2011) state that Mn and Zn (also included in functions of DCA) are useful indicators of the origin of tuna due to their intensive feeding regimes and transport, and to their migration routes and alimentation, respectively. Thus, the control of these two elements in tuna diets could help improve this information and identify the batches and thus the origin of specimens. However, a disadvantage of the use of this tissue is the confusion between batch 1 and 2 specimens (Table 3), probably due to their similar diet of small frozen pelagic species (De la Gándara et al., 2010).

Finally, for brain (a small organ in tunas), there was a high percentage of discrimination (82.2 %), which thus allows us to identify specimens from the batch 2 (sea cage tunas, 93.3 % of success), and a low percentage of uncertainty between fish of batches 1 and 2 (Table 3). However, the inherent difficulties involved in obtaining this tissue and the possibilities of confusion between reared and wild tuna make this tissue of little use for analysing the batch of specimens.

5. Conclusion

The essential elemental composition in soft tissues in ABFT could be used to discriminate different tuna batches. For some elements, ANOVA tests using results for all tissues reveal differences between batches of fish, while a PCA can differentiate groups of specimens using tissue from kidney and muscle. A DCA can generate formulas for identifying the possible batch of specimens. Muscle appears to be the best tissue to be used with this tool and wild specimens can be readily identified (PCA and DCA), although in reared specimens the differences between fish are more complicated. In muscle, the elements selected for analysis are the essential, present in high enough concentrations to guarantee good analytical results. Future research into the elemental composition of tuna diet and different origins of fishes could provide fresh data that can be used to identify juvenile tunas.

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Declaration of Competing 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 data that has been used is confidential.

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