Elemental composition in feathers of a migratory passerine for differentiation of sex, age and molting areas

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Abstract

The bulk analysis of single feathers of 263 feathers belonging to 238 individuals of a migratory passerine (collared flycatcher, *Ficedula albicollis,* originating from a breeding population in the Pilis-Visegrád Mountains in Hungary) by inductively coupled plasma sector field mass spectrometry (ICP-SF-MS) for determination of elements after proper dissolution allowed the quantitative determination of 38 elements. Calcium, Mg, Mn, Fe and Zn were found to have a quantitative determination frequency larger than 80% and a concentration greater than 100 μg/g. Among ecotoxicologically relevant elements, Ni, Cd, Hg and Pb could be determined in more than 55% of the tail feather samples. The concentration of Hg with a quantification limit of 0.006 μg/g and Pb with that of 0.015 μg/g, was higher than 1 μg/g and 10 μg/g, respectively, in more than 80% of the investigated samples, but generally lower than levels that could cause adverse behavioral effects. The principal component analyses of elemental concentration data followed by the application of general linear models revealed that, for male collared flycatchers, the concentration of Sn, Pb, Ni, Sr, Mg, Zn, Ba and Sc differed significantly in the wing and tail feathers collected from the same individuals. With females, only the Ca and Sc concentration showed a significant difference between wing and tail feathers. Moreover, the concentration of rare earth elements, V, Fe, Sr, Mg, Mn, Zn, Pb and Ba in tail feathers allowed differentiation between sexes, while the concentration of Se, Bi and Sc, between yearling and adult male individuals. At the same time, Sc differentiated age categories in females. Distribution of major elements along the rachis of feathers could be monitored by laser ablation ICP-SF-MS after normalization of the intensities to either ^{13}C or ^{34}S signals.

Keywords: breeding, elemental distribution, habitat, inductively coupled plasma mass spectrometry, laser ablation, migratory birds, rare earth elements.

Highlights

Among the 38 elements investigated, toxic elements (i.e. Ni, Cd, Hg and Pb) occurred in more than the 55% of the tail feather of the collared flycatchers at the μg/g level.

Differentiation between sex, age and molting areas of the collared flycatcher was possible by determination of feather elemental composition.

Time-resolved distribution of major elements along the rachis of feathers by laser ablation ICP-SF-MS and normalization of the intensities to $^{13}C/^{34}S$ signals.

1. Introduction

Tracking dispersal and migratory movements of birds is a challenge, since it influences population dynamics and may affect evolutionary patterns (Nathan 2001; Akesson 2002; Webster et al. 2002). The relationship between breeding, molting and overwintering locations of birds affects their life history. From the study of migratory connectivity, information can be obtained on breeding strategies and arrival dates of males / females to breeding grounds (Webster et al. 2002; Martin et al. 2007).Moreover, preservation strategies can be developed to protect declining populations (Webster et al. 2002; Martin et al. 2007).

Conventional procedures for tracking migratory birds (e.g. ringing) often bring poor results due to low recovery rates and insufficient data for identifying wintering areas of specific populations (Cochran and Wikelski 2005). The satellite tracking methods are efficient to reveal the migratory connectivity of birds (Ouwehand et al. 2016), however, they are expensive and not suitable for small passerines yet (González-Solís et al. 2007; Semmens et al. 2007). Therefore, there has been a great interest in alternative methods that rely on the genetic and/or biogeochemical composition of individuals (Rubenstein and Hobson 2004). Biogeochemical markers such as stable isotopes (^{2}H, ^{13}C, ^{15}N, ^{34}S) (Caccamise et al. 2000; Ofukany et al. 2012) and trace elements derived from the environment are incorporated into animal tissues through water and food chain. The prerequisites for the use of biogeochemical markers for monitoring purposes are as follows: i) the synthesis of animal tissues to be monitored should occur at the particular location of interest; ii) the concentration of an element once incorporated into a certain tissue cannot be altered (i.e. by environmental or physiological effects); and, iii) the elemental abundances should show geographic patterns in the environment (Poesel et al. 2008). Recent studies using biogeochemical markers attempted to link wintering and breeding populations of different bird species (Wassenaar and Hobson

2001). However, it should be noted that interpreting concentrations and origins of (in)organic contaminants in tissues of migratory birds is a real challenge, since exposure to toxic elements may occur also during migration (Dietz et al. 2009; Hargreaves et al. 2010). Determination of the elemental composition of feathers may be a promising tool not only for ecotoxicological purposes, but also for tracking avian migration. The use of feathers for monitoring has the advantage of being a non-invasive sampling technique (Kim and Oh 2014; Abdullah et al. 2015) that can be easily repeated (Adout et al. 2007). During their period of growth, feathers are connected to blood-vessels, and the metals supplied by food may be built into the feather keratin. Once developed, feathers become metabolically inert; therefore, they reflect the composition of regional food (Szép et al. 2003; Donovan et al. 2006; Norris et al. 2007). However, several artifacts can be created by ignoring the physiology of feathers, for example, some elements enter the developing cells in proportion to their abundance in the bloodstream (Bortolotti 2010). The elemental profile of a feather may reveal information on the geographic origin of a bird provided that there are elemental differences between molting areas. Factors such as age, sex, species, metabolism and molting locations were demonstrated to influence the trace element composition in feathers (Bortolotti and Barlow 1988; Bortolotti et al. 1990; Boncompagni et al. 2003; Szép et al. 2003; Donovan et al. 2006). The stable isotope composition mainly differs on continental or regional habitat scale (Hobson 2005). Birds excrete considerable amounts of metals such as Cd, Cr, Cu, and Cu through feather molt (Malik and Zeb 2009; Zamani-Ahmadmahmoodi et al. 2010). It is supposed that differences in Hg concentration between sexes are mainly due to egg-laying decontamination in females although Hg is deposited in the feathers of both sexes (Ramos et al. 2009).

Several analytical techniques have been used to determine elemental concentrations in feathers such as flame, graphite furnace atomic absorption spectrometry (Markowski et al. 2013), cold vapor atomic absorption spectrometry (CV-AAS) for Hg (Carvalho et al. 2013;

Carravieri et al. 2014; Mashroofeh et al. 2015), inductively coupled plasma optical emission (Szép et al. 2003; Donovan et al. 2006; Szép et al. 2009) or inductively coupled plasma-mass spectrometry (ICP-MS) (Adout et al. 2007; Norris et al. 2007; Rubio et al. 2016) and instrumental neutron activation analysis (Haskins et al. 2011). All these techniques require sample digestion or dissolution prior to the analysis. The use of micro-destructive techniques such as laser ablation (LA)-ICP-MS may supply information from a single feather (Jensen et al. 2002; Ek et al. 2004; Kaimal et al. 2009). Using synchrotron radiation analysis, several elements can be detected (e.g. Ca, Fe, Sr, Zn) that are integral parts of the feather structure. Moreover, some metals (e.g. Fe, Zn) are efficiently sequestered by melanins (Martin et al. 2013), while others (e.g. Pb) are distributed across both the melanized and non-melanized portions of the feather.

The use of trace elements to establish migratory connectivity of birds is limited to a few studies (Parrish et al. 1983; Szép et al. 2003; Donovan et al. 2006; Norris et al. 2007). As a model for our study, we have chosen the collared flycatcher (*Ficedula albicollis*) (Figure 1S, online resources), a long-distance migratory, hole-nesting, insectivorous passerine that breeds mainly in deciduous woodlands of Central Europe and it winters in sub-Saharan Africa (Cramp and Perrins 1993, Hölzinger 1993; Adamík et al. 2016). This species is ideal for long-term ecological studies, as its individuals can easily be captured in artificial nestboxes, and they have a high breeding-site fidelity (Hegyi et al. 2013). Therefore, the main objective of the present study was to investigate the suitability of elemental determination in feathers of collared flycatchers to distinguish sexes, age categories as well as breeding and wintering habitats as an alternative to elemental isotope determination. Additionally, the study of the distribution of elements along the rachis was also aimed to follow the variability of the incorporation of chemical elements at a finer time-resolved scale, compared to that conferred by analysis of whole feathers after proper dissolution.

2. Materials and methods

2.1. Sample origin

The field work was carried out in a Hungarian breeding population of the collared flycatcher in the Pilis-Visegrád Mountains close to Budapest, Hungary (47°43′N, 19°01′E), where about 800 artificial nestboxes had been set up in the 1980s to allow a long-term field study of hole-nesting passerines. The study plot is part of a continuous, unmanaged, sessile oak (*Quercus petraea*) dominated woodland, a protected area of Duna-Ipoly National Park. The woodland has clayed brown forest soil on volcanic stones (pH = 5–6). The study area of the nestbox plot is of approximately 72 ha. As flycatchers prefer artificial nestboxes to natural cavities, and density of nestboxes was relatively high (> 8.1 nestboxes/ha), while dead trees were very scarce in the study area, we assumed that the number of collared flycatcher pairs breeding in natural holes in the area could be very low. The average breeding density in the study area, calculated from breeding densities between 1982 and 1991, was 5.2 pairs/ha.

After arriving at the breeding sites, males immediately occupy nestboxes and establish small territories around the boxes where they start singing and displaying. Adult males arrive in the first half of April, while females arrive approximately one week later and settle with a chosen male. Females build nests alone and lay as well as incubate eggs (clutch size varies between 4 and 8 eggs). Both sexes provide parental care at the nestling stage. Nestlings start to fledge 14 days after hatching. After fledging, birds start to prepare for migration, and leave for the sub-Saharan wintering site in early autumn.

The age of individuals was determined by ringing data or plumage color (Svensson 1992). The nuptial plumage of collared flycatcher males is black and white with a prominent

white collar, a forehead patch, and wing patches. The growth rate of feathers is about 2.5 mm/day (Hargitai et al. 2012). Collared flycatchers molt tertials, body feathers and tail feathers in their winter quarters (Svensson 1992). This means that tail feathers plucked in the breeding habitat had been grown in Africa. Feather samples were collected in the breeding seasons of 2008 to 2010.

We collected two tail feathers (second outermost on left and right sides) from 238 individuals (n = 42/18 and n = 76/102 yearling and adult females/males, respectively) at the end of the nestling feeding period (May-June) (Table 1). Moreover, 25 of these individuals (n = 2/2 and n = 9/12 yearling and adult females/males, respectively) were also sampled with regards to wing feathers (left and right tenth primaries) (Table 1). For this study, we used only the right side feathers. For LA studies, in total, 12 tail and wing feathers (six of each type), originating from six additional individuals were separately used.

2.2. Reagents

Throughout the investigation, deionized water with resistivity of 17 MΩ × cm produced by PUR1TE Still Plus (Thame, Oxfordshire, UK) was used. Laboratory grade Triton X-114 and acetone were purchased from Sigma Aldrich (St. Louis, USA). Suprapure quality of 65% and 30% by weight of nitric acid (HNO_3) and hydrogen peroxide (H_2O_2), respectively, were supplied by Merck (Darmstadt, Germany). For calibration, ICP Multielement standard solution VI as well as 1 g/L Hg, Sb, Sn and Pt individual standard solutions - all purchased from Merck - were used. For the determination of rare earth elements (REEs), AccuTrace™ Reference Standard Calibration Standard 1 solution containing REEs in a concentration of 10 mg/L each and purchased from AccuStandard Inc. (New Haven, USA) was used. Indium (In) ICP standard in a concentration of 1 mg/L was used as internal standard for all calibration as

well as sample solutions. Working solutions were prepared daily by appropriate dilution of the aforementioned stock solutions.

2.3. Sample preparation

In order to remove suet layer and contaminants from the surface of feather, different washing procedures commonly found in the literature were applied (i.e. water, acetone, Triton X-114 as well a combination of these latter two) In all cases, samples were sonicated for 20 min. The washed samples either underwent microwave-assisted (MW)-digestion or were used for LA-ICP-MS analysis without any further sample treatment. However, each feather had to be cut into three parts with a ceramic scalpel in order to fit into the LA chamber.

For bulk analysis, feather samples were exsiccated after washing with Triton X-114. Then, they were weighed and placed into quartz vessels and 5 mL of cc. HNO_3 solution was added to each. After two hour-stay at room temperature, 2 mL of H_2O_2 solution was added to the samples and left for additional two hours in a fume cupboard. After this step, samples were subjected to MW-digestion for 20 min with a nominal power value of 600 W / seven vessels (from which, six samples and one blank). The maximum allowable temperature was limited to 160 °C. After cooling, the digested solutions were transferred to 15-mL polypropylene test tubes. The residual total organic carbon in the samples was less than 0.5%.

2.4. Instrumentation

Weighing of feathers was done by using an AB 135 S/Fact balance supplied by Mettler Toledo Ltd. (Budapest, Hungary) with a readability of 0.01 mg. The effectiveness of the washing procedures of feathers was checked by using an Amray 1830I/T6 scanning

electron microscope (SEMTech Solutions Inc., North Billerica, MA, USA). Microwave-assisted digestion was performed in temperature controlled Ethos 1 system (Milestone, Sorisole, Italy) equipped with seven PTFE vessels. Complete digestion was achieved by placing 20-mL quartz inserts into each vessel. For sonication, an Elmasonic S40 ultrasonic bath (Elma GmbH, Singen, Germany) was used. Total organic carbon was determined by C/N Analyzer Type 2100 S of Analytik Jena, Germany.

Bulk analysis was performed on an Element 2 sector field (SF) ICP-MS (hereafter ICP-SF-MS) instrument (ThermoFisher, USA) with low and medium resolutions of 300 and 4000, respectively. Instrument tuning with a multielemental standard solution of 1 μg/L for each element was performed on a daily basis before analyzing the samples.For LA, a UP 213 instrument (New Wave Research Inc., Fremont, CA, USA) equipped with a Nd:YAG laser source of 1064 nm attenuated to 213 nm was used. The LA unit was hyphenated to the ICP-SF-MS instrument with polyethylene tubes and tuning was performed before analyzing the samples. The performance of LA-ICP-SF-MS was tested using National Institute of Standards and Technology glass reference material SRM 612 by its continuous ablation to provide maximum sensitivity for ^{208}Pb and maintaining low oxide formation CeO/Ce. The operating conditions of the (LA)-ICP-SF-MS instrument have been compiled in Table 1S (online resources). Since the magnetic analyzer of the sector field arrangement changes slowly between different m/z ranges, intensities of the monitored isotopes were recorded sequentially by applying lateral sweeping. Background intensities were monitored before and after each measurement to correct memory effects caused by ablation.

2.5. Statistical methods

Differences in toxic element concentrations of tail feathers as a function of sex were investigated by Mann–Whitney U tests. Pearson's correlation was applied for intensity data originating from the LA spots on the samples (n = 250) by LA-ICP-SF-MS as well as for Hg and Se concentration bulk analysis data.

To reduce dimensions and create orthogonal variables from the 38 elements, we conducted two separate principal component analyses (PCAs) on element concentrations.

At first, principal components (PCs) were prepared on pooled elemental concentration data for wing and tail feathers originating from individuals (n = 25) that provided both wing and feather tails to the present study. Only those elements were included in the PCA that could be quantified in both feather types in at least 50% (i.e. quantification frequency, QF > 50%). Thus, finally, concentration data of 21 elements (Ba, Ca, Ce, Cu, Fe, Hg, La, Mg, Nd, Ni, Pb, Pr, Rb, Sc, Sr, Sm, Sn, Th, V, Y and Zn) were used.

In the second PCA, we used the elemental concentrations of only tail feathers collected from 238 individuals. Applying the same criteria for data inclusion, besides the aforementioned elements, concentration data of Bi, Co, Mn, Sb and Se were also used. Furthermore, wing-tale and tail PC axes (hereafter wing-tail PCs and tail PCs, respectively) were rotated by applying varimax raw algorithm to better explain variances. According to the Kaiser's criterion, only those PCs were used for the subsequent analyses the eigenvalues of which were greater than one.

General linear models (GLMs) with backward stepwise model selection were applied to investigate the relationship between elemental concentrations and feather type, sex, and age. For these analyses, two age categories were used: one-year old breeders (hereafter, yearlings) and two-year old or older ones (hereafter, adult). Using GLMs, the effects of predictor variables are controlled on each other. In the case of wing-tail PCs, one PC was the dependent variable, and feather type and sex were used as categorical predictors.

Additionally, these models contained the two-way interaction of the categorical predictors (i.e. 'feather type × sex'). We did not include age as categorical predictor into these analyses because of the low sample size of yearlings. When tail PCs were analyzed in relation to sex and age, one PC was used as dependent variable, and sex and age as categorical predictors. Furthermore, the 'sex × age' interaction was also included in the models.

The frequency distributions of the continuous variables were tested for normality. The PCs were 1/square-root transformed if it was necessary to fit into normal distribution. An alpha level of 0.05 was used. All statistical methods were performed in Statistica software package (V 10.0).

3. Results and discussion

3.1. Optimization of washing procedure for feathers

Several commonly used washing procedures were tested for the removal of suet from the surface of feather, namely i) ultrapure water; ii) acetone as well their combination (Vallner et al. 2009; Carvalho et al. 2013); iii) Triton X-114 and iv) mixture of acetone and Triton X-114. Efficacy of suet removal was checked by SEM and back scattered electron (BSE) imaging. Washing with water was not effective at all. Treatment with acetone removed suet but several particles with high atomic numbers (practically, dust) could not be removed from the surface of feather. Washing with Triton X-114 *cf.* Adout et al. (2007) proved to be the most effective treatment according to the recorded BSE images (Figure 2S a & b, online resources) and further washing with acetone did not increase the efficiency of cleanliness.

3.2. Elemental characterization of feather samples by bulk analysis

In total, 38 elements could be quantified in the feathers of collared flycatchers. This analytical performance is similar to that of Poesel et al. (2008), who reported on the determination of 34 elements in the feathers of white-crowned sparrows (*Zonotrichia leucophrys pugetensis*) by ICP-MS and wet chemical analysis. Quantification limit values for each studied element can be seen in Table 2S (online resources). The relative standard deviation (RSD) of the concentration data for major elements was between 1% and 3%, while for minor elements, RSD could achieve even 20%. In the lack of a feather standard reference material, quality assurance followed standard methods using BCR 414 plankton certified reference material (Quevauviller et al. 1993). To achieve the method validation in a range comparable to the dry weight of the feather samples, 10 mg of reference material BCR 414 was subjected to the preparation procedure described above. The recovery rates for the certified elements were between 80% and 110% of the certified values for BCR 414 plankton.

Box-and-whisker plots with median and quartiles for elemental concentration in tail and wing feathers of male and female collared flycatcher including their quantification frequencies (QF) for the present study can be seen in Figures 3S-10S (online resources). The most abundant elements with a quantification capability larger than 80% and relatively large concentration (> 100 μg/g) were Ca, Fe, Mg, Mn and Zn (Figure 3S-5S, online resources). Comparing our results to elemental concentration in plumage of a hirundine species, sand martin (*Riparia riparia*) breeding in Eastern Hungary, Ca, Fe, Mg, and Mn could also be determined in concentrations larger than 100 μg/g (Vallner et al. 2000; Szép et al. 2003). Also comparing at the regional scale, the elemental levels of the present study are in good agreement with those reported by Veerle et al. (2004) for feathers of free-living great tits (*Parus major*). Although QF of Ba and Cu was remarkably high (> 75%) in the present study, their typical concentration values hardly exceeded 10 μg/g. In the case of these major

elements in the collared flycatcher, their concentration in tail feathers (assuming to be grown in the wintering habitat according to Svensson 1992) were greater than in the wing ones (grown in the breeding habitat) roughly by factor 2 (Figures 3S-5S, online resources). The QF of REEs was also remarkably high (cca. 90%) with typical concentration values of about 1 μg/g.

Among toxic elements included in the priority list of contaminants of 2013/39/EU directive for surface water, Cd, Hg, Ni and Pb could be determined in more than 55% in the investigated tail samples (Figure 1). Concentrations of these ecotoxicologically relevant elements did not significantly differ in tail feathers of female and male individuals (all $P>0.07$). Results of the present study are in good agreement with literature data recently tabulated by Abdullah et al. (2015) for As, Cd, Cu, Cr, Fe, Li, Mn, Ni, Pb and Zn. Moreover our results also fit in the reference ranges for Cd, Cu, Cr, Hg, Ni, Pb and Zn and Zn in feathers of song sparrows (*Melospiza melodia*) by Lester and van Riper III (2014). For Hg, comprehensive literature data for birds breeding in subantarctic, subtropical and tropical regions have been just recently compiled by Carravieri et al. (2014). Ofukany et al. (2012) found that feathers of double-crested cormorants (*Phalacrocorax auritus*) grown on Lake Winnipeg had greater Hg concentrations (mean = 4.26 ± 1.47 μg/g fresh weight; $n = 20$) than winter-grown feathers (3.19 ± 1.64 μg/g; $n = 19$).

As for other examples, the 90th percentile for Cd, Hg, Pb, Cu and Zn concentration in the little egret (*Egretta garzetta*) breeding in Spain was 0.02 μg/g, 1.63 μg/g, 0.4 μg/g, 26.9 μg/g and 122 μg/g, respectively (Rubio et al. 2016) These values are also in good agreement with data reported here (Figure 1 and Figure 11S as well as Figures 3S-5S of the online resources). Surprisingly, the concentration data of toxic metals included in the priority list of contaminants in surface water by the EU were not reported in tail feathers of sand martin population breeding in Hungary (Szép et al. 2003). The mean Pb base concentration level in a

similar passerine (blue tit, *Cyanistes caeruleus*) feathers sampled in Poland was about 4 μg/g (Markowski et al. 2013). Similar Ni content was determined in feather samples taken from black-tailed godwit (*Limosa limosa*) breeding in the Netherlands (Roodbergen et al. 2008). Authors concluded that Cd, Hg and Pb were transferred from the soil to godwits even though this species spends only a few months in the breeding area during the year (Roodbergen et al. 2008). In conclusion, in spite of the fact that some of literature data refer to fish eating birds, similar concentrations were obtained for insectivorous passerines such as the collared flycatcher.

Mercury levels in feathers that are associated with adverse reproductive effects in birds are 5 μg/g (Burger et al. 2015). The median Hg levels in the feathers of collared flycatcher individuals in this study were 0.4 μg/g – 0.5 μg/g for the time period between 2008 and 2010. Adverse effects in birds occur at Pb levels of 4 μg/g in feathers (Burger et al. 2015). In the present study, median Pb levels were about 1.5 μg/g. The feather Cd levels that can cause adverse behavioral effects range from 0.1 μg/g (shearwaters) to 2 μg/g in terns (Burger 1993). In our study, the median Cd concentrations were about 0.15 μg/g. Nickel content in birds can reach 5 μg/g (Eisler 1981). In our study, the median value for Ni in feather was about 2 μg/g - 2.5 μg/g (Figure 1). Nickel concentrations in avian tissues in excess of 10 μg/g dry weight (DW) kidney or 3 μg/g DW liver are sometimes associated with adverse effects (Eisler 1981). Excess Ni is reported to affect feather molting (Malik and Zeb 2009).

3.3. Differentiation between sex, age and feather types

For wing-tail PCA, data were compressed into five PCs (wing-tail PC1-PC5) that explained 87.36% of total variance among individuals (Table 2). The PCA of elemental

concentration data in tail feather resulted in six PCs (tail PC1-PC6) explaining 74.38% of total variance (Table 2). All of the strong loadings (>0.5) showed positive direction that conveys a very clear information about the raw data corresponding to element concentrations of the feather samples (Table 2). Wing-tail PC1 strongly associated with concentrations of Ce, Fe, La, Nd, Pr, Sm, Th, V and Y. In wing-tail PC2, Sn, Pb and Ni had higher positive factor loadings, while in PC3, Ba, Mg, Zn and Sr (Table 2), whereas in PC4, Ca and Sc showed strong loadings. Because these four PCs were 1/square-root transformed before the analyses, higher transformed PC scores refer to lower element concentrations. The higher wing-tail PC5 (there was no transformation) score reflected larger Hg concentrations. Based on the loadings, tail PC1 represented Ce, Fe, La, Nd, Pr, Sm, Th and Y. The PC2 was related to Pb, while PC3 was related to Ba, Mg, Mn, Sr and Zn. The PC4 was associated with Ca, Cu, Ni, and Rb, whereas PC5 with Bi and Se. At last, PC6 could be associated only with Sc. Tail PC1-PC5 were 1/square-root transformed before the analyses, thus higher transformed PC score refers to lower element concentrations, while tail PC6 was not transformed, so higher PC6 score reflects higher Sc concentration. Despite that partly different elements were included into the two PCAs and different sample sizes were included into the two PCAs, the patterns of the parallel variations of element concentrations were highly similar in the two cases suggesting that these results are robust.

None of the variance homogeneities of the standardized wing-tale PCs differed between groups (Levene's tests, all P>0.17). We found that wing feathers had significantly lower values for PC2 ($t=-2.27$, $df=1,48$, $P=0.03$) and PC4 ($t=-3.31$, $df=1,48$, $P=0.002$), but higher scores for PC3 ($t=3.47$, $df=1,48$, $P=0.001$).

In the case of wing-tail PCs, we revealed that PC1 was significantly related to sex ($F=16.28$, $df=1,48$, $P=0.0002$), because males had higher PC1 scores. Although PC2 could be significantly associated with feather type ($F=5.07$, $df=1,48$, $P=0.03$), the 'feather type × sex'

interaction showed a marginally significant effect (F=3.66, df=1,47, P=0.061) on PC2, thus, we investigated the feather type differences separately for females and males. We found that PC2 differed between feather types only in males (F=10.88, df=1,26, P=0.003) (Figure 2a), namely wing feathers had lower PC2 scores. In the model of PC3, we found significant effect of 'feather type × sex' interaction on the dependent variable (F=7.45, df=1,46, P=0.009), and the main effects were also significant (feather type: F=13.18, df=1,46, P=0.0007; sex: F=9.40, df =1,46, P=0.004). Because of this interaction, we also conducted separate GLMs for sexes for this case. The PC3 differed between feather types only in males (F=24.77, df=1,26, P=0.00004) having higher scores for wing feathers (Figure 2b). The PC4 varied with feather type regardless of sex (F=10.21, df=1,48, P=0.002) (Figure 2c). All of the other relationships were non-significant (all P>0.09). When the aforementioned GLMs were run excluding the samples of the four yearling individuals, we got almost identical results (details not shown here).

In conclusion, concentration of Ba, Ca, Mg, Ni, Pb, Sc, Sn, Sr and Zn differed significantly in the wing and tail feathers of male collared flycatchers. In females, only Ca and Sc concentration differed between wing and tail feathers.

The first three tail PCs differed between sexes: PC1 was higher in males (F=11.52, df=1,236, P=0.0008) (Figure 3a); PC2 was also higher in males: (F=6.97, df=1,236, P=0.009) (Figure 3b), while PC3 was higher in females (F=158.26, df=1,236, P<0.0000) (Figure 3c). However for PC5, the main age effect was significant (F=4.88, df=1,236, P=0.03), but the 'sex × age' interaction also had a marginally significant, weaker effect (F=2.93, df=1,234, P=0.088). According to this, we prepared additional GLMs separated for sexes, and we revealed age differences only in males (F=7.27, df=1,118, P=0.008), as adult males had higher PC5 scores. Adult birds had higher PC6 values (F=4.20, df=1,236, P=0.04) (Figure 3d). The other relationships were non-significant (all P>0.11).

Thus, it can be concluded that concentration of REEs, Ba, Fe, Mg, Mn, Pb, Sr, Th, V and Zn in tail feathers enabled differentiation between sexes. Concentrations of Bi, Sc and Se were significantly different for yearlings and adults in males, while only Sc discriminated yearlings and adults in females. Assuming that wing and tail feathers were primarily grown in the breeding and wintering habitats, respectively, with different geochemical background, differences in the elemental profile of feathers can be understood. The collared flycatcher is assumed to overwinter in savannah zones of tropical Africa (Cramp and Perrins 1993; Hölzinger 1993). According to observations based on ringing data made by Hölzinger (1993), its wintering habitat is clustered in the region of Eastern-Central Africa near the Equator, i.e. Uganda, Tanzania, Burundi, Rwanda, and east DR Congo (Hölzinger 1993). Recently, Adamík et al. (2016) has reported on the prevalence of the Congo basin as wintering habitat of the collared flycatcher by using light-level geolocators. Interestingly, dissolution of trace elements (e.g. Sc, REEs) into the Congo River and its main tributaries (Dupré et al. 1996) as well as their accumulation in clay-rich sediments in Uganda (Nyakairu and Koeberl 2001) have been reported. On a world scale, the concentrations of trace elements in the Congo River are among the highest measured (Dupré et al. 1996).

The recommendation of Donovan et al. (2006) to focus such type of research on a single species across relatively smaller geographic extents (e.g. breeding location of small size) was taken into consideration by designing our experimental set-up. At the regional scale, Szép et al. (2003) found large differences in the composition of feathers of sand martin grown in Africa versus Europe presumably due to the differences in the elemental profile of food sources.

For the successful application of the elemental profiles in feathers for discrimination between geographical locations, in the present study, it was taken into account that feather chemistry varies among age and sex classes (Bortolotti et al. 1989). It was also suggested that

variations in chemical composition of back and tail feathers in bald eagles (*Haliaeetus leucocephalus*) attributable to sex have a physiological basis, while age variation could be explained by diet (Bortolotti and Barlow 1988). However, arctic shorebird species differed in almost every element detected (As, Be, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Se, Tl, V, and Zn) in feather samples, but there were no differences between males and females in these shorebirds (Hargreaves et al. 2010).

Element signatures (e.g. Ce, Cu, Mg, Na, Nd, P, Rb, Sn and Th) allowed discrimination between sites in the US (even more than 1,200 km apart) within species such as Eastern bluebird (*Sialia sialis*), ovenbird (*Seiurus aurocapilla*), tree swallow (*Tachycineta bicolor*) and wood thrush (*Hylocichla mustelina*) (Donovan et al. 2006). Poesel et al. (2008) found differences in trace elemental profiles (Ga, I, Mg, Nd, Sr, Y and Zn) even between closely spaced (as close as 40 km apart) populations in the white-crowned sparrow. This result could be explained by diverse fine-scale environmental heterogeneity caused by the effects of microclimatic conditions and local human activity (Poesel et al. 2008). Moreover, seven species within the same site could be discriminated by feather element concentrations (e.g. Ca, Cd, Cr, Fe, Mg, Na, P, S and Sn) (Donovan et al. 2006). Similarly, elemental profile (such as Al, Cd, Cr, Cu, Fe, Mn, Ni, Pb and Se) in penguin species could be used for defining different populations within or between species at a given site (Jerez et al. 2011).

The importance of exogenous contamination on heavy and toxic metal levels was also demonstrated by elemental analysis in great tit tail feathers (Veerle et al. 2004). Although atmospheric deposition might be responsible for increased concentration of Ag, Al, As, Cd, Co, Cu, Fe, Mn, Pb and Tl in feathers, this was not the case for Zn needed for feather formation and Hg. In the latter case, Hg detoxification through its evaporation from the feather in methyl Hg form may be the reason for not being deposited onto the feather surface (Veerle et al. 2004).

In the present study, due to the relatively small size of the breeding habitat, high variations in the diet of the collared flycatcher were not expected compared to the vast sub-Saharan wintering habitat. However, for a better interpretation of the outcomes of PCA-GLM, further studies aiming at determination of elemental profile of soil and food web of the breeding and wintering habitats of the collared flycatcher would be needed.

3.4. Determination of the elemental profile in feather by single sample LA-ICP-MS analysis

For LA-ICP-MS measurements, isotopes were selected upon preliminary results of bulk analysis. The number of isotopes monitored by LA-ICP-MS was influenced by the lower sensitivity of this technique compared to bulk analysis and the slow scanning speed of the SF spectrometer. Thus, the main criteria for selection of isotopes were: i) large concentration according to bulk analysis; ii) high variation in the element concentration; iii) minimal variance of the isotope abundances of the same element.

Feather samples were ablated along the rachis. In the lack of a proper certified reference material, quantitative determination of the elemental distribution in feathers was not possible. Intensities of the monitored elements had to be normalized with the counts of the matrix elements (e.g. ^{13}C for organic matrices). Our experience was that the intensity of ^{13}C was nearly constant only in the lower one third and two thirds of rachis, since in the upper third, wall thickness and density of rachis influenced the counts of the ablated material (Figure 4 a). At the same time, signal intensity of ^{34}S along the rachis was similar to those of ^{13}C, indicating a uniform distribution of S in keratin (Figure 4 b). At the same time, signal intensity of ^{44}Ca increased from the hollow shaft towards the tip of the rachis. Therefore, signal intensities of isotopes recorded in low and medium resolutions were related to the counts of ^{13}C and ^{34}S, respectively. Surprisingly, Kaimal et al. (2009) reported on quantitative

determination of trace elements (relative to SRM 612 made of glass) using ^{42}Ca as the internal standard assuming a homogenous distribution of this element in feathers of mallard (*Anas platyrhynchos*) to estimate the origin of migratory birds despite of the discrepancy in the matrix composition of feathers and glass.

The LA-ICP-SF-MS technique enabled spatial distribution of elements along the rachis. Intensities of the monitored isotopes usually increased along the rachis from the hollow shaft towards the tip indicating the incorporation of these elements through feedstuff in higher amounts with the development of the individual in time. Intensities of some isotopes (e.g. ^{208}Pb and ^{55}Mn) were higher in the tail feathers than in the wing ones (Figure 4 b & c). The scarcity of data dealing with feather analysis by LA-ICP-MS may be due to the difficulties arisen at quantification. Therefore, Ek et al. (2004) could only determine that external metal contamination of feathers prevailed by acquiring Cd, Cu, Pb, Pd, Pt, Rh and Zn profiles along feather rachis of raptor and other bird species by LA-ICP-MS.

Significant correlation could be observed only for alkaline earth metals (Ca, Sr and Ba) intensities along the rachis (Figure 5 a). Moreover, Mn showed good correlation with Sr ($r = 0.78$) but correlation was also significant with Ca ($r = 0.65$) and Ba ($r = 0.52$). This latter correlation indicates that the oxidation state of Mn in rachis is +II as for alkaline earth metals. Intensities of Hg and S correlated at a lesser extent (Figure 5 b). However, Se (0.24 µg/g - 14.18 µg/g) and Hg (0.22 µg/g - 1.44 µg/g) concentrations, determined by (CV-)AAS after proper sample dissolution, showed a positive correlation in growing feathers of spectacled petrels of the genus *Procellaria* (Carvalho et al. 2013). In our study, correlation between Hg and Se concentration levels could be performed only for bulk analysis data. Thus, correlation was observed between Hg and Se concentration data of the present study ($r=0.21$, $P=0.001$, $n=263$).

4. Conclusions

Bulk analysis of individual feathers by inductively coupled plasma sector field mass spectrometry for determination of elements after proper sample preparation and followed by statistical data evaluation of the obtained concentration data sets allowed discrmination between breeding and wintering habitats of a passerine migratory bird species as well as the differentiation between age and sexes. Distribution of major elements in the feathers determined by laser ablation inductively coupled sector field plasma mass spectrometry enabled the assessment of the variability of the incorporation of chemical elements at a time-resolved scale with increased resolution compared to that conferred by the bulk analysis of individual feathers grown for about 2-3 weeks after proper dissolution. Therefore, the high resolution lateral analysis of feathers can be considered as a complimentary technique to the bulk one. The drawback of the former technique is that fewer elements can be monitored. As a future prospect from instrumental point of view, the combination of laser ablation with a more efficient time-of-flight analyzer is aimed. The faster mass sweep would dramatically reduce the acquisition time and the cost of the measurements. Due to the normalization of the intensities to ^{13}C signals, reliable screening of the feathers along the rachis could be achieved.

Nevertheless, an exhaustive geochemical study extended over the food web of the breeding and wintering habitats would be needed for a finer topographic characterization through the determination of elemental composition and distribution in feathers.

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Tables

Table 1.

Sample sizes of feathers collected from collared flycatcher (*Ficedula albicollis*) individuals at the breeding site between 2008 and 2010

Sex	Feather type	Binary age[1]	Feather N°/year			*Sum*	Individual N°
			2008	2009	2010		
male	tail	1	6	8	4	*18*	120[2]
		>1	28	26	48	*102*	
	wing	1	2	0	0	*2*	
		>1	2	10	0	*12*	
Sum	*tail*		*34*	*34*	*52*	*120*	
	wing		*4*	*10*	*0*	*14*	
female	tail	1	19	15	8	*42*	118[2]
		>1	18	25	33	*76*	
	wing	1	0	2	0	*2*	
		>1	0	9	0	*9*	
Sum	*tail*		*37*	*40*	*41*	*118*	
	wing		*0*	*11*	*0*	*11*	
Overall sum						**263**	**238**[2]

[1] yearling (one-year old) or adult (more than one-year old individual);

[2] Tail and wing feathers sampled simultaneously from 14 and 11 male and female individuals, respectively.

Table 2. Loadings of the principal components (PCs) with the raw concentration data for elements in wing and tail feathers of collared flycatcher individuals. In wing-tail PCs, data were pooled for wing and tail feathers. Loading factors larger than 0.5 are in bold. For more details, see section 2.5.

	wing-tail PCs					tail PCs					
Element	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC1**	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**
Ca	0.21	0.07	0.26	**0.81**	-0.27	0.25	0.19	0.29	**0.66**	0.00	0.22
Rb	**0.57**	0.32	0.04	0.12	-0.24	0.46	0.24	0.02	**0.68**	-0.05	0.00
Sr	0.09	0.04	**0.84**	0.22	0.11	0.14	0.17	**0.76**	0.08	-0.03	0.12
Sn	-0.01	**0.94**	0.03	-0.02	0.03	0.11	0.47	-0.04	0.46	0.05	0.03
Sc	0.01	0.03	0.07	**0.92**	0.24	0.10	0.20	0.05	0.09	0.03	**0.88**
Y	**0.97**	0.00	0.08	0.17	-0.02	**0.88**	0.13	0.21	0.17	-0.01	0.00
La	**0.99**	0.01	0.07	0.01	-0.01	**0.97**	0.09	0.11	0.03	-0.08	0.02
Ce	**0.99**	0.00	0.05	-0.04	0.04	**0.97**	0.04	0.05	0.04	-0.03	-0.03
Pr	**0.99**	0.00	0.06	0.01	0.01	**0.97**	0.07	0.10	0.02	-0.04	0.04
Nd	**0.98**	0.08	0.07	0.02	-0.05	**0.95**	0.09	0.08	0.09	-0.05	0.03
Sm	**0.94**	-0.04	0.12	0.20	0.00	**0.93**	0.08	0.15	0.12	-0.01	0.12
Hg	0.20	0.00	0.17	0.10	**0.84**	0.13	0.14	0.08	0.18	0.42	0.44
Pb	0.06	**0.97**	-0.01	0.05	-0.01	0.11	**0.95**	0.10	0.13	-0.03	0.09
Th	**0.93**	-0.02	0.02	0.19	-0.03	**0.83**	0.07	-0.03	0.13	0.11	0.36
Mg	0.10	-0.17	**0.88**	0.05	-0.18	0.08	-0.04	**0.77**	0.38	-0.03	0.09
V	**0.87**	0.16	-0.01	-0.03	0.27	**0.77**	0.14	0.07	0.03	0.20	0.07
Fe	**0.92**	0.22	0.04	-0.23	0.14	**0.80**	0.01	0.00	0.07	-0.09	-0.19
Ni	0.04	**0.95**	0.06	-0.05	-0.02	0.12	0.46	0.01	**0.68**	-0.06	0.03
Cu	0.14	0.37	0.47	0.29	-0.44	0.10	0.40	0.18	**0.64**	-0.09	0.14
Zn	-0.09	0.18	**0.86**	0.16	0.16	0.06	0.27	**0.59**	0.29	0.12	0.48
Ba	0.42	-0.04	**0.73**	-0.16	0.19	0.34	0.24	**0.79**	-0.12	-0.10	-0.03
Se						-0.07	-0.03	0.26	0.04	**0.73**	-0.38
Sb						0.09	0.11	0.05	0.12	0.14	0.15
Bi						-0.04	-0.08	-0.11	-0.06	**0.85**	0.16
Mn						0.22	0.10	**0.73**	-0.03	0.01	-0.17
Co						0.12	0.39	0.36	-0.14	0.08	0.29
Explained variance (%)	38.37	21.65	13.54	8.16	5.64	27.20	13.39	10.84	8.42	8.29	6.24

Figure captions

Figure 1. Box-and-whisker plots with median and quartiles for Ni and Pb (a) as well as for Cd and Hg (b) concentration in tail feathers of male (n = 120) and female (n = 118) collared flycatcher (*Ficedula albicollis*) individuals.

Figure 2. Feather type differences in wing-tail PC scores (mean ± standard error, SE) of the collared flycatcher. PC2 (a) and PC3 (b) differed between feather types only in males, while PC4 (c) differed between wing and tale regardless of sex. All of these PCs were 1/square-root transformed before the analyses. See text for statistical details.

Figure 3. Sex and age differences in tail PC scores (mean ± standard error, SE) of the collared flycatcher. PC1 (a), PC2 (b) and PC3 (c) showed sex differences in tail feathers, while PC6 (d) differed between age categories regardless of sex. All but PC6 were 1/square-root transformed. See text for statistical details.

Figure 4. Signal intensities of ^{13}C (a), ^{34}S (b) along the rachis of tail and wing feathers as well as those of ^{208}Pb (c) and ^{55}Mn (d) normalized to ^{13}C and ^{34}S, respectively.

Figure 5. Correlation of Mn, Sr, Ca and Ba (a); as well as that of Hg and S (b) along the avian rachis

Figures

Figure 1

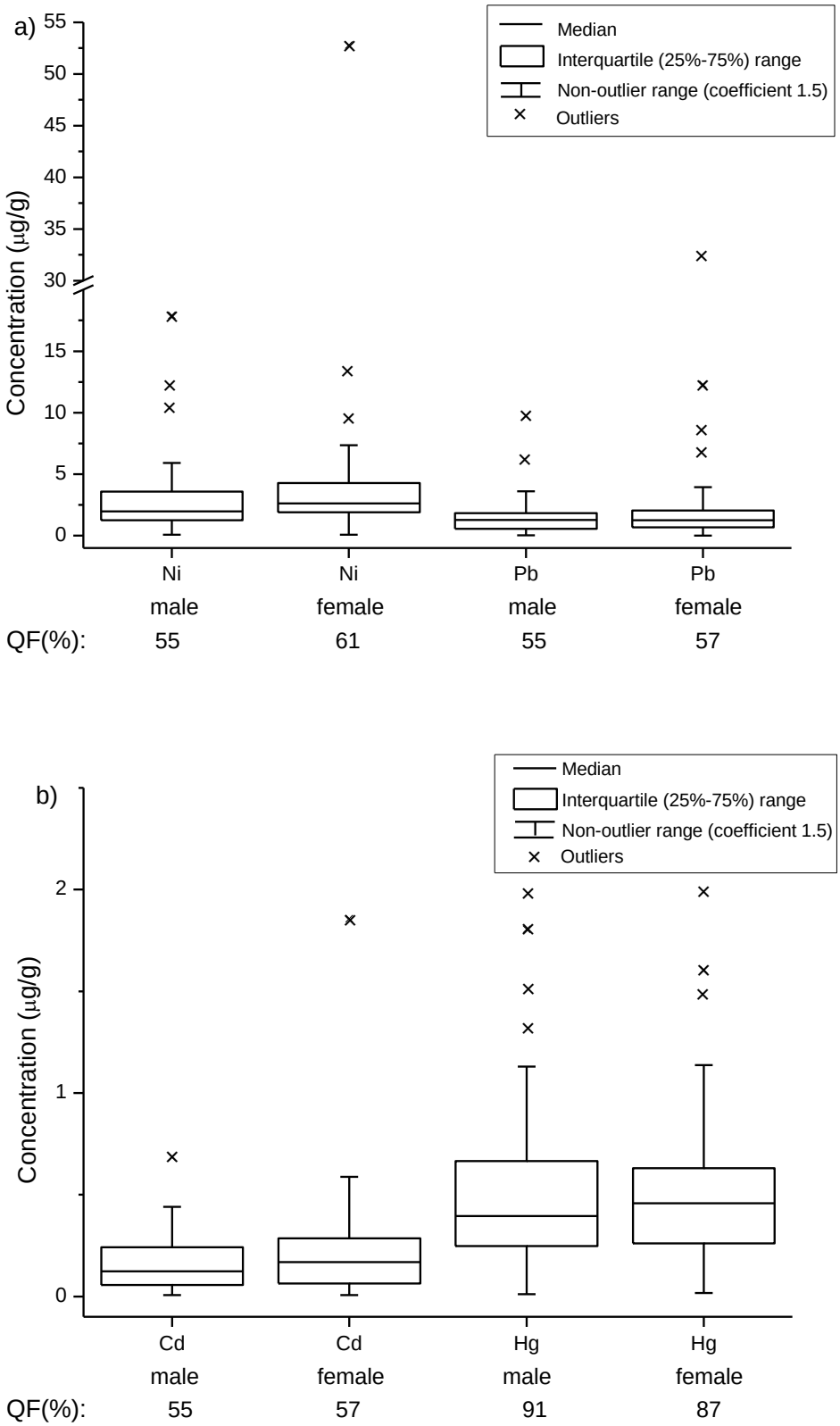


Figure 2

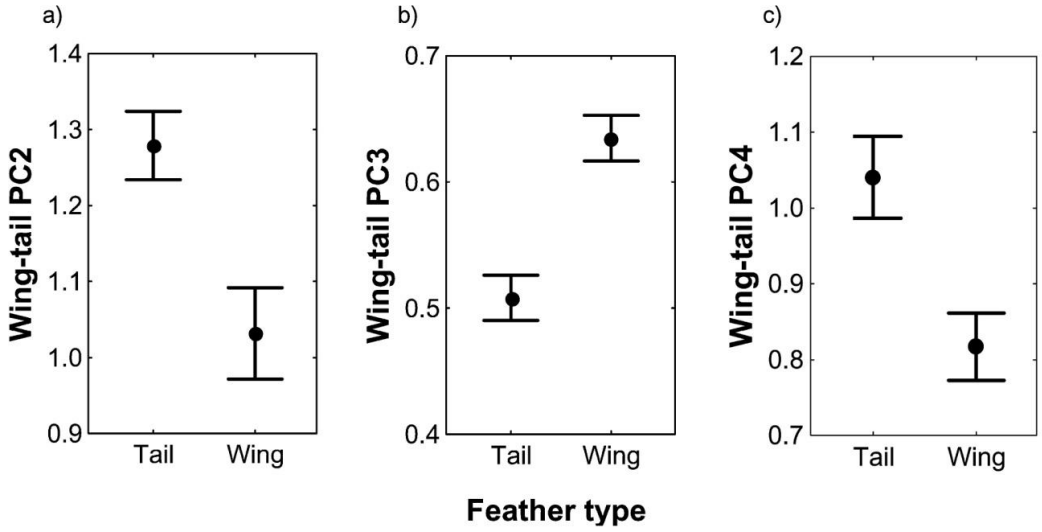


Figure 3

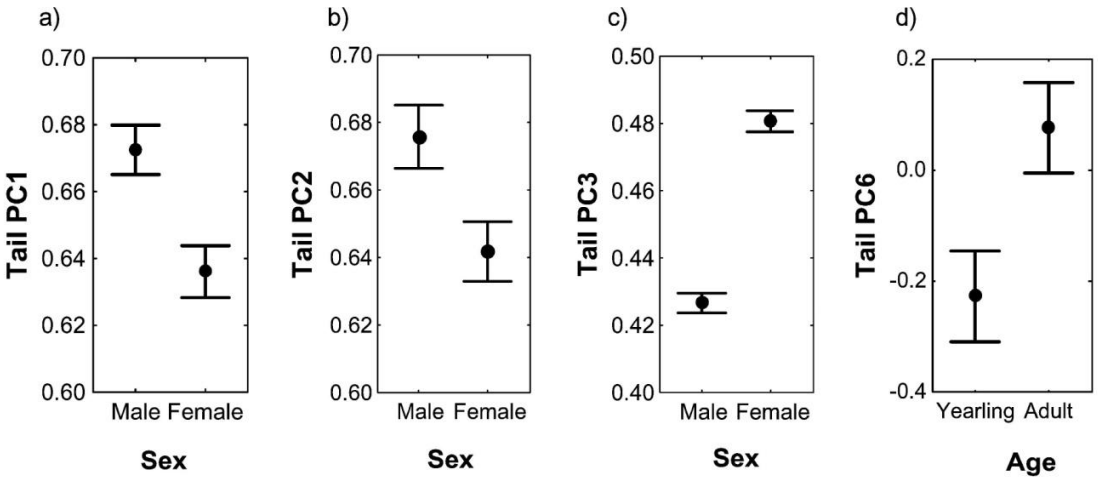
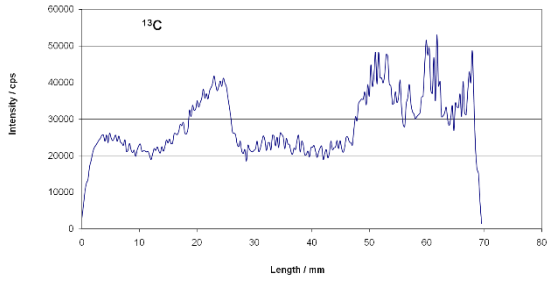


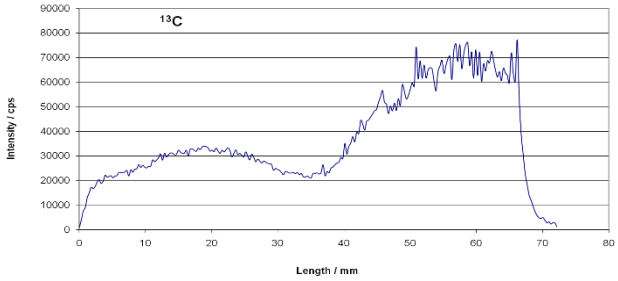
Figure 4

a)

tail

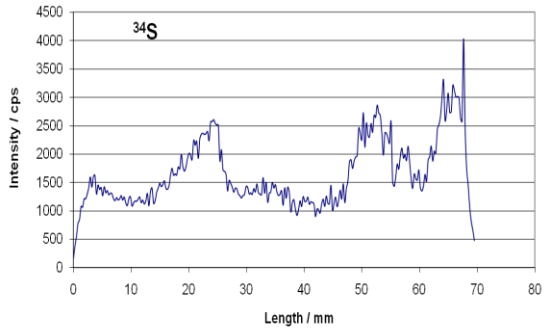


wing



b)

tail



wing

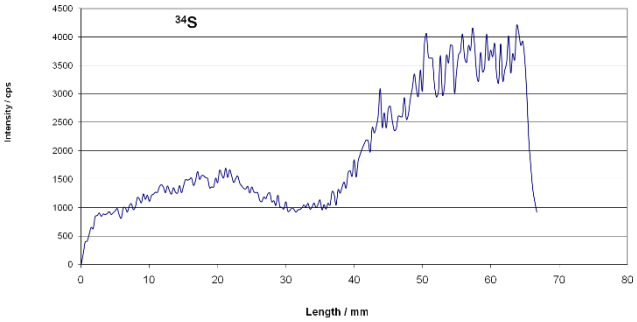
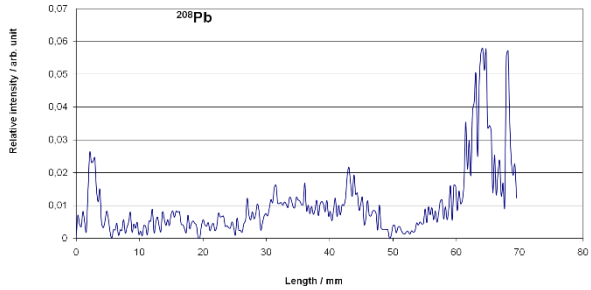


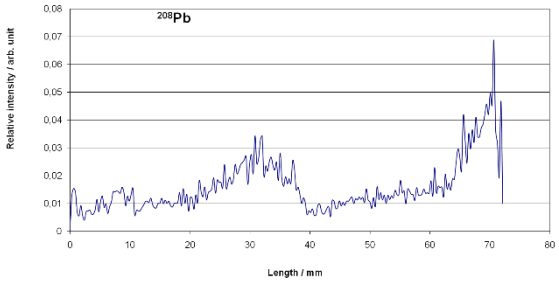
Figure 4 (continued)

c)

tail

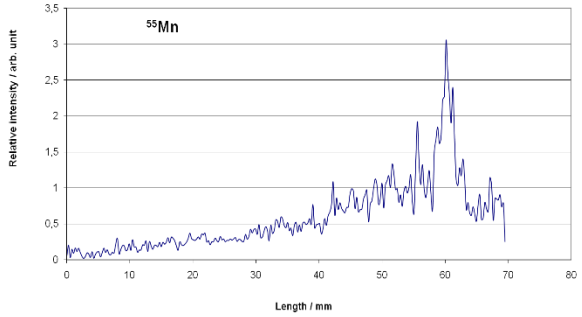


wing



d)

tail



wing

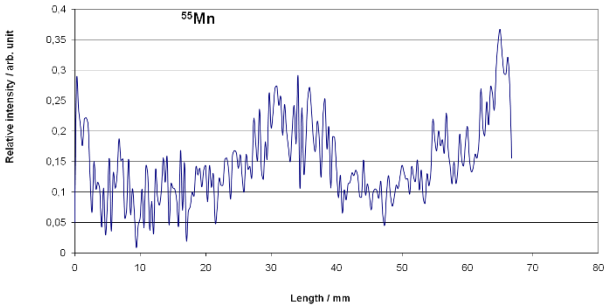
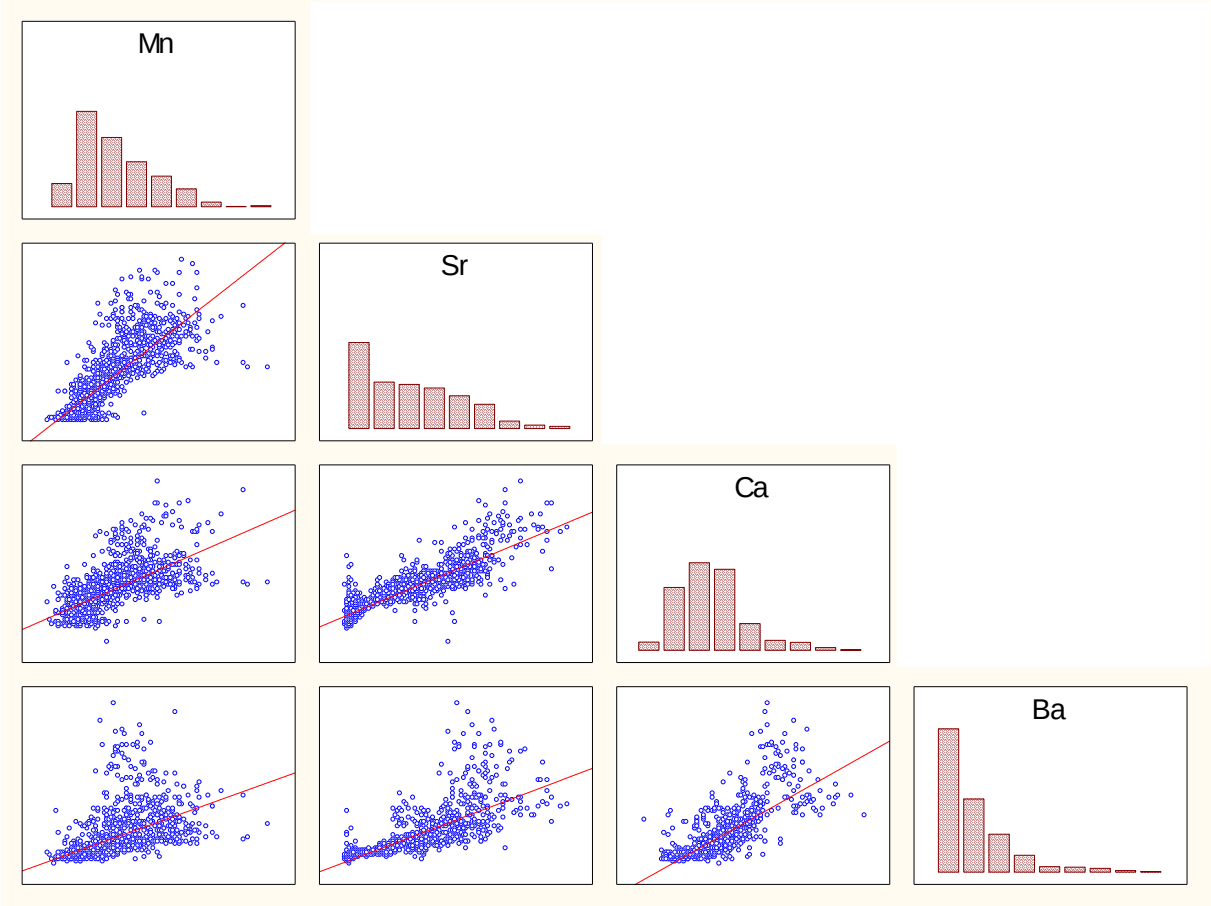
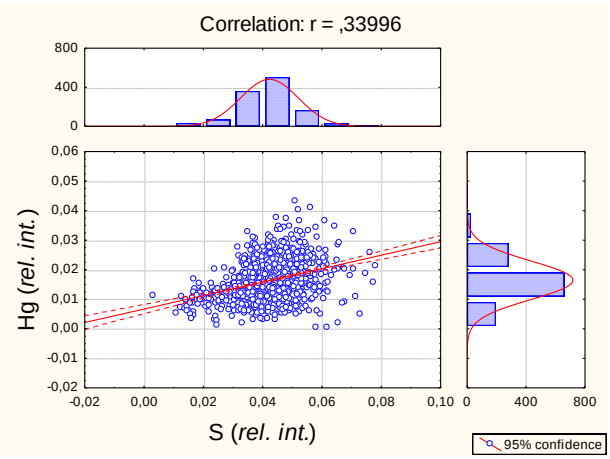


Figure 5

a)


Mn
Sr
Ca
Ba

b)


Correlation: r = ,33996
800
400
0
0,06
0,05
0,04
0,03
0,02
0,01
0,00
-0,01
-0,02
Hg (rel. int.)
-0,02
0,00
0,02
0,04
0,06
0,08
0,10
0
400
800
S (rel. int.)
95% confidence