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Unveiling Ecosystem Health through Animal and Plant Bioindicators, while Exploring the Phytotherapeutic and Nutritional Potential of Plants

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General introduction

In the last century, the growing anthropic pressure on ecosystems has highlighted the urgent necessity to develop an integrated methodology to evaluate and monitor environmental health. Pollution, climate alterations, biodiversity loss, expansion of urban areas, agricultural practices, and industrial activities have deeply transformed ecological balances. [1,2] This led to a decrease in ecosystem resilience and repercussions that extend to all the environmental compartments. To assess this complex scenery, the One Health paradigm is an interdisciplinary approach that enhances the relationship between human, animal, and ecosystem health. One Health is not only a theoretical vision; it encompasses an operative framework that proposes to address global challenges by integrating skills from different disciplines. The health of wild animals cannot be separated from their habitat quality, as human health is strictly connected to the integrity of ecosystems and their resources. This methodology is proving to be essential to assess complex problems such as contaminants exposure or habitat degradation. [3] In this work, the One Health principle is concretized by using vegetal and animal bioindicators to monitor environmental pollution, with nutritional analysis. Plants and animals are not the only victims of anthropic pollution; they can be used as tools to observe the ecological status of an area. The bioaccumulation of mineral elements and their physiological and metabolic responses are valuable data that reflect the quality of soil, water, air, and nutrition. [4] An additional element of strength of this work is the non-invasive method of studying animal species, minimizing the impact of research on individuals, which is especially important for endangered species. Analyzing fur, feathers, or feces allows researchers to gain insight into animal and ecosystem health without compromising the organisms' survival. [5,6] Animal and vegetal bioindicators, in addition to well-defined protocols and advanced analytical techniques, permit evaluating ecosystem states from multiple perspectives. This methodology allows us to identify environmental risks for biodiversity and animal health while offering useful tools to ensure the food and phytotherapy safety of vegetal products often used in traditional practices and still poorly regulated in local contexts. [7] In this scenario, this work presents a series of studies that, despite focusing on different organisms and matrices, share the same aim: to contribute to the understanding of ecosystem health through an integrated, multidisciplinary approach consistent with the principles of One Health.

The main objective of this PhD thesis is to investigate ecosystem health through a multidisciplinary approach, exploring the potential of plants and animals as bioindicators, focusing on essential and non-essential elements, and evaluating the element content in traditional officinal plants. The entire work pursues the goal of offering practical and replicable tools to monitor the environment, biodiversity conservation, and the evaluation of the safety of plant species of dietary or therapeutic interest. The common thread is represented by the One Health approach, which guides the choice of matrices, analytical techniques, and application contexts.

This thesis is structured in four experimental contributions, each of which assesses a complementary aspect of the central aim. The first chapter regards the development of an on-site voltammetric method to quantify lead (Pb) and cadmium (Cd) in officinal plants. It introduces an analytical portable system designed to be simple and economical to quantify Cd and Pb in the leaves used for phytotherapy practices. The aim is to offer an accessible method to contexts with limited resources, useful for food safety and environmental evaluation. The second work concerns the evaluation of bioaccumulation of essential and non-essential elements in officinal endemic plants (*Ficus lutea*, *Ficus reflexa*, and *Uapaca Thouarsii*) of Madagascar and their implication for traditional medicine and biomonitoring. The aim is to estimate the spatial variability of mineral element content in endemic officinal plants of Madagascar, comparing their accumulation patterns with environmental anthropic influence, particularly in the proximity of mining activity, to evaluate both the safety of plants' consumption, from the point of view of the element content, taking into account their role as traditional medical remedies, and their potentialities as bioindicators. The third chapter deals with the potential of a biomonitoring multi-matrix and non-invasive method in four endangered lemur species (*Lemur catta*, *Eulemur macaco*, *Eulemur mongoz*, and *Eulemur rubriventer*) in two different zoological contexts with ecological and geochemical different characteristics. The comparative analysis of three biological matrices (fur, feces, and blood) permits us to observe how the environmental condition influences element accumulation while also providing valuable insights for guiding management decisions in zoological structures, in an integrated and sustainable perspective inspired by the principles of One Health. The last chapter assesses the element content in African penguin (*Spheniscus demersus*) feathers and their ecological implication, especially their influence on molt. This study's scope is to evaluate how element content reflects the environmental exposition and physiological process regarding molt, contributing to the estimate of the ecological state of the habitat of African penguins.

The four chapters collectively aim to contribute to the interpretation of the health of organisms as a reflection of environmental quality. They report the development of analytical methods and accessible approaches in both the conservation field and the phytotherapy, food, and health sectors. Despite those studies regard different biological, ecological, and methodological contexts, they develop coherently along a single thematic axe: the evaluation of ecosystem health through the integrative contribution of animals and vegetals, with a strong focus on contamination by essential and non-essential elements, bioaccumulation dynamics, and the ethical and sustainable conservation of biological data. The mutual complementarity of the studies manifests in multiple levels: technical-analytical, biological-ecological, and applicative. The first element is the application of highly sensitive analytical techniques to quantify trace elements in complex biological matrices. In particular, the first chapter illustrates a simple, economical, and portable voltammetric method developed to determine Cd and Pb in leaves. This approach, optimized with a rigorous experimental design, represents an important tool potentially applicable in developing countries or in remote contexts where access to equipped laboratories is limited. In the other studies, high-sensitivity instrumentation is used for both vegetal and animal matrices, allowing an inter-matrix and inter-specific comparison of elemental patterns. This cross-cutting approach makes possible a multilevel assessment of contamination and exposure, reinforcing the methodological integration of the thesis. Another common aspect is the choice to conduct ethical research using a non-invasive approach (collecting leaves, fur, and feces), demonstrating the applicability of an ethical biomonitoring strategy that aligns with conservation and animal welfare principles. In addition, all chapters emphasize the direct connection between environmental quality and organism health. A further strength of the work is the wide applicability of the results, ranging from the quality control of medicinal plants to the use of sentinel species for conservation, up to the development of analysis tools in contexts with low technological availability. The obtained results can be the starting point to support new environmental policies, conservation programs, ethical management of zoological structures, and strengthen international scientific cooperation, as demonstrated by the active collaboration between Italy and Madagascar.

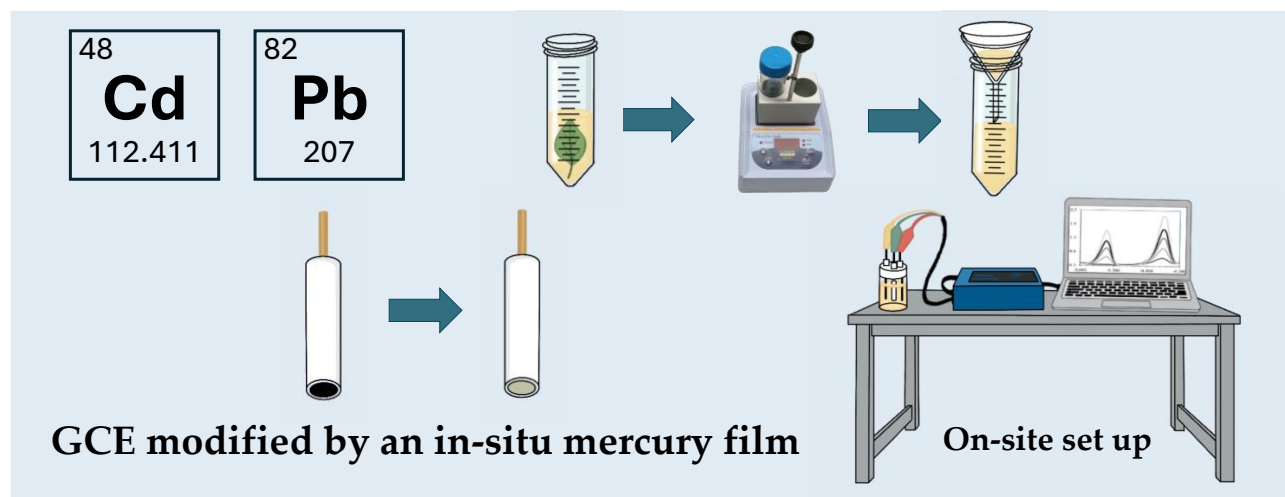
This thesis stands out for its integrated and cross-sectoral approach applied to study ecosystem health, combining the tools of environmental chemistry, phytochemistry, and biology in a framework consistent with the multidisciplinary character of the Sustnet PhD school and the principles of One Health. The novelty is the implementation of advanced methodologies, sometimes

adapted in a portable and accessible key, to real context and unusual complex biological matrices, with constant attention to sustainability and research ethics. Overall, the thesis proposes a flexible, replicable, and scientifically robust operating model to study the interaction between living organisms and the environment, underlining how the health of ecosystems is a fundamental key to understanding and protecting environmental compartments.

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Experimental Design Optimization of a Simple and Cost-Effective Voltammetric Method for On-Site Determination of Lead and Cadmium in Officinal Plant Leaves



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DP-ASV; Lead; Cadmium; Modified GCE; Experimental Design; On-Site

Abstract

This study introduces an improved method for the on-site quantification of cadmium and lead in officinal plants using differential pulse anodic stripping voltammetry (DP-ASV) with a glassy carbon electrode (GCE) modified by an in-situ mercury film (iMF-GCE). Initial experiments, conducted with one-variable-at-the-time (OVAT) approach, have provided suboptimal recovery rates and linearity, factors refined later through a systematic experimental approach involving two different designs: Plackett-Burman and Face Centered Composite Design (FCCD). Certified reference materials confirmed the method's reliability and its potential for effectiveness. The key parameters were optimized, achieving an E_{dep} of -1.20 V and a t_{dep} of 195 s for optimal performance. The results obtained allowed substantial improvements in accuracy, with recovery rates of 85.8% for Cd and 96.4% for Pb, and significantly lowered detection limits from $1.54 \mu\text{g L}^{-1}$ to $0.63 \mu\text{g L}^{-1}$ for Cd and from $0.15 \mu\text{g L}^{-1}$ to $0.045 \mu\text{g L}^{-1}$ for Pb. Validation through field applications demonstrated the method's suitability for the analysis of official plants. Other applications are for environmental monitoring and food safety control.

1 Introduction

The World Health Organization (WHO) highlights the significant reliance on traditional herbal medicines worldwide.[1] While Western societies typically favor synthetic drugs for their rapid effects, there has been a recent resurgence in the use of phytotherapy, driven by pharmacophobia and the perception that herbal remedies are inherently safe. However, herbal medicines pose risks from intrinsic plant toxicity and contamination with pesticides and potentially toxic elements (PTEs).[2–6] Among the latter, cadmium and lead are potentially toxic even at very low concentrations[7–9]. Therefore, their content within herbs and herbal preparations must be determined accurately and with a simple and inexpensive procedure.

Various techniques exist for detecting these metals, including X-ray fluorescence spectrometry (XRF)[10], inductively coupled plasma optical spectroscopy or mass spectrometry (ICP-OES, ICP-MS)[11–16], microwave plasma atomic emission spectroscopy (MP-AES)[17], atomic absorption spectroscopy (AAS)[17–20] Typically, these methods are carried out in laboratory settings requiring specialized equipment and trained personnel. However, purchase and maintenance costs often render them inaccessible. Presently, there is a growing trend toward developing cost-effective field methods that can be used in various settings[21–23]. These methods are adaptable for on-site analysis, reducing the need to transport samples to a laboratory. This renders it highly practical for real-time monitoring of diverse contexts. The portability of the equipment guarantees that it can be deployed in remote areas, delivering instant results and enabling swift decision-making processes.

Furthermore, even small and medium companies can buy a portable instrument and use it to check the quality of their products without the need to set up a laboratory. Among the techniques that can be performed on-site is XRF; although it is portable and easy to perform, it lacks sensitivity for low contamination levels. Voltammetric techniques offer highly sensitive and low-cost portable analyzers that are suitable for field applications. Using ASV, it is possible to detect trace levels of various substances in diverse matrices, including plant and environmental samples. [19,20,24–26] Conventional analytical method optimization typically employs a one-variable-at-a-time (OVAT) approach, which involves adjusting parameters individually [27–29]. However, this univariate method often leads to suboptimal results because it fails to consider the interactions between factors. The use of statistical design of experiments (DoE) methods has been proposed to optimize the performance of electrochemical sensors, allowing for the identification of optimal conditions with a reduced number of experiments. DoE methods enable the generation of regression equations highlighting the effects and importance of individual factors and their interactions, which classical methods cannot easily achieve[30]. Some studies have successfully applied response surface methodology (RSM) to optimize voltammetric signals, demonstrating its utility and effectiveness in this context[31]. In a previous paper, we applied voltammetry for on-site determination of Cu in waters and soils[32–35]. This research study aims to develop a portable on-site procedure able to quantify Cd and Pb in medicinal plants using ASV with comparable performance to traditional laboratory measurements. A glassy carbon electrode (GCE) modified with a mercury film was adopted as working electrode (WE). This kind of WE is extensively used for laboratory analysis[20,36–41], but few methods are dedicated portable methods[42]. In particular, the performances of in situ mercury film (iMF-GCE) and ex-situ mercury film (eMF-GCE) were compared, and the former was selected as the most suitable for the objectives of the study. Mercury electrodes have some environmental concerns, but they do offer very significant analytical advantages, in particular for complex matrices such as those presented by medicinal plants. Such matrices present challenges from high organic content and potential interference. The use of mercury electrodes is superior in such cases, due to the properties of sensitivity and selectivity that are gained through the formation of amalgams, which allows for narrow peak resolution and ultra-low detection limits. For example, the paper of S. Titretir Duran et.al. highlights the reliability of mercury films in delivering high precision even in the analysis of complex biological matrices. [43] Compared to conventional carbon-based electrodes, bismuth is a relatively greener option; however, it tends to exhibit wider peaks and lower sensitivity, especially during multi-analyte detection in

difficult matrices. For example, the work of S. Shi et.al. stresses the advantages of mercury electrodes over bismuth ones in low-concentration applications, to guarantee accurate and reliable quantification of trace metals.[44] For the detection of Cd and Pb concentrations below legal limits in our study, extremely low detection limits must be ensured that were reliably obtained with mercury thin-film electrodes. In addition, the volume of mercury applied in each analysis was small, and stringent waste treatment protocols were employed to minimize any potential environmental hazard. Mercury electrodes were the optimal candidates for this application due to their excellent analytical performance and sensitivity. Initially, the OVAT method was used for preliminary exploration, but owing to unsatisfactory outcomes, DoE was employed as a more effective approach. A Plackett-Burman design was used for factor screening, followed by a FCCD to analyze and optimize the effects of significant factors. Finally, the developed method was successfully applied to the analysis of officinal plants.

2 Materials and Methods

2.1. Reagents and Instruments

All solutions were prepared in high purity water (HPW) produced with Millipore Milli-Q Plus system (18.2 MΩ cm). All the reagents were of analytical grade. Standard solutions of Cd, Pb, and Hg were prepared from 1000 mg L⁻¹ Sigma-Aldrich solutions. Nitric acid (HNO₃, 65%, Sigma Aldrich), hydrogen peroxide (H₂O₂, 30%, Sigma Aldrich), hydrochloric acid (HCl, 37%, Sigma Aldrich), acetic acid (CH₃COOH, 99%, Sigma Aldrich) and potassium acetate (CH₃COOK) were also used. On-site sample pretreatment was performed with a mini dry bath (Starlab International, Hamburg, Germany), which can be used in the field combined with a portable battery.

The analyses were carried out using a PalmSens4 portable potentiostat (PalmSens, Houten, Netherlands). As an electrochemical cell for this study, an on-site three-electrode system was designed. It consisted of GCE as WE with an Ag/AgCl reference electrode and a platinum counter electrode. To improve the sensitivity and selectivity for the detection of Cd and Pb, the working electrode was coated with a thin mercury film. The fact that the assembly used few reagents and equipment also made it cost-effective. It was interfaced with a tablet or laptop computer and the software PSTrace5.9 was used to set the process parameters. The potentiostat was connected to a KIA-Topolino magnetic stirrer and fed with a portable battery.

2.2. Samples

Certified reference materials (CRM) were analyzed to evaluate the extraction efficiency and electrochemical determination accuracy using different GCE modifications. Two different CRMs were analyzed, one for Pb (NIST® SRM® 1573a– Tomato Leaves) and one for Cd (ERM-CD281-Rye

Grass), as there were no CRMs available that contained both elements above the limit of detection of the technique.

The developed method was evaluated on natural samples on site to verify the efficiency of the procedure under field conditions with various plant species. This paper reports samples in which at least one of the two analytes was detectable. The plant species analyzed were *Petroselinum crispum*, *Taraxacum officinale* and *Uapaca thouarsii*. *Taraxacum officinale* and *Petroselinum crispum* samples were collected in Turin, Italy, close to highly trafficked streets, whereas *Uapaca thouarsii* (a plant traditionally used for phytotherapy by Malagasy people) was sampled in Maromizaha forest, in Madagascar. The three plants were analyzed in Valentino Park, a park located in Torino, where the portable analyzer and the other items necessary for the analysis were placed on a simple picnic table.

2.3. Procedures

2.3.1 Extraction using a mini dry bath

The extraction process in the mini dry bath was carried out with 0.5 g of each sample treated with 3 mL of H₂O₂ and 5 mL of HNO₃, according to our previous work[23]. The whole procedure was carried out (for laboratory experiments) under a hood and the vessels containing the various samples were inserted into the mini dry bath and allowed to heat at 70 ± 3 °C for 2h. Later, filtration was carried out with Whatman filters, and each solution was diluted to 15 mL with HPW. Blanks (matrix solution), prepared only with the two reagents in the same amounts, were also run following the same pretreatment and analysis protocols.

2.3.2 Voltammetric Measurements

The analyses were carried out using ASV, keeping the solution under mechanical stirring during the deposition step and then performing the voltammetric scan in differential pulse mode (differential pulse voltammetry, DPV). The working electrode was a GCE modified with Hg film through in/ex-situ deposition (see chapters 2.4.2 and 2.4.2). Sensitive Voltammetric Method for Rifampicin" (2021, Sensors). The use of mercury thin-film electrodes in this study is justified by their superior analytical performance, especially for detecting low analyte concentrations in complex matrices. They provide unparalleled sensitivity and selectivity through their ability to form amalgams with target metals, enhancing peak resolution and reducing detection limits. This capability is particularly critical for analyzing samples like medicinal plants, where the complexity of the matrix and the presence of trace-level concentrations represent a significant analytical challenge. Previous studies have validated the efficacy of mercury-based electrodes in similar contexts. For instance, the work of L. López-Solís et.al. demonstrated their quantification of trace zinc in biological fluids, while the paper written by M. Szłósarczy et.al. highlighted their sensitivity in detecting rifampicin in pharmaceutical formulations.[45,46] These findings underscore the unique advantages of mercury thin-film electrodes in achieving high analytical performance, particularly in scenarios where alternative materials, such as bismuth, may face limitations in sensitivity or resistance to fouling.

Before proceeding with the optimization using experimental design and with the analysis of the samples, tests were carried out on synthetic solutions and two certified materials to assess the applicability of the whole procedure.

The synthetic solutions were analyzed with 10 mL of supporting electrolyte (6×10^{-2} mol L⁻¹ HCl). The process for analyzing the samples and the corresponding blanks (matrix solution) was analogous, despite 9 mL of supporting electrolyte and 1 mL of sample or matrix solution being used within each cell. Procedural blanks were prepared with the reagents used for the pretreatment (HNO₃ and H₂O₂) and processed following the same method developed for plant samples. Voltammetric measurements were performed to detect any possible contribution of analytes present in the reagents or arising from contamination during the pretreatment process. The voltammograms of procedural blanks did not show any visible signal in the potential areas of the Cd and Pb peaks, suggesting that blank analytes concentrations were below the LODs (after the optimization, the LODs were 0.629 µg L⁻¹ for Cd and 0.045 µg L⁻¹ for Pb). Three successive additions of 5 µg L⁻¹ of Pb and Cd standard solutions were carried out into the cell and voltammograms were recorded after each addition to generate the calibration curve. After each measurement, an electrochemical cleaning stage of the electrode surface was made by applying a positive potential, essentially to remove residuals of analytes possibly left undissolved during the stripping phase. The parameters used in the measurements are shown in the next chapters (Section 2.4 for preliminary experiment). In each measurement, after a pre-concentration step at a negative potential (from -0.80 V to -1.20 V potential were tested) the potential was varied to more positive values, and the voltammogram was recorded; Pb and Cd gave rise to peaks at about -0.45 V, and -0.65 V, respectively. The peak area was considered for quantitative analysis instead of peak height, as it was found to provide a more accurate measure of the total analyte concentration

The electrode surface was polished and cleaned once a week. Firstly, the glassy carbon surface was rubbed on a velvet pad, on which a few drops of 0.05 µm-alumina suspension were placed. Secondly, the electrode was washed, by immersion, in ethanol and HPW three times.

2.4 Preliminary Experiments

2.4.1 *In situ mercury film (iMF-GCE)*

The analyses were conducted in synthetic solutions, adding an aliquot of Hg standard (final concentration 0.7 mg kg⁻¹), necessary for forming the film *in situ* during the deposition step. Supplementary Table 1 shows the parameters adopted in the preliminary phase of this work. After recording the voltammogram of the blank, aliquots of Cd and Pb standard solutions were added, and the corresponding signals were recorded. All experiments were performed in duplicate.

2.4.2 *Ex situ* mercury film (eMF-GCE)

The use of eMF-GCE requires several steps. The first one consisted of the deposition of the Hg film, using 30 mL of 1×10^{-2} mol L⁻¹ spiked with 0.7 mg L⁻¹ Hg standard solution while keeping the solution stirred. Supplementary Table 1 reports the voltammetric parameters adopted for deposition in the preliminary phases of this work. After the deposition of the film, an electrochemical cleaning stage was carried out, aimed at removing any impurities present on the film. After cleaning, the analyses were carried out with the same supporting electrolyte adopted for the in-situ film using the parameters reported in the same Table. It is crucial to emphasize that, in contrast to the iMF-GCE, the eMF-GCE requires a more intricate preparation process, involving additional procedural steps and increased reagent consumption.

2.5 Statistical Tool

CAT Chemometric Tool [47] was employed to examine the data collected from experimental designs hereafter described.

2.6 Experimental Design

After the preliminary study, the on-site method was optimized through an experimental design to quantify Pb and Cd in plants using iMF-GCE and DP-ASV. The goal is to optimize the ratio of high-quality information about the influence of variables on the chemical system to achieve the best performance possible. To this aim, a Plackett-Burman design, followed by a FCCD, were employed. The experiments were conducted in random order for both experimental designs.

2.6.1 *Plackett-Burman Design Screening of Independent Variables*

During the screening design, experiments were carried out on synthetic solutions (section 2.3.3). Six factors (variables) and the domain of the selected design of experiment were chosen based on previous experimental criteria, out of all the variables that could potentially influence the method examined. Three variables regarded the in-situ formation of the mercury film (concentration of Hg, deposition time, and deposition potential), and three the DP-ASV scan (pulse and step potential, and pulse duration). Supplementary Table 2 reports the values adopted in the preliminary study and the interval chosen for each factor for the Plackett-Burman design. To measure the quality of the method performed during the experiments, linearity (R^2), and repeatability (RSD%), both for Pb and Cd were monitored as mentioned in section 2.3.2 (Supplementary Table 3). This procedure identified three factors as capable of influencing the method responses. Their values were optimized as reported in chapter 2.6.2.

2.6.2 Optimization of DP-ASV Parameters using Face Centered Composite Design

Once the most significant variables were identified, a FCCD, with 3 variables (t_{dep} , E_{dep} , and E_{step}) at three levels, was used to refine the parameters for quantifying Cd and Pb. Therefore, a new set of 20 experiments including five repetitions of the central point, was performed using 9 mL of supporting electrolyte (6×10^{-2} mol L⁻¹HCl), and 1 mL of blank solution (section 2.3.2). The values assigned to the three factors in this model are presented in Table 1. The graphical representation of the design of experiments is reported in Figure 1, while Supplementary Table 5 collects the values of factors and responses of each experiment. To measure the quality of the method performed during the experiments, linearity (R^2), repeatability (RSD%), and peak area (A) for both Pb and Cd were monitored as described in section 2.3.2. Design matrix with coded values of each experiment and response values obtained with the FCCD are shown in Supplementary Table 5. Rows highlighted in grey represent repetitions of the central point within the experimental design.

3 Results and Discussion

3.1 Preliminary Experiment Results

In the first step of the study, iMF-GCE and eMF-GCE were compared. The linearity of the analytical response was investigated by adding five subsequent spikes of 5 $\mu\text{g L}^{-1}$ of Cd and Pb and measuring the corresponding signals. The equations of the calibration curves and the R^2 values were obtained. The LOD was determined based on the residual of the linear regression, which served as an estimate of experimental variability. The accuracy of the results was evaluated by analyzing the above-mentioned CRMs. Repeatability was evaluated with 10 replicates of 10 different cells containing 20 $\mu\text{g L}^{-1}$ of Cd and Pb. The relative standard deviations (RSD%) of the peak heights were computed. Table 1 collects the equations of the calibration curves and the R^2 values together with the other figures of merit of the two methods. The repeatability and LOD values obtained were satisfactory and comparable to literature values for similar methods used to quantify metals in water and synthetic solutions. [27,29,48] The performance of both electrodes was adequate but not entirely satisfactory. Upon closer examination, it became evident that the iMF-GCE exhibited slightly superior performance for Cd ($R^2 = 0.9954$) and Pb ($R^2 = 0.9940$) compared to the eMF-GCE (0.9952 and 0.9903, respectively). While both iMF-GCE and eMF-GCE have their strengths, iMF-GCE was selected for its overall superior performance in terms of sensitivity, linearity, precision for Pb, and recovery for Pb. Additionally, its ease of use and shorter analysis time make it the preferred choice for the on-site are of Cd and Pb in plant samples. Although the outcomes were promising, the accuracy and linearity were lower than expected, particularly concerning the recovery rates and the LOD for Cd (70.2 %), suggesting that the results obtained using the OVAT method were not sufficient. Therefore, the procedure was implemented using an experimental design as explained in

the following section. The preliminary study proved beneficial in understanding the boundaries of the experimental domain and selecting the variables to investigate further.

Table 1 Repeatability, linearity, LOD, and accuracy obtained in preliminary experiments for iMF-GCE and eMF-GCE in CRMs

	Analyte	Repeatability (RSD %)	Linearity	LOD (μgL^{-1})	Accuracy (% recovery)
iMF-GCE	Cd	14.40%	$y = 0.0262x - 0.1555$ $R^2 = 0.9954$	1.54	70.17
	Pb	7.60%	$y = 0.0711x - 0.1531$ $R^2 = 0.9940$	0.15	86.33
eMF-GCE	Cd	6.33%	$y = 0.1421x + 0.0837$ $R^2 = 0.9952$	0.27	69.2
	Pb	11.93%	$y = 0.0077x - 0.0032$ $R^2 = 0.9903$	0.22	84.5

3.2 Plackett-Burman Design

The results obtained from the experiments are reported in Supplementary Table 3, as described in chapter 2.6.1. To identify a single response from the four obtained, a Principal Component Analysis was performed. The aim was to identify a linear combination that effectively collects the information present in various responses. The results revealed that the variables were not correlated; consequently, no principal component that could describe them was identified. Therefore, each response was treated individually to understand the significance of the factors. Figure 1 presents bar plots showing the importance of the model's coefficients (Supplementary Table 5), one for each collected response.

These charts illustrate which variables have a statistically significant impact and the level of confidence associated with them. Typically, a p-value is linked to each coefficient, corresponding to the probability that the variation in the response caused by that term is a result of random error. The higher the probability that the coefficient is significant, the lower the p-value. The values are significant if larger than the semi-amplitude of the green error bar, while asterisks represent the significance level of statistical analysis (with one, two, or three asterisks corresponding to p-values less than 0.05, 0.01, and 0.001, respectively)[30]. The results revealed that only three (t_{dep} , E_{dep} , and E_{step}) out of the six variables examined exhibited a significant impact on the outcome of the analysis and deserved further investigation. The other experimental parameters did not substantially impact instrumental performance, so they were set to the most practical values based on analysis time. Only the response from the Cd peak appeared to be influenced by the variation in the factors. Since Cd did not produce satisfactory results during the preliminary experiments, the impact of significant

factors was investigated with the aim of improving the performance of the method for the determination of this element and of observing its effects on Pb response.

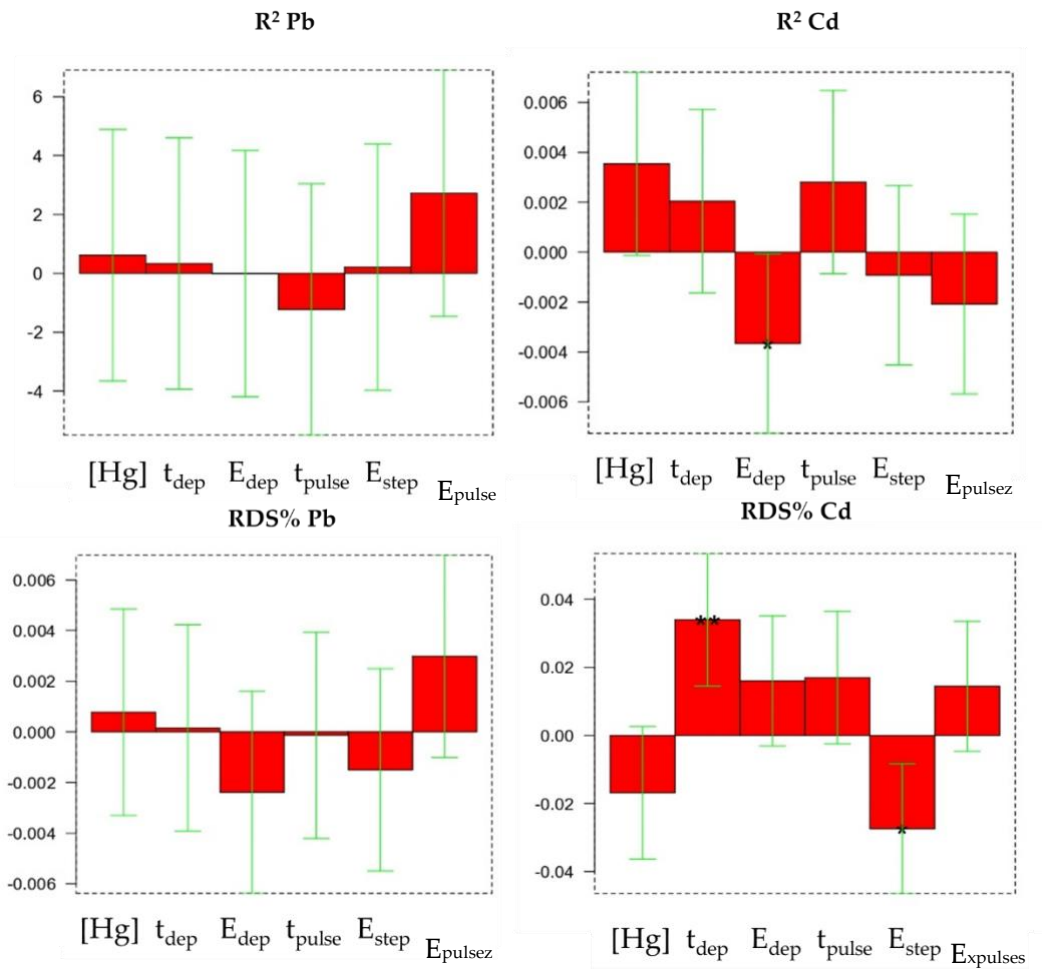


Figure 1 Bar plots representing the coefficients significances of the Plackett-Burman model.

3.3 Face Centered Composite Design

The results collected from the experiments carried out following the FCCD are reported in Supplementary Table 5. Furthermore, each of the six responses was analyzed independently to identify the optimal level of factors and their corresponding parameters by examining the distinctive response curves (Figure 3). Figure 2 presents bar plots showing the importance of the model's coefficients, one for each collected response (Supplementary Table 7). The significant coefficients are the linear terms E_{dep} and t_{dep} , their interaction, and the quadratic term of t_{dep} . The graphs indicate that E_{dep} and t_{dep} had different impacts on all the results obtained except for the repeatability of Cd analysis (RSD%), which does not appear to be influenced by the variation of these two parameters. Contrary to the results obtained through the Plackett-Burman Design, E_{step} does not seem to have any impact on the responses obtained from the model and therefore was not considered as a parameter to be optimized in the method. The degree and type of influence of factors on response variables are represented in the contour plots in Figure 3. The isoresponse plot, also known as a contour plot, is a two-dimensional graph that displays all the points with the same response on lines. The x and y axes of the plot represent the two selected variables (E_{dep} and t_{dep}), and the value of the desired response is typically indicated above the lines. This type of plot is useful for identifying the conditions that produce the desired response.[49] By observing the isoresponse curves, a point in the graph was found where R^2 and the peak height were adequate area for the peaks of Cd and Pb, and the RSD% was less than 20%. It corresponds to an E_{dep} of -1.20V and a t_{dep} of 195s.

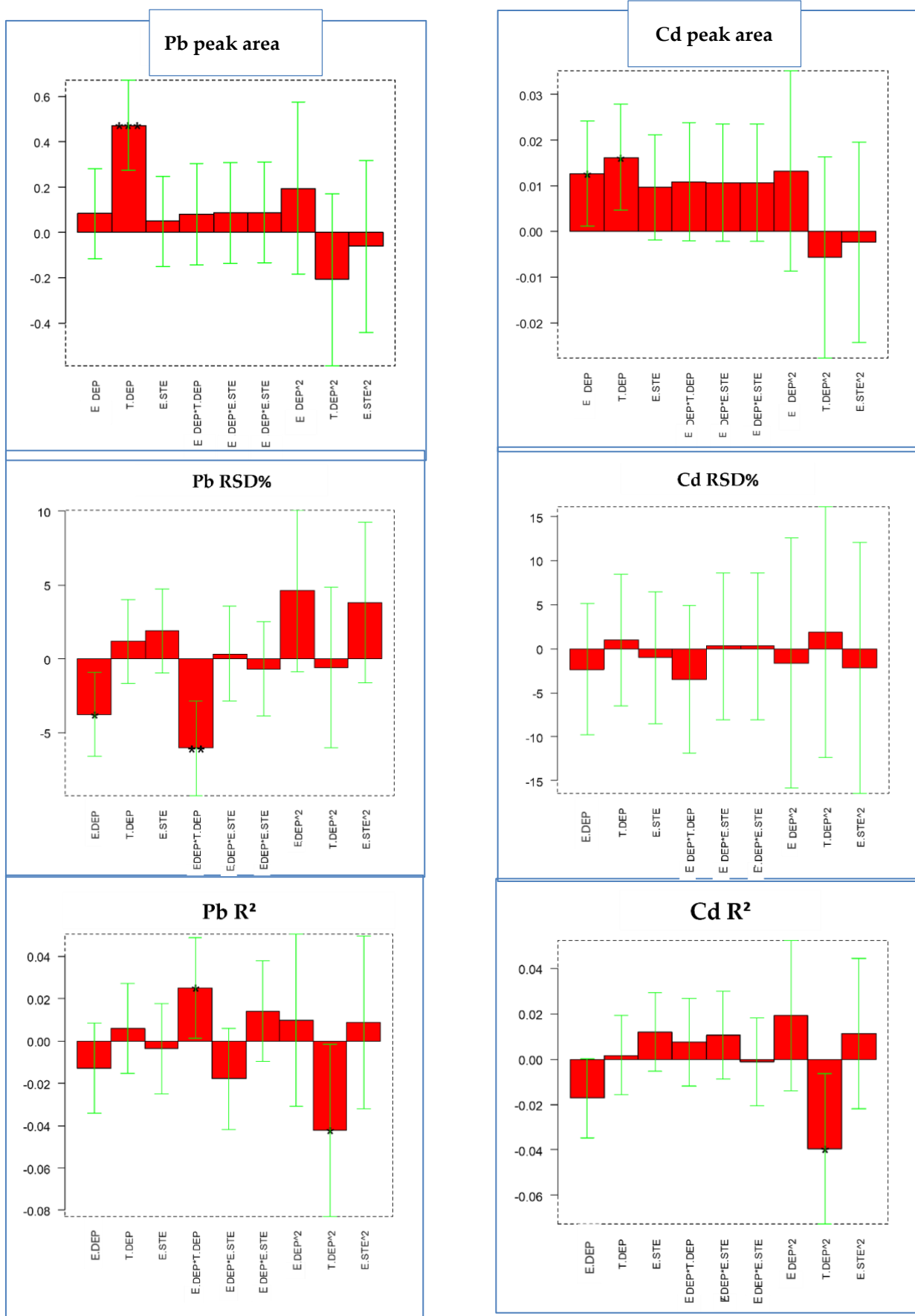


Figure 2 Bar plots representing the coefficients significances of the Face Centered Composite Design model.

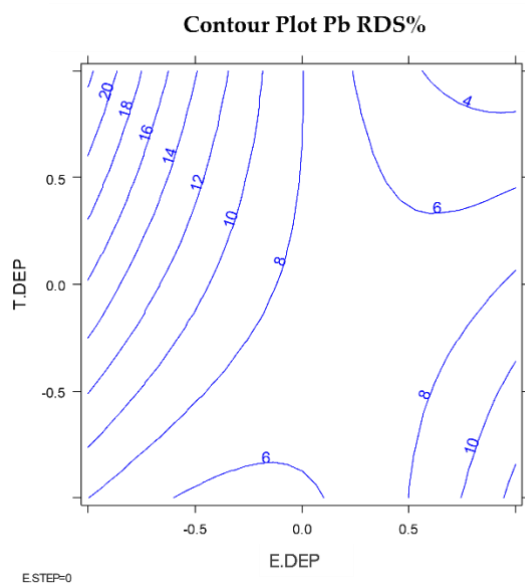
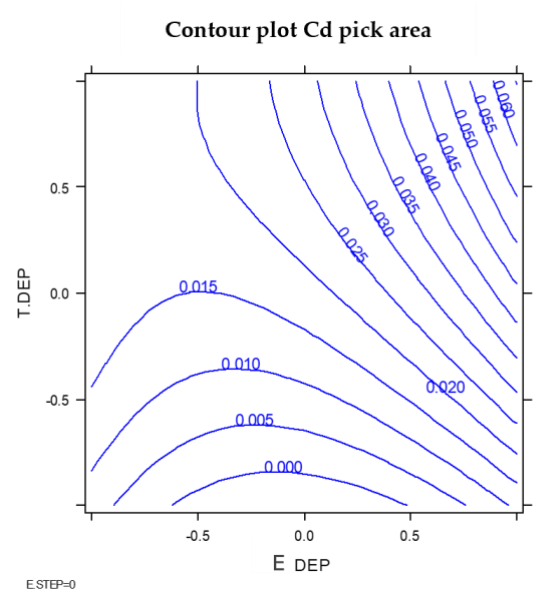
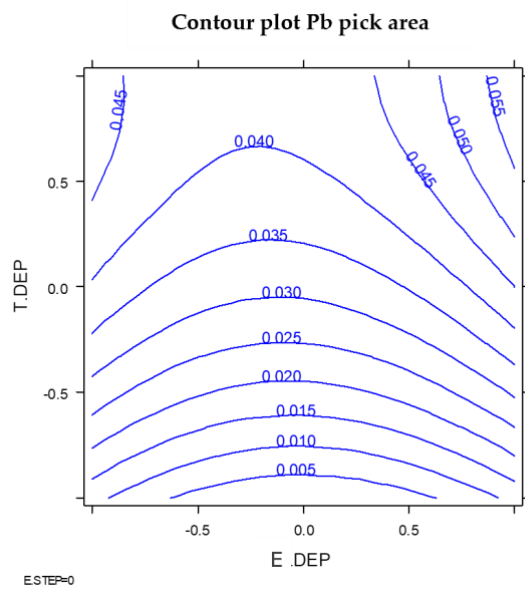
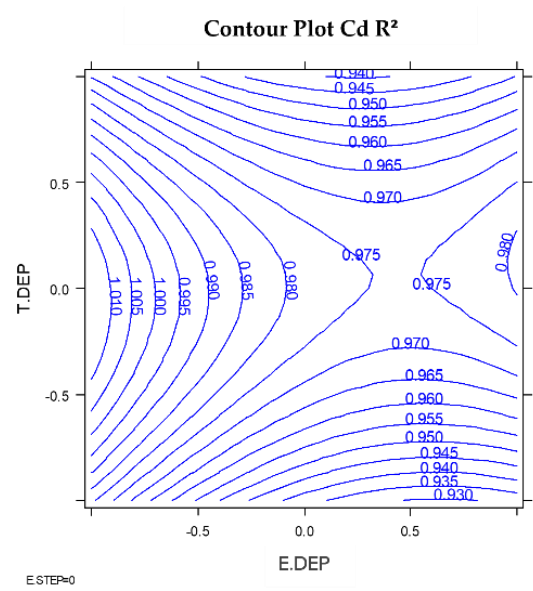
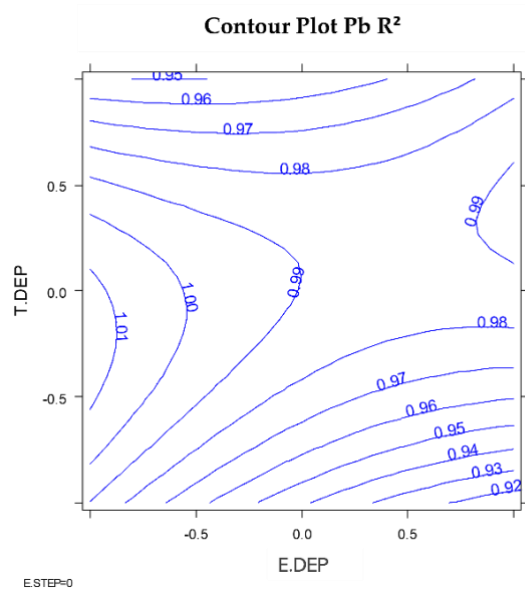


Figure 3 Contour plot representing the isoresponse curves obtained from Face Centered Composite Design.

3.4 Performance Tests and On-Site Real Sample Analysis

Thanks to the experimental design methods, it was possible to identify the optimal conditions for the quantification of Cd e Pb. The accuracy of the optimized method was tested as described in section 2.2. The results obtained are summarized in Table 2. The improvement in the recovery of Cd was significant, rising from 70.2 %, obtained in the preliminary analysis, to 85.8 %. Similarly, the improvement in the recovery of Pb was notable, increasing from 86.3 % to 96.4 %. LOD values, likewise, were drastically reduced, especially for Cd, decreasing from $1.54 \mu\text{gL}^{-1}$ to $0.629 \mu\text{gL}^{-1}$. The LODs expressed in mg/kg are 4.5×10^{-5} for Pb and 6.29×10^{-4} for Cd, taking into account the sample amounts weighed and the solution volume in the cell. The obtained results indicate LODs significantly below the regulatory thresholds established by European legislation, specifically under Regulation (EU) 2021/1317 (for lead with limits such as 0.60 mg/kg for dried fruit-based spices and up to 2.00 mg/kg for bark-based spices, and cadmium, set at 0.20 mg/kg for fresh herbs)[50]. In addition, significant progresses were achieved in linearity and repeatability, which in turn enabled a straightforward on-site application with very good performance.

Finally, the procedure so optimized was tested for on-site analysis of real plant samples. These analyses were conducted at Valentino Park in Turin, Italy. The voltammograms are shown in Figure 4 and the results obtained are collected in Table 3. For comparison, the same samples were analyzed in the laboratory: the concentrations found were in good agreement with on-site values. However, even though the optimized method has satisfying LOD, we did not find plant samples with detectable concentrations of both analytes. Then the results of samples for which at least one of the two analytes was above the detection limit were reported. No detectable signals corresponding to Cd and Pb were measured in blanks' voltammograms during both preliminary and optimization experiments, suggesting that the presence of the analytes in the procedural blanks was below LODs: this finding supports the method's robustness and applicability. Additionally, the sample signals were at least one order of magnitude higher than these thresholds, further confirming this observation.

Table 2 Result obtained from the analysis of Certified Reference Materials

Responses	Tomato leaves (Cd)	Rye grass (Pb)
Certified concentration (μgkg^{-1})	1520 $\pm$ 30	1670 $\pm$ 100
Measured concentration (μgkg^{-1})	1350 $\pm$ 20	1730 $\pm$ 200
Recovery (%)	85.8	96.4
R ²	0.999	0.999
RSD% (%)	2.8	15.9
Peak area	0.0038	0.0089
LOD (μgL^{-1})	0.629	0.045
LOD (μgKg^{-1})	377	27

Table 3 Result obtained from the analysis of natural samples conducted on-site

Sample	Cd (μgKg^{-1})	Pb (μgKg^{-1})
<i>Ulapaca thouarsii</i>	<LOD	744 ± 7
<i>Petroselinum crispum</i>	<LOD	198 ± 3
<i>Taraxacum officinale</i>	<LOD	990 ± 8

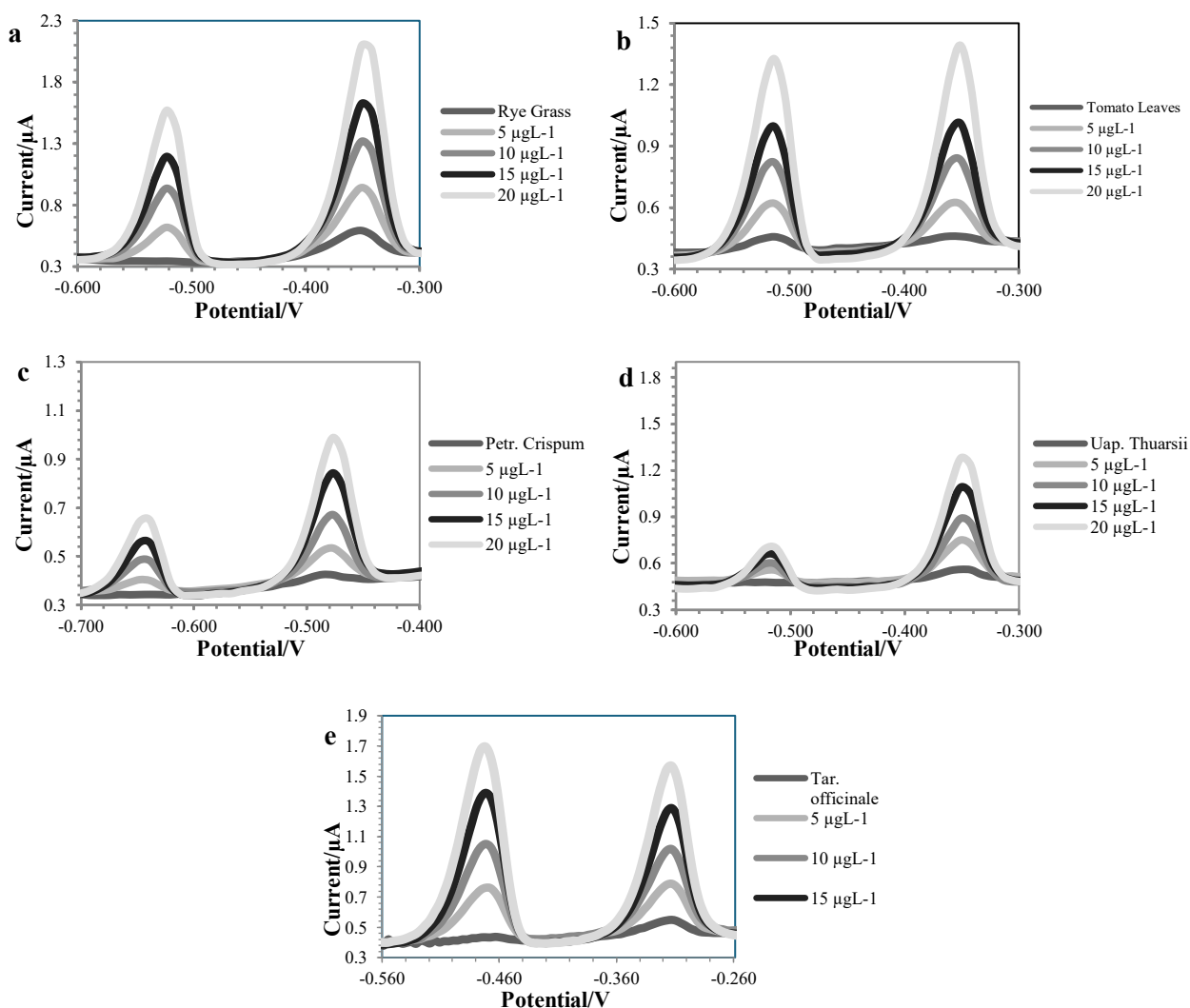


Figure 4 Voltammogram obtained from on-site analysis of Cd (first peak) and Pb (second peak) in the CRMs (a Rye Grass and b Tomato Leaves) and real samples (c *Petroselinum crispum*, d *Taraxacum officinale* and e *Ulapaca Thuarsii*).

4. Conclusions

This paper illustrates a simple, cost-effective method for the on-site quantification of Cd and Pb in plant samples using DP-ASV with either an iMF-GCE or an eMF-GCE. Despite acknowledge the environmental and toxicity limitations of mercury electrodes, and we are committed to exploring alternative materials, mercury electrodes remain unparalleled in precision and sensitivity. This is particularly important in the case of complex matrices and detection at the trace level. For the

purpose of the current study, namely, the analysis of complex acid matrices and the achievement of sub-legal detection limits for both Cd and Pb, mercury film electrodes proved to be a valid and efficient solution. Although this study paves the way to greener alternatives for future research, the unmatched analytical performance of the mercury electrodes becomes an essential and indispensable tool for the tight requirements of both this study and beyond. The procedure involves the pretreatment of samples through a mini dry bath to extract Cd and Pb from the matrix. Preliminary experiments conducted with the OVAT method revealed that iMF-GCE exhibited a slightly superior performance than eMF-GCE. In addition, the iMF-GCE procedure is more straightforward and requires fewer reagents, making it more suitable for on-site applications, so it was chosen for the subsequent experiments. However, the preliminary experiments using the OVAT method did not yield enough information to improve the operative conditions, thus leading to the adoption of an experimental design approach. Through systematic optimization by the help of Plackett-Burman and FCCD steps, deposition parameters for the optimized method were further improved with optimal conditions of $E_{\text{dep}} = -1.20 \text{ V}$ and $t_{\text{dep}} = 195 \text{ s}$, showing great accuracy and sensitivity improvements and recovery rates for Cd of 86.3 % and Pb of 96.4 %. At the same time, LOD values significantly decreased both for Cd (from $1.54 \mu\text{g L}^{-1}$ to $0.629 \mu\text{g L}^{-1}$) and Pb (from $0.15 \mu\text{g L}^{-1}$ to $0.045 \mu\text{g L}^{-1}$), highlighting the method's sensitivity. The absence of measurable peaks in blanks' voltammograms highlights the method's reliability in quantifying trace element concentrations in samples. Validation through CRMs confirmed the method's consistency, and the analysis of the same samples in the laboratory and field proved its applicability for accurate on-site analysis.

This work highlights the efficacy of combining sample pretreatment, method optimization through experimental design, and simple electrochemical techniques: the iMF-GCE coupled to DPASV provides a practical, sensitive, and accurate analytical method that can be easily deployed under various field conditions, making it a valuable tool for the determination of trace elements in official plants. The procedure involves minimal reagents, cost-effective apparatus, and straightforward laboratory requirements, making it highly accessible for many users. In particular, the method can be of use for control agencies and small-medium companies to assess plant safety. Finally, the method can find applications in other fields requiring the analysis of plant matrices, such as environmental monitoring and food testing. This work contributes to forward the accessibility to a high-quality analytical tool for different applications, providing an economical, portable, and reliable solution.

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Supplementary Material

Supplementary Table 1 Parameters adopted during the preliminary analysis with iMF-GCE, eMF-GCE and during the electrochemical cleaning.

	Parameter		iMF-GCE	eMF-GCE	Electrochemical cleaning of the eMF-GCE
Pretreatment Settings	Deposition potential (V)	E_{dep}	-1.00	-0.15	-1.2
	Deposition time (s)	t_{dep}	1200	300	180
DPV Settings	Equilibration time (s)	t_{eq}	15	15	10
	Initial potential (V)	E_{begin}	-1.00	-0.90	-1.20
	End potential (V)	E_{end}	-0.9	-0.15	-0.3
	Step potential (V)	E_{step}	0.004	0.004	0.004
	Pulse potential (V)	E_{pulse}	0.01	0.02	0.02
	Pulse duration (s)	t_{pulse}	0.015	0.015	0.015
	Scan Rate (Vs^{-1})	-	0.01	0.01	0.05

Supplementary Table 2 Levels and values for the factors included in the Plackett–Burman design.

Factor		Preliminary value	-1	1
Hg concentration (mg/kg)	[Hg]	0.7	0.5	1
Deposition time (s)	t_{dep}	180	60	240
Deposition potential (V)	E_{dep}	-1.2	-0.8	-1.2
Pulse duration (s)	t_{pulse}	0.015	0.009	0.021
Step potential (V)	E_{step}	0.004	0.002	0.009
Pulse potential (V)	E_{pulse}	0.02	0.01	0.03

Supplementary Table 3 Design matrix with coded values of each experiment and response values obtained with the Plackett–Burman design

							Cd		Pb	
Experiment	[Hg]	t_{dep}	E_{dep}	t_{pulse}	E_{step}	E_{pulse}	R^2	RSD%	R^2	RSSD%
1	1	-1	-1	1	-1	1	0.998	9.97	0.999	8.08
2	1	1	-1	-1	1	-1	0.999	3.12	0.991	6.19
3	1	1	1	-1	-1	1	0.994	18.52	0.999	14.23
4	-1	1	1	1	-1	-1	0.992	0.86	0.987	2.06
5	1	-1	1	1	1	-1	0.998	7.40	0.990	6.08
6	-1	1	-1	1	1	1	0.998	7.42	0.999	10.97
7	-1	-1	1	-1	1	1	0.973	6.18	0.988	8.19
8	-1	-1	-1	-1	-1	-1	0.997	5.84	0.999	8.44

Supplementary Table 4 Levels and values for the factors included in the FCCD

Factor		-1	0	1
Deposition time (s)	t _{dep}	60	150	240
Deposition potential (V)	E _{dep}	-1	-1	-1,2
Step potential (V)	E _{step}	0	0,1	0,01

Supplementary Table 5 Design matrix with coded values of each experiment and response values obtained with the FCCD

Experiment	t _{dep}	E _{dep}	E _{step}	Cd			Pb		
				R ²	RSD%	A	R ²	RSD%	A
1	-1	-1	-1	0,98	15,48	0	0,98	8,81	0
2	-1	1	-1	1	25,06	0,02	0,96	29,35	0,05
3	-1	-1	1	1	13,92	0,01	1	16,03	0,01
4	-1	1	1	0,99	20,36	0,02	1	26,5	0,04
5	1	-1	-1	0,92	16,43	0	0,95	14,44	0,01
6	1	1	-1	0,95	7,779	0,02	0,99	3,458	0,04
7	1	-1	1	0,96	11,79	0,01	0,86	15,71	0,01
8	1	1	1	1	8,623	0,11	1	9,297	0,08
9	-1	0	0	1	6,502	0,01	1	11,4	0,05
10	1	0	0	0,97	13,45	0,04	1	11,58	0,04
12	0	-1	0	0,96	10,63	0	0,99	7,215	0,01
13	0	1	0	0,89	16,36	0,01	0,9	5,364	0,03
14	0	0	-1	0,96	9,488	0,01	1	6,958	0,03
15	0	0	1	0,99	9,392	0,01	0,99	14,44	0,02
16	0	0	0	0,98	12,82	0,03	0,98	15,42	0,03
17	0	0	0	1	6,141	0,03	1	4,022	0,04
18	0	0	0	1	13,14	0,02	0,99	8,208	0,02
19	0	0	0	1	9,255	0,04	1	6,223	0,05
20	0	0	0	0,99	35,33	0,02	0,99	7,181	0,02

Supplementary Table 6 *p*-values of factors calculated for each response of Plackett-Burman Design.

Variable	Cd R ²	Pb R ²	Cd RSD%	Pb RSD%
b ₀	0.0000	0.0000	0.0000	0.0025
[Hg]	0.0564	0.6669	0.0800	0.7896
T _{DEP}	0.2296	0.9298	0.0044	0.8043
E _{DEP}	0.0471	0.2013	0.0878	0.9614
T _{PULSE}	0.1138	0.9402	0.0785	0.5509
E _{STEP}	0.5645	0.4037	0.0115	0.9743
E _{PULSE}	0.2150	0.1204	0.1167	0.1818

Cd R ²					Pb R ²				
Variable	Coef.	Std.dev.	Conf.Int.	p-value	Variable	Coef.	Std.dev.	Conf.Int.	p-value
b₀	0.9937	0.001	0.0037	0.0000	b₀	0.994	0.001	0.0041	0.0000
[Hg]	0.003	0.001	0.0037	0.0564	[Hg]	0.001	0.001	0.0041	0.6669
T_{DEP}	0.002	0.001	0.0037	0.2296	T_{DEP}	0.0001	0.001	0.0041	0.9298
E_{DEP}	-0.003	0.0015	0.0036	0.0471	E_{DEP}	-0.002	0.001	0.0040	0.2013
T_{PULSE}	0.003	0.001	0.0037	0.1138	T_{PULSE}	-0.0001	0.0001	0.0041	0.9402
E_{STEP}	-0.001	0.0015	0.0036	0.5645	E_{STEP}	-0.001	0.001	0.0040	0.4037
E_{PULSE}	-0.002	0.0015	0.0036	0.2150	E_{PULSE}	0.003	0.001	0.0040	0.1204

Cd RSD%					Pb RSD%				
Variable	Coef.	Std.dev.	Conf.Int.	p-value	Variable	Coef.	Std.dev.	Conf.Int.	p-value
b ₀	0.217	0.008	0.0195	0.0000	b ₀	815337450	20	41.828	0.0025
[Hg]	-0.017	0.008	0.0195	0.0800	[Hg]	0	20	41.828	0.7896
T _{DEP}	0.034	0.008	0.0195	0.0044	T _{DEP}	0	20	41.828	0.8043
E _{DEP}	0.016	0.008	0.0191	0.0878	E _{DEP}	0	20	40.983	0.9614
T _{PULSE}	0.017	0.008	0.0195	0.0785	T _{PULSE}	-110821346	20	41.828	0.5509
E _{STEP}	-0.0270	0.008	0.0191	0.0115	E _{STEP}	0	20	40.983	0.9743
E _{PULSE}	0.014	0.008	0.0191	0.1167	E _{PULSE}	815337450	20	40.983	0.1818

Supplementary Table 7 p-values of factors calculated for each response of FCCD.

Variable	Cd peak A	Pb peak A	Cd R ²	Pb R ²	Cd RSD%	Pb RSD%
b0	0.009	0	0	0	0.002	0.009
E _{DEP}	0.03	0.52	0.05	0.21	0.5	0.03
T _{DEP}	0.01	0.0007	0.83	0.55	0.8	0.01
E _{ST}	0.09	0.87	0.15	0.71	0.8	0.09
E _{DEP} *T _{DEP}	0.09	0.56	0.42	0.04	0.4	0.09
E _{DEP} *E _{ST}	0.1	0.29	0.26	0.12	0.9	0.1
T _{DEP} *E _{ST}	0.1	0.61	0.88	0.22	0.9	0.1
E _{DEP} ²	0.21	0.2	0.23	0.61	0.8	0.21
T _{DEP} ²	0.58	0.25	0.02	0.04	0.8	0.58
E _{ST} ²	0.009	0	0	0	0.002	0.009

Cd Peak Area					Pb Peak Area				
Variable	Coef.	Std.dev.	Conf.Int.	p-value	Variable	Coef.	Std.dev.	Conf.Int.	p-value
b0	0.018	0.006	0.013	0.010	b0	0.031	0.005	0.010	0.000
E _{DEP}	0.013	0.005	0.012	0.034	E _{DEP}	0.003	0.004	0.010	0.523
T _{DEP}	0.016	0.005	0.012	0.011	T _{DEP}	0.021	0.004	0.010	0.001
E _{ST}	0.010	0.005	0.012	0.090	E _{ST}	0.001	0.004	0.010	0.875
E _{DEP} *T _{DEP}	0.011	0.006	0.013	0.089	E _{DEP} *T _{DEP}	0.003	0.005	0.011	0.564
E _{DEP} *E _{ST}	0.011	0.006	0.013	0.096	E _{DEP} *E _{ST}	0.005	0.005	0.011	0.285
T _{DEP} *E _{ST}	0.011	0.006	0.013	0.097	T _{DEP} *E _{ST}	0.003	0.005	0.011	0.607
E _{DEP} ²	0.01	0.01	0.022	0.211	E _{DEP} ²	0.011	0.008	0.018	0.205
T _{DEP} ²	-0.01	0.01	0.022	0.575	T _{DEP} ²	-0.010	0.008	0.018	0.247
E _{ST} ²	-0.002	0.001	0.022	0.810	E _{ST} ²	-0.003	0.008	0.018	0.676

Cd R ²					Pb R ²				
Variable	Coef.	Std.dev.	Conf.Int.	p-value	Variable	Coef.	Std.dev.	Conf.Int.	p-value
b0	0.979	0.009	0.019	0.000	b0	0.99	0.01	0.023	0.000
E _{DEP}	-0.017	0.008	0.017	0.051	E _{DEP}	-0.01	0.01	0.021	0.210
T _{DEP}	0.002	0.008	0.017	0.829	T _{DEP}	0.01	0.01	0.021	0.550
E _{ST}	0.012	0.008	0.017	0.154	E _{ST}	-0.004	0.001	0.021	0.709
E _{DEP} *T _{DEP}	0.007	0.009	0.019	0.419	E _{DEP} *T _{DEP}	0.02	0.01	0.024	0.040
E _{DEP} *E _{ST}	0.010	0.009	0.019	0.259	E _{DEP} *E _{ST}	-0.02	0.01	0.024	0.123
T _{DEP} *E _{ST}	-0.001	0.009	0.019	0.883	T _{DEP} *E _{ST}	0.01	0.01	0.024	0.218
E _{DEP} ²	0.02	0.015	0.033	0.231	E _{DEP} ²	0.01	0.02	0.041	0.611
T _{DEP} ²	-0.04	0.015	0.033	0.024	T _{DEP} ²	-0.04	0.02	0.041	0.044
E _{ST} ²	0.01	0.015	0.033	0.470	E _{ST} ²	0.01	0.02	0.041	0.639

Cd RSD%					Pb RSD%				
Variable	Coef.	Std.dev.	Conf.Int.	p-value	Variable	Coef.	Std.dev.	Conf.Int.	p-value
b0	16	4	8.115	0.002	b0	7	1	3.105	0.000
E.DEF	-2	3	7.465	0.503	E.DEF	-4	1	2.856	0.015
T.DEF	1	3	7.465	0.773	T.DEF	1	1	2.856	0.380
E.ST	-1	3	7.465	0.768	E.ST	2	1	2.856	0.170
E.DEF*T.DEF	-3	4	8.346	0.375	E.DEF*T.DEF	-6	1	3.193	0.002
E.DEF*E.ST	0	4	8.346	0.937	E.DEF*E.ST	0	1	3.193	0.816
T.DEF*E.ST	0	4	8.346	0.939	T.DEF*E.ST	0	1	3.193	0.641
E.DEF^2	-2	6	14.235	0.802	E.DEF^2	5	2	5.447	0.089
T.DEF^2	2	6	14.235	0.775	T.DEF^2	0	2	5.447	0.812
E.ST^2	-2	6	14.235	0.740	E.ST^2	4	2	5.447	0.150

Bioaccumulation of Trace Elements in Malagasy Medicinal Plants: Implications for Environmental Monitoring and Traditional Medicine Safety

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Abstract

Madagascar is one of the largest hotspots of global biodiversity with high levels of endemics, with many of its plants having been or being used in traditional medicine. Despite its natural richness, Madagascar is one of the poorest nations in the world, with plants being a valuable resource, including in medicine. The increase in anthropogenic activities with the lack of control of the environment raises concerns about the impact of heavy metal bioaccumulation on the quality of the environment and human well-being. The present study examined the accumulation of essential and non-essential elements in three Malagasy medicinal plants (*Ficus reflexa*, *Ficus lutea*, and *Uapaca thouarsii*) sampled in three areas with different degrees of anthropogenic impact: the primary forest of Maromizaha (low impact), Mitsinjo Park (moderate impact), and the Forest of Torotorofotsy (high impact, vicinity of the Ambatovy mine). The elemental content was determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) after the application of microwave-assisted acid digestion. The results showed that Cr, Co, and V contents in the soil samples were higher than the Italian legal thresholds, while no accumulation of the mentioned elements was found in the plant tissues, showing the presence of metal uptake regulatory physiological mechanisms. The arsenic content in the *Uapaca*

thouarsii of the Torotorofotsy site (0.026 mg/kg) was higher than the EU maximum level permitted in general food vegetal based commercialize in powder form (0.020 mg/kg), showing the need for continuous monitoring. This type of study results is crucial in regions where, in the absence of adequate systematic monitoring of ecosystem health and awareness among populations and institutions, conservation strategy efforts may be compromised.

1. Introduction

Madagascar is a biodiversity hotspot that is rich in endemic flora. The diverse botanical richness of this island includes numerous plant species with cosmetic and medicinal properties. Rural populations treat lots of common diseases with traditional medicine based on plant remedies, in particular for the cure of gastrointestinal disorders, malaria/fever, and as remedy for rheumatisms, colds, skin illnesses, and inflammations.[1] Plants are essential resources for impoverished nations as food, as well as in cosmetics and medicine since indigenous populations in developing countries extensively utilize botanical assets. In countries like Madagascar, where modern healthcare is often unavailable or unaffordable, reliance on plant-based remedies is particularly significant. The therapeutic properties of Malagasy endemic plants, such as *Ficus reflexa*, *Ficus lutea*, and *Uapaca thouarsii*, have long been recognized in medicinal practices. Fioccardi et al. (2022) confirmed that these species possess high levels of polyphenols and flavonoids, suggesting that their antioxidant and anti-inflammatory properties underlie their widespread mainstream use.[2]

In general, the economic downturn in developing nations has increased pharmaceutical prices, making traditional medicine and medicinal plants more appealing alternatives. In these cases, people sometimes rely on traditional knowledge of local plants without considering the quality of the raw material, the dosage of intake, and its possible toxicity. [2–4] Studying these plant species could reveal the scientific basis of traditional medicinal practices, understand the medicinal and cosmetic potential of endemic plants already used by local populations, and provide tangible benefits to disadvantaged populations.[2,4,5] On the other hand, the impact of anthropogenic activities can alter the quality of these resources, including chemical composition, and potentially compromise their safety. In this context, studying the bioaccumulation of essential and non-essential elements in therapeutically relevant plants is crucial not only for understanding ecological dynamics but also for assessing the potential health risks associated with their use and evaluating their role as bioindicators of environmental quality. [6–8] In these areas, climate change's adverse effects may

also increase anthropogenic pressures' impacts on the ecosystem, including chemical pollution. In this context, the already fragile infrastructures and limited resources may fall under the load of environmental hazards.[9] In particular, the knowledge about pollution in the sub-Saharan ecosystem is minimal, despite the fragility of this area.[10]

In addition, the legislation on pollution and conservation in developing countries, such as Madagascar, is lacking.[11] This gap in the literature highlights a potential underestimation of the risks associated with biodiversity loss in Madagascar, potentially spoiling the efforts on ecosystem conservation and wildlife protection. As a matter of fact, the identification and assessment of chemical pollution has been defined as a priority issue according to the National Health and Pollution Action Plan of Madagascar.[12] Furthermore, since plants passively absorb elements from the surrounding soil and water, their elemental composition reflects environmental conditions, making them effective tools for biomonitoring pollution levels in affected ecosystems. By analyzing the accumulation of both essential and non-essential elements in medicinal plants, this study aimed to determine whether contamination from anthropogenic sources influences the safety of traditional herbal remedies. Understanding these dynamics is key not only for safeguarding public health but also for implementing conservation strategies and promoting the sustainable use of Madagascar's rich botanical heritage. and A further aim of the study is to evaluate the possible role of Malagasy medicinal plants in environmental biomonitoring. Three species of phytotherapeutic interest were selected: *Ficus reflexa*, *Ficus lutea*, and *Uapaca thouarsii*. Sampling was conducted between June 2022 and February 2023 in three areas with varying anthropogenic impact: the primary forest of Maromizaha (low impact), Mitsinjo Park (intermediate exposure), and Torotorofotsy Forest (high contamination, proximity to the Ambatovy mine). Leaves, soil, and water were collected and analyzed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) or Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), following microwave-assisted acid digestion, to quantify the present elements. Statistical data processing enabled the evaluation of differences in elemental composition between species and collection sites, providing useful data for the safe use of medicinal plants and environmental monitoring.

2. Materials and Methods

2.1. Instrument and reagent

The study was conducted using highly sensitive and selective- analytical instrumentation and analytical-grade reagents to ensure the accuracy and reliability of elemental analysis. The microwave-assisted digestion system used for sample mineralization was a Milestone MLS-1200 Mega, equipped with high-pressure TFM (modified PTFE) vessels, allowing for controlled temperature and pressure conditions to optimize the decomposition of organic matrices while preventing the loss of volatile elements. The elemental analysis was performed using three complementary techniques: Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Thermo Scientific iCAP 7000), Inductively Coupled Plasma Mass Spectrometry (ICP-MS, Thermo Element XR), and Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS, Perkin Elmer XXXX). In all cases external calibration was carried out using multi-element standard solutions at different concentrations prepared from certified 1000 mg/L stock solutions (Sigma-Aldrich, USA). For sample preparation, all reagents were selected to minimize risks of contamination. Nitric acid (HNO_3 , 65%), hydrogen peroxide (H_2O_2 , 30%), and hydrochloric acid (HCl , 37%) (all from Sigma-Aldrich, trace metal grade) were used for mineralization of the biological and environmental samples. Ultrapure water HPW (18.2 M Ω -cm) was obtained from a Millipore Milli-Q system and was used for all dilution steps and sample rinsing to prevent contamination.

2.2. Sample collection and sampling sites

The sampling campaign was conducted between June 2022 and February 2023 in the Marais de Torotorofotsy (Ambatovy), Mitsinjo Park, and Maromizaha primary forest. These three sites are located in Eastern Madagascar's Alaotra-Mangoro region, specifically in the Moramanga district. This region of Madagascar is characterized by a tropical/subtropical climate tempered by altitude with high rainfall and a specific rainy season. The primary forest of Maromizaha (18°56'49" S; 48°27'55" E) is a protected area spanning the rural municipalities of Andasibe and Ambatovola. It is surrounded by three forest blocks: the Special Reserve Analamazaotra to the northwest, the Vohimana forest to the northeast, and the Vohidrazana forest to the east. Mitsinjo Park (18°56'30" S; 48°24'30" E) is a community-managed protected area, established as part of a conservation initiative; it consists of secondary and regenerating forests adjacent to the Andasibe-Mantadia National Park. Mitsinjo experiences moderate anthropogenic pressure due to its proximity to human

settlements and ecotourism activities while still serving as a refuge for Madagascar's unique biodiversity. The Torotorofotsy forest (18°55'30" S; 48°25'30" E) is a Ramsar-designated site recognized for its ecological significance (Ramsar Sites are wetlands of international importance designated under the Convention on Wetlands). This area consists of swamp forests, grasslands, and open water bodies. Situated in proximity to the Ambatovy nickel and cobalt mine, Torotorofotsy is subject to varying levels of anthropogenic influence, making it a key area for environmental monitoring and conservation efforts. In particular, the Ambatovy mine, located near the Torotorofotsy Forest, is one of the largest producers of nickel and cobalt globally, with an annual production of approximately 36,085 tons of nickel and 3,387 tons of cobalt. Given the scale of operations, continuous monitoring is essential to assess the potential environmental impact of these mining activities on local ecosystems and medicinal plants. [13]The sampling campaign was performed by employing expert local guides following a standardized protocol applied consistently at each site. Plant species selected for the analysis included *Uapaca thouarsii*, *Ficus reflexa*, and *Ficus lutea*, identified for their role in Malagasy traditional medicine (Table 1). Leaves of these plants were collected at each site and stored in labeled, clean plastic bags. Additionally, soil and water samples were obtained from each area to evaluate the concentrations of analytes in the plant environment. A 10 cm layer of soil was taken at three different points in each park and stored in labeled clean plastic bags, while two water samples were collected from surface water sources located near the analyzed plants and stored in Polyethylene (PE) containers.

Table 1 List of medicinal plants sampled in the study areas

Vernacular Name	Scientific Name	Family
Nonoka Keliravina	<i>Ficus reflexa</i> Thunb.	Moraceae
Nonoka Beravina	<i>Ficus lutea</i> Vahl.	Moraceae
Voapaka Keliravina	<i>Uapaca thouarsii</i> Baill.	Euphorbiaceae

2.3. Pretreatment

Vegetal samples were weighed and dried at 60°C in a heater to determine the humidity content. Dried leaves were ground using a ball mill to obtain a homogenous powder. Mineralization was

performed using acid digestion. Samples (0.5 g) were weighed and processed in triplicate to assess repeatability. Before digestion, each aliquot was pre-wetted by adding 2 mL HPW, followed by the addition of 6 mL HNO₃ and 2 mL H₂O₂. The mixture was allowed to react for 30 min at room temperature to enhance pre-oxidation of the organic matrix. After 30 min under a fume hood, mineralization was carried out following a five-step microwave digestion program. The process began with a preheating phase at 250 W for 2 min, followed by a stabilization period of 0 W for 2 min. The power was then increased to 250 W for 6 min, followed by a ramp-up phase at 400 W for 5 min, and concluded with a high-power digestion step at 600 W for 5 min, ensuring the complete mineralization of the organic matrix. The resulting solutions were filtered with 0.45µm Whatman filters and diluted with HWP at 50 mL. This procedure was also applied to prepare blank samples for quality control.

Soil samples were first sieved to divide the fraction of solid particles with grain sizes larger than 0,075 mm and quantify the percentage of the latter in total. The soils were then ground in a mill and homogenized. 0,1g of the resulting powder of each sample was mineralized in a microwave oven with 5 mL of aqua regia (the solution comprised a 3:1 mixture of hydrochloric acid and nitric acid, respectively). The program began at 250 W for 3 min, followed by 250 W for 5 min, and 550 W for 3 min. A final ventilation phase of 90 min at 0 W was applied to allow for controlled cooling and pressure release. The obtained solutions were filtered and diluted to 50 mL with HPW.

2.4. Element Analysis

2.5. Statistical analysis

Chemometric treatments were performed to explore the correlation between all variables and the similarities or differences between samples. Data were pretreated and processed using XLSTAT software (Addinsoft, Paris, France). For elements with concentrations below the limit of detection (LOD) in more than 40% of the measurements, the variables were not considered for statistical analysis. In the case of a few missing data (concentrations lower than the LOD), a random value was set between zero and the detection limit. Principal Components Analysis (PCA) and Hierarchical Clusters Analysis (HCA) were employed to assess differences in metal concentrations in plants of the three species and between samples from different origins, allowing for the simultaneous analysis of elements considered as dependent variables. This multivariate approach captures patterns and interactions between metals and samples that might be overlooked in univariate analyses, providing a comprehensive view of how geographic origin influences metal profiles.[14] PCA was employed to reduce the dimensionality of the dataset and uncover correlations among chemical variables in different environmental compartments. HCA was employed to classify the samples based on their elemental composition, uncovering homogeneous groups regarding their origin and the

environmental conditions of the sampling locations. [15] A one-way ANOVA was performed to compare the mean concentrations of all analytes among the three site and plant species. Following a significant ANOVA result ($p < 0.05$), a Tukey HSD (Honestly Significant Difference) post-hoc test was applied to determine pairwise differences between groups. To ensure reliability of analytical data, quality control procedures were followed, including analysis of procedural blanks for the verification of the absence of detectable contamination, using certified reference material for verification of the accuracy of measurements, and replicating analysis of at least 10% of the samples to verify the repeatability of the results. Limits of detection (LOD) and quantification (LOQ) were estimated for each analyzed element respectively, with the data obtained meeting international standards of analytical validity.

3. Results and Discussion

3.1. Soil Samples

In terms of absolute concentrations, the elements detected in the soil samples, ordered from highest to lowest, were as follows: Fe > Al > Ti > Mg > Ca > K > V > Mn > Cr > Ce > Na > Ni > Zn > Cu > Ba > Nd > Pb > Co > Gd > Y > As > Pr > Sm > Dy > Yb > Er > Tb > Cd > Eu > Ho > Tm (Supplementary Table 1). Among these, macronutrients essential for plant growth include K (260 mgkg^{-1}), Ca (621 mgkg^{-1}), and Mg (913 mgkg^{-1}), which play fundamental roles in plant metabolism, including enzyme activation, photosynthesis, and osmoregulation. [16,17] The high average concentrations of Fe (37060 mgkg^{-1}) and Al (33090 mgkg^{-1}) suggest their abundance in the parent rock material, although their essentiality varies among plant species, as excessive levels may lead to toxicity in certain conditions. [18,19] Micronutrients, which are required in trace amounts for enzymatic functions and metabolic processes, include Mn (166 mgkg^{-1}), Zn (25.2 mgkg^{-1}), Cu (24.2 mgkg^{-1}), and Co (12.1 mgkg^{-1}). [20] While these elements are detected at relatively moderate concentrations in the soil, their availability for plant uptake is subject to complex biochemical and physicochemical factors. As there are no regulations regarding PTEs levels in Malagasy soils, the data obtained were compared to the Italian D.Lgs. 152/2006 threshold values. [21] The regulated elements (As, Cd, Co, Cr, Cu, Ni, Pb, V, and Zn) were then compared with the respective limits to identify the possible exceedances and potential influence of the Ambatovy mine on soil contamination. Different samples of soil collected from the three areas were found to exceed the reference limits. Among the elements determined, Cr, Co, and V exceeded Italian legal limits in multiple samples. Cr exceeded the 150 mgkg^{-1} limits in the samples collected in the locality of Mitsinjo, with a value up to 178 mgkg^{-1} , hypothesizing a potential

local source of contamination. V recorded high values in many localities, with a peak of 597 mgkg^{-1} at Torotorofotsy, which was well above the 90.0 mgkg^{-1} limit. Co, a metal exploited in the Ambatovy mine, recorded concentrations above regulatory levels (20.0 mgkg^{-1}) in several samples, with the peak concentration at Torotorofotsy (56.5 mgkg^{-1}), where Ni, instead, never exceeded the limit of 120 mgkg^{-1} . Such high concentrations of PTEs could unveil an anthropogenic contribution; in particular, one can hypothesize a proximity impact due to the presence of the Ambatovy mine where Co is exploited, as mirrored by the high concentration of the latter recorded in the samples. However, further investigations should be performed to account for the influence of the territory's natural geochemical variability, which could influence the observed values.

1.1 Literature comparison

Monitoring studies of environmental pollution in the Sub-Saharan region are limited, and this lack of information could compromise the conservation efforts, as discussed before. In this context, comparing data obtained in Madagascar with data collected in other areas of East Africa could be useful for understanding accumulation and environmental pollution dynamics in tropical environments. Comparative data were obtained from previous studies (Supplementary Table 2 and 3): one of these pertains to an analysis conducted in the same area of Madagascar examined in the present work, while the others refer to metal content in soils and sediments from Zambia and Uganda. The concentrations obtained in the present study highlight some similarities and differences concerning the few environmental biomonitoring data available for sub-Saharan Africa. Cr and Co concentrations result above the Italian law limits in Mitsinjo and Torotorofotsy but are comparable to literature data regarding the region of Chingola, Zambia (Cr in a range of $14\text{--}42 \text{ mgkg}^{-1}$ and Co in a range of $9\text{--}35 \text{ mgkg}^{-1}$) while data of Lake Victoria in Uganda showed similar levels of Cr (49 mgkg^{-1}) but lower concentration of Co (1.8 mgkg^{-1}), suggesting an inferior anthropic impact. Similarly, Ni concentrations are higher in Torotorofotsy (67.2 mgkg^{-1}) than in Zambia (25 mgkg^{-1}) or in Uganda (15.3 mgkg^{-1}) supporting the hypothesis that Ambatovy mine in Torotorofotsy could have an active influence on the element content on the area. Results obtained for PTEs in this study suggest a direct influence of the local mine activity as already observed in Zambia and Uganda. [22–28] These findings underline the importance of consistent and continuous monitoring of ecosystem pollution to ensure population, animal, and plant health.

3.2. Plant Samples

The elements were detected in plant samples in descending order of concentration as follows: Na > Ca > K > Mg > Sr > Mn > Al > Fe > Ba > Ni > Zn > Cu > Ti > Co > Sc > rare earth elements (REEs), which were present in trace amounts (Supplementary Table 4). The component at highest concentration in the samples, among the investigated analytes, was Na, followed by Ca, K, Mg, and Sr. These elements, except Sr, are macronutrient components of crucial importance to the physiology of plant organisms. Na and K are required for osmoregulation and ion homeostasis control, Ca for the strengthening of the cell wall structure, Mg for the core of the pigment of the photosynthetic apparatus, and as enzyme co-factor. Sr, although non-essential for the diet, can be absorbed by the roots due to its structural analogy with Ca. [29–33]

Of the other components, Mn, Al, Fe, and Ba were at the intermediate levels of concentration. Mn has a role in photosynthesis and redox processes, Fe in the electron transport of the chloroplast and mitochondria. Al, although non-essential for the diet, influences the growing ability of the roots; Ba has no known biological effects. [18,19,34,35] Among the transition metal components, Ni, Zn, Cu, Ti, Co, and Sc are present at trace concentrations. These elements have the following roles in plants: Ni for the participation in the enzyme urease, Zn for the protein synthesis and the enzyme activating capability, Cu for the oxidative stress co-factor capability Co for the nitrogen fixative capability; Ti and Sc have no known biological effects, even if Ti influences positively the growing ability of the plants. [20,36–39] REEs were detected at the lowest concentrations ($<10\mu\text{gkg}^{-1}$); in particular La, Ce, and Nd have been investigated for their role in plant metabolism, although they are not classified as essential elements their influence and function remain mostly undefined. [7]

Among the essential and non-essential elements, the levels were relatively low, with most of the elements below the LOD values. Since there are no Malagasy regulations for the levels of these elements in food or medical plants, the results were compared to European legislation. Regulation (EU) 2023/915 establishes maximum levels of contaminants in various categories of plant-based foods. Although there are no specific limits for medicinal plants or tree leaves, it is possible to compare the results obtained with general ranges. The concentrations of Pb and Cd in the analyzed samples were below the LOD values ($\text{LOD}_{\text{Cd}} = 1.8 \times 10^{-5} \text{ mgkg}^{-1}$, $\text{LOD}_{\text{Pb}} = 3.3 \times 10^{-5} \text{ mgkg}^{-1}$). According to regulatory standards, the maximum permissible level of Cd is 0.020 mgkg^{-1} in spinach leaves and similar vegetables, whereas for leafy vegetables, the limit is set at 0.050 mgkg^{-1} . Detectable concentrations of As were found only in *Uapaca thouarsii* collected in Torotorofotsy (0.026 mgkg^{-1}),

and *Ficus reflexa* collected in Mitsinjo and Maromizaha (0.0005 mgkg⁻¹ and 0.015 mgkg⁻¹). EU regulation fixed the limit for general food powder at 0,020 mgkg⁻¹ and at 0.15-0.30 mgkg⁻¹ for rice and cereal-based products. These results indicate that the As concentrations in the plant samples analyzed in the present work are largely within acceptable safety standards, as stipulated by EU regulations. Only *Uapaca thouarsii* sampled in Torotorofotsy just exceeded the 0.020 mgkg⁻¹ limit stipulated for general food powders but was well below the less restrictive limits stipulated for rice and cereal products (0.15–0.30 mgkg⁻¹)[40]. Although these concentrations do not pose an immediate threat for human health, the presence of measurable As levels, particularly in *Uapaca thouarsii*, highlights the necessity for continued monitoring, especially in areas affected by mining activity, to ascertain the degree of potential long-term accumulation and environmental risk.

Notably, although some PTEs in the soils were found to be higher than the threshold values, plant analyses did not reflect the same pattern, suggesting the presence of regulatory mechanisms that control metal assimilation. [7]These findings emphasize the importance of integrating soil and plant analyses when evaluating elemental bioavailability and potential contamination risks. Soil composition alone is insufficient to predict plant metal accumulation, as physiological mechanisms regulate nutrient uptake and exclude or compartmentalize excess elements. Future research should focus on detailed speciation studies to assess the mobility and chemical forms of PTEs in the soil matrix as well as a broader assessment of plant tolerance strategies to refine environmental risk evaluations.

3.3. Statistical comparison

Elemental analysis of *Ficus lutea*, *Ficus reflexa*, and *Uapaca thouarsii* indicated a homogeneous chemical composition between samples, with few statistically significant differences between species and sampling areas. ANOVA, followed by the post-hoc Tukey HSD test, showed that only Zn, Ti, and Sc showed notable variance among the groups. Zn and Ti concentrations differed among the sampling areas (Zn p=0.043, Ti p=0.045) (Supplementary Tables 5, 6, and 7). Tukey's test showed that Zn content was significantly higher in leaves collected from Mitsinjo than in those collected from Maromizaha (p=0.00365) (Supplementary Table 5). In contrast, Ti exhibited dissimilarity between the Mitsinjo and Torotorofotsy groups (Supplementary Table 5). The ANOVA test revealed a significant difference in Sc concentrations (p=0.0493) difference among the investigated species (Supplementary Table 6). The Tukey test revealed that *Ficus lutea* had higher Sc concentrations than *Uapaca thouarsii* (p=0.0497) collected in the same site, suggesting different accumulation capacities

for the two plants. However, *Ficus reflexa* did not show significant differences compared to the other two species ($p > 0.12$), indicating a less-defined accumulation pattern for Sc (Supplementary Table 7).

Comparative analysis of soil and plant element concentrations in the three areas made it possible to evaluate the significant relationship between the geochemistry of the soils and the accumulation of elements in the plant species studied, even if, as discussed in chapter 3.2, metal assimilation is controlled by regulatory mechanisms. The results highlight that Mitsinjo soils have higher concentrations of Ba, Zn, and Ni than Maromizaha and Torotorofotsy, (Supplementary Tables 8,9, and 10). ANOVA confirmed the statistical significance of the differences in Ba ($p = 0.0019$) (Supplementary Table 8). The Tukey test allowed us to identify which specific group differences were significant: in particular, Ba concentrations were found to be significantly higher in Mitsinjo than in Maromizaha ($p = 0.0036$), while Torotorofotsy had substantially lower concentrations than Mitsinjo ($p = 0.0029$), suggesting an elevated variability of this element between the experimental sites (Supplementary Table 8). However, despite these variations in soil composition, no significant differences were detected in plant tissues for these elements. This suggests that the uptake mechanisms of these species may regulate metal absorption and mitigate potential environmental effects. These observations underscore the relevance of determining elemental concentrations in both plants and underlying soils for a clearer understanding of the absorption regimes and sources of pollution. Future research will involve chemical speciation examination of elements, as well as a full study of the soil mineralogical composition, to more clearly understand the observed behavior. The ANOVA for Ni and Co did not reveal statistically significant differences between locations, either in plants or soil (Supplementary Tables 10 and 11). Although the mean values suggest a higher accumulation of Ni and Co in plants from Mitsinjo and Torotorofotsy than in those from Maromizaha, these differences were not significant. This finding is particularly interesting, given that these sites are at different distances from the Ambatovy mine (varying degrees of environmental impact from mining activities). The limited variability observed for most elements suggests that the pedological conditions in the three localities are relatively similar and that the studied species can buffer chemical fluctuations in the substrate through physiological regulation mechanisms.

The absence of a clear separation between groups in multivariate analyses, namely PCA and HCA, is a positive outcome as it indicates that human activities, including the presence of the Ambatovy mine, do not appear to significantly alter the chemical composition of plants in the different study

areas (Figures 2 and 3). This result is particularly significant considering that Torotorofotsy represents the most degraded and anthropized area, Mitsinjo is a park located near villages and infrastructures, and Maromizaha is a protected primary forest with minimal anthropogenic impact. The fact that no substantial differences emerged among the plants from these areas suggests that element availability is regulated by relatively stable environmental factors, such as soil composition, pH, and biological processes affecting element mobility, rather than by anthropogenic activities. [6,8] Although some statistically significant differences emerged in ANOVA and Tukey test, these were not pronounced enough to determine clear patterns at a global level, confirming that plants can buffer potential chemical variations in the substrate through physiological regulatory mechanisms. To gain further insights into these dynamics, future studies should investigate the chemical speciation of elements to evaluate their actual bioavailability, utilize isotopic analyses to trace their mobility, and expand the number of examined plant species to verify whether some possess different accumulation capacities. Additionally, long-term monitoring efforts will be conducted in the coming years, with repeated sampling in the same areas, to track potential temporal variations in soil and plant chemical composition. These data will allow for a more precise evaluation of the evolution of element levels over time and the potential emergence of bioaccumulation or contamination patterns. Such investigations could further confirm the resilience of local ecosystems and provide useful indications for the management and conservation of tropical forests in contexts subject to anthropogenic pressures.

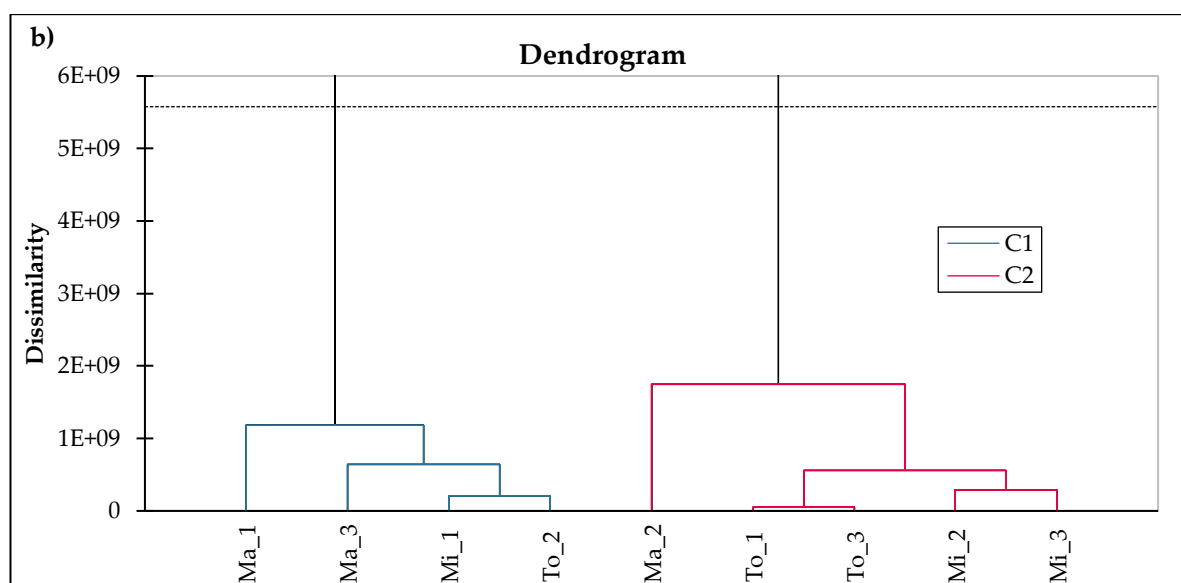
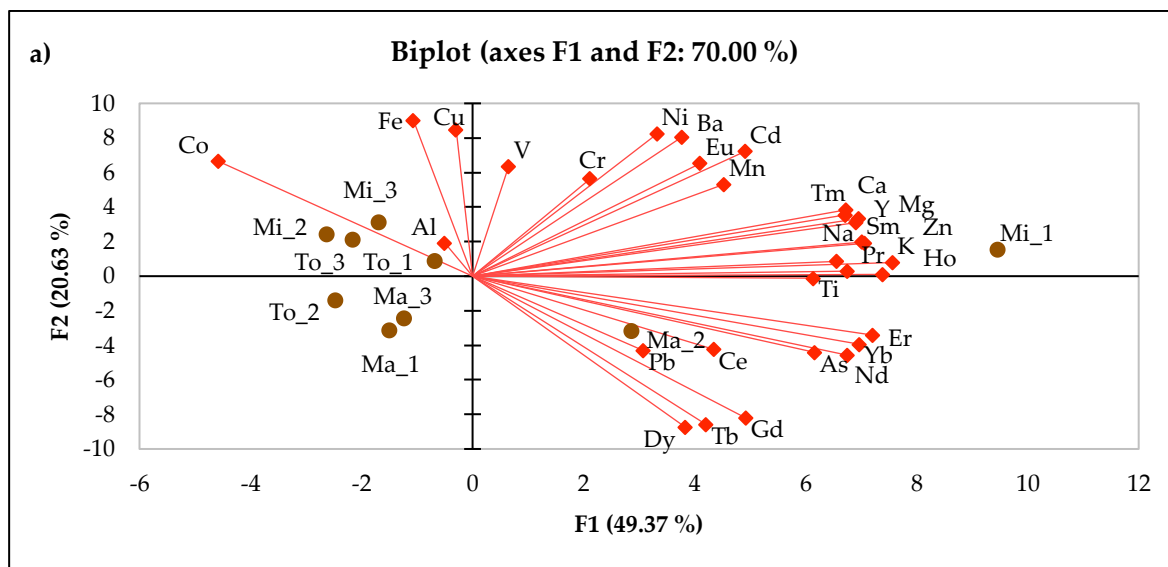


Figure 2 Biplot of scores and loadings for PCA (a) and Q-mode HCA dendrogram (b) of soil samples
(Ma_=Maromizaha forest, Mi_=Mitsinjo park, To_=)

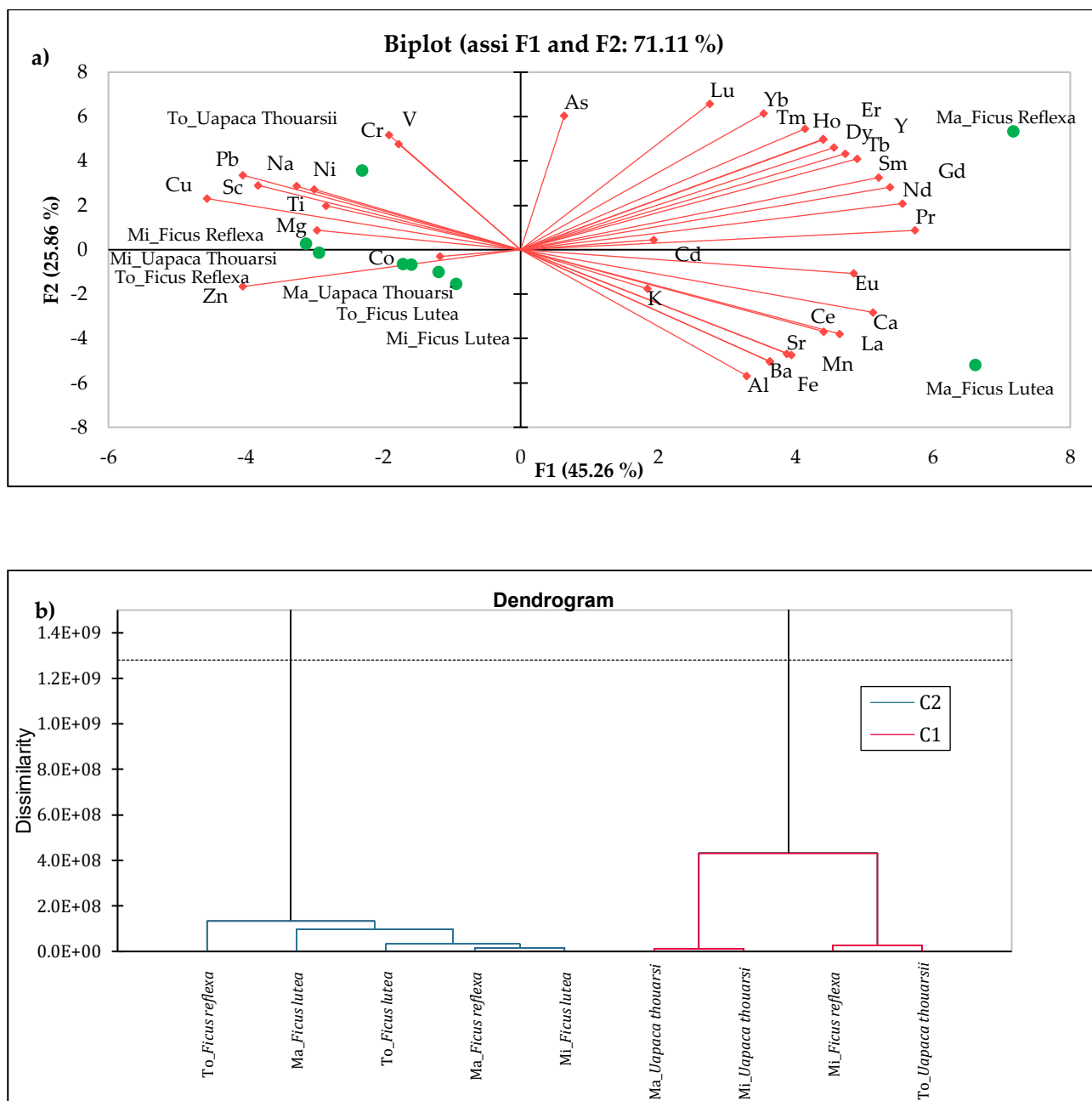


Figure 3 Biplot of PCA analysis (a) and dendrogram of Q-mode HCA analysis (b) of plant samples (Ma_=Maromizaha forest, Mi_=Mitsinjo park, To_=Torotorofotsy

4. Conclusion

This study focused on the quantification of mineral elements in three species of plants (*Ficus reflexa*, *Ficus lutea*, and *Uapaca thouarsii*) collected in three areas with different levels of anthropic impact: the primary Forest of Maromizaha, the Mitsinjo Park, and the Torotorofotsy Forest. The analysis of

soils revealed a relative homogeneity among the sampling sites; ANOVA highlighted that only Ba, Zn, and Ni concentrations were significantly different between the sites. Furthermore, among the elements determined, Cr, Co, and V exceeded Italian legal limits for multiple samples. Cr exceeded limits in the samples collected in the locality of Mitsinjo, while V recorded high values in many localities, with a peak of 597 mgkg⁻¹ at Torotorofotsy. Co, a metal exploited in the Ambatovy mine, recorded concentrations above regulatory levels in several samples, with the peak concentration at Torotorofotsy (56.6 mgkg⁻¹). Such high concentrations of PTEs could unveil an anthropogenic contribution; in particular, one can hypothesize a proximity impact due to the presence of the Ambatovy mine where Co is exploited, as mirrored by the high concentration of the latter recorded in the samples. Despite the high levels of some PTEs in soil samples, the concentrations of the same elements in plants do not underline an accumulation pattern derived from environmental pollution, suggesting the presence of plant regulatory mechanisms that control metal assimilation. The majority of leaf samples analyzed here is largely within acceptable safety standards, as stipulated by EU regulations. Only *Uapaca thouarsii* sampled in Torotorofotsy (0.026 mgkg⁻¹) just exceeded the 0.020 mgkg⁻¹ limit stipulated for general food powders but was well below the more restrictive limits stipulated for rice and cereal products (0.15–0.30 mgkg⁻¹) [40]. Although these concentrations do not pose an immediate toxicological threat, the fact that measurable As is present, particularly in *Uapaca thouarsii*, highlights the necessity for continued monitoring, especially in areas affected by mining activity, to ascertain the degree of potential long-term accumulation and environmental risk. Despite the ecological and socio-economic importance of these areas, knowledge of the biogeochemical cycle of elements in soils and plant tissues remains fragmentary. The lack of knowledge about regulatory mechanisms that control metal assimilation in Malagasy plants may give rise to an underestimation of environmental risk. At the same time, the limited information about the sub-Saharan environment and the lack of systematic studies regarding the adaptivity and resiliency of endemic Malagasy flora is an obstacle to the success of the conservation strategies applied in these areas. In addition, the fragility of the local infrastructure, an economy based primarily on agriculture, and the absence of adequate environmental regulations emphasize the vulnerability of this region and the effect of anthropogenic pollution. Without adequate systematic monitoring of the ecosystem health and sensibilization among populations and institutions, the efforts on conservation strategy could be compromised.

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Supplementary Material

Supplementary Table 1 Concentrations (mgkg⁻¹) and standard errors (SE) of analytes in soil samples collected in Maromizaha Forest, Mitsinjo Park, and Torotorofotsy Forest

Sample n°		1	2	3	4	5	6	7	8	9
Location		Maromizaha Forest			Mitsinjo Park			Torotorofotsy Forest		
Al	Mean	1980	9.82×10 ⁴	5.4×10 ⁴	2.45×10 ⁴	5.09×10 ⁴	6.42×10 ⁴	7.25×10 ⁴	2.42×10 ⁴	7.17×10 ⁴
	SE	50	3000	3000	1000	3000	4000	9000	3000	2000
As	Mean	3.8	4.1	2.7	4.9	1.7	2.0	1.4	1.9	2.5
	SE	0.5	0.5	0.3	0.3	0.2	0.2	0.1	0.1	0.2
Ba	Mean	4.2	15.0	11.0	55	40.9	35.6	12.8	5.8	4.9
	SE	0.15	0.4	0.8	3	0.6	1.5	1.2	0.4	0.3
Ca	Mean	330	1030	49	2900	74.2	1480	56	37.8	48.7
	SE	20	14	2	400	0.1	90	6	10.1	0.7
Cd	Mean	<LOD	<LOD	<LOD	0.163	0.0806	0.032	<LOD	<LOD	0.068
	SE	<LOD	<LOD	<LOD	0.008	0.001	0.001	<LOD	<LOD	0.004
Ce	Mean	<LOD	249	14.7	87	13.1	10.4	27	<LOD	9.0
	SE	<LOD	6	0.8	5	0.5	0.4	2	<LOD	0.1
Co	Mean	0.75	0.85	6.23	1.2	10.0	18.8	1.4	1.23	56.6
	SE	0.01	0.02	0.06	0.2	0.8	0.7	0.1	0.171	1.24
Cr	Mean	1.6	10	3.2	190	61	160	330	80.5	0.67
	SE	0.2	1	0.5	20	3	3	20	6.2	0.0289
Cu	Mean	2.6	5.2	0.430	19.3	18	17.7	36.3	3.93	46.9
	SE	0.3	0.2	0.002	0.5	2	0.7	0.5	0.0626	1.24
Dy	Mean	2.28	2.4	2.1	2.4	0.63	0.61	2.27	2.09	0.628
	SE	0.04	0.1	0.3	0.2	0.02	0.03	0.09	0.171	0.0258
Er	Mean	1.02	1.83	1.2	2.5	0.48	0.437	1.2	0.80	0.77
	SE	0.08	0.08	0.2	0.1	0.07	0.004	0.1	0.03	0.03
Eu	Mean	0.18	0.38	0.25	0.60	0.245	0.44	0.31	0.23	0.63
	SE	0.01	0.02	0.04	0.02	0.008	0.02	0.02	0.03	0.04
Fe	Mean	2.5×10 ⁴	3.8×10 ⁴	1.8×10 ⁴	3.5×10 ⁴	7.1×10 ⁴	5.1×10 ⁴	7.5×10 ⁴	1.6×10 ⁴	8.5×10 ⁴
	SE	2000	4000	2000	3000	3000	2500	4000	1500	2000
Gd	Mean	6.9	9.1	7	8.7	2.1	2.2	7	6.4	2.6
	SE	0.3	0.6	1	0.8	0.2	0.1	1	0.6	0.2
Ho	Mean	0.16	0.60	0.3	0.966	0.16	0.161	0.254	0.17	0.37
	SE	0.01	0.03	0.045	0.004	0.02	0.008	0.004	0.01	0.03
K	Mean	370	820	330	1800	230	480	460	130	85
	SE	15	60	50	200	20	20	70	10	7
Mg	Mean	220	166	<LOD	6600	84	800	330	58	51
	SE	10	5	<LOD	800	8	20	50	5	4
Mn	Mean	10.2	260	33	450	139	510	126	70	0.26
	SE	0.2	10	4	30	5	20	5	10	0.01
Na	Mean	70	34	26	143.0	31	38	38.2	11	7.9
	SE	6	2	4	0.6	4	2	0.6	2	0.6
Nd	Mean	15.8	22.4	17	30	6.8	7.9	17	15	12
	SE	0.8	0.5	1	2	0.2	0.3	1	0.9	0.2
Ni	Mean	<LOD	10.2	<LOD	61	28	43.8	67	11.4	19.0
	SE	<LOD	0.4	<LOD	3	4	0.4	7	0.5	0.8
Pb	Mean	1.56	58	9.4	13.1	6.31	6.1	11.9	1.3	9.9
	SE	0.0363	2	0.4	0.5	0.09	0.3	0.6	0.2	0.8

	Sample n°	1	2	3	4	5	6	7	8	9
	Location	Maromizaha Forest			Mitsinjo Park			Torotorofotsy Forest		
Pr	Mean	1.62	3.55	2.3	5.4	1.160	1.5	2.05	1.74	3.46
	SE	0.08	0.05	0.1	0.8	0.002	0.1	0.21	0.12	0.05
Sm	Mean	0.67	3.6	1.8	6.1	1.20	1.61	1.3	0.94	3.0
	SE	0.09	0.4	0.3	0.5	0.065	0.03	0.08	0.07	0.1
Tb	Mean	1.01	1.12	0.9	1.1	0.29	0.289	1.0	0.93	0.31
	SE	0.05	0.03	0.15	0.1	0.03	0.001	0.15	0.08	0.03
Ti	Mean	50	2310	1160	2900	1360	810	1320	1110	218
	SE	6	90	20	100	10	30	100	70	2
Tm	Mean	<LOD	<LOD	<LOD	0.30	0.0395	0.024	<LOD	<LOD	<LOD
	SE	<LOD	<LOD	<LOD	0.01	6×10 ⁻⁴	0.001	<LOD	<LOD	<LOD
V	Mean	4.8	170	70.0	390	62	340	640	303	6.3
	SE	0.5	5	0.9	10	90	30	20	10	0.3
Y	Mean	<LOD	2.63	<LOD	8.56	0.603	0.574	<LOD	<LOD	3.1
	SE	<LOD	0.137	<LOD	0.53	0.00297	0.00788	<LOD	<LOD	0.0762
Yb	Mean	0.9	1.68	1.08	2.2	0.440	0.34	1.2	0.91	0.745
	SE	0.1	0.06	0.02	0.1	0.005	0.01	0.1	0.06	0.006
Zn	Mean	35	55	20	107	28.0	52.0	33	10	10.0
	SE	1	3	1.5	7	0.1	0.7	1	2	0.2

Supplementary Table 2 Concentrations (mgkg⁻¹) of elements in soil samples of East Africa found in literature part 1

Element	Location	Mean (mgkg ⁻¹)	Source
Al	Maromizaha, Madagascar	77300	[24]
As	Chingola, Zambia	2.00	[28]
	Kabwe, Zambia	7.00	[28]
	Kabwe, Zambia	7.10	[23]
	Lusaka, Zambia	4.00	[28]
Ca	Maromizaha, Madagascar	4.27	[24]
Cd	Chingola, Zambia	0.050	[28]
	Kabwe, Zambia	1.00	[28]
	Kabwe, Zambia	0.780	[23]
	Lusaka, Zambia	0,080	[28]
Co	Chingola, Zambia	35.0	[28]
	Kabwe, Zambia	13.0	[28]
	Lake Victoria, Uganda	1.80	[25]
	Lusaka, Zambia	9.00	[28]
	Maromizaha, Madagascar	6.08	[24]
Cr	Chingola, Zambia	14.0	[28]
	Kabwe, Zambia	42.0	[28]
	Lake Victoria, Uganda	49.4	[25]
	Lusaka, Zambia	39.0	[28]
Si	Maromizaha, Madagascar	279	[24]
Sn	Maromizaha, Madagascar	3.17	[24]
Ti	Maromizaha, Madagascar	2210	[24]
V	Maromizaha, Madagascar	67.4	[24]
Zn	Chingola, Zambia	33.0	[28]
	Kabwe, Zambia	607	[28]
	Kabwe, Zambia	898	[23]
	Lake Victoria, Uganda	293	[26]
	Lusaka, Zambia	48.0	[28]
	Maromizaha, Madagascar	55.5	[24]

Supplementary Table 3 Concentrations (mgkg⁻¹) of elements in soil samples of East Africa found in literature part 2

Element	Location	Mean (mgkg ⁻¹)	Source
Ni	Chingola, Zambia	6.00	[28]
	Kabwe, Zambia	12.0	[28]
	Kabwe, Zambia	25.0	[23]
	Lusaka, Zambia	21,0	[28]
	Maromizaha, Madagascar	17.4	[24]
P	Maromizaha, Madagascar	287	[24]
Pb	Chingola, Zambia	54.0	[28]
	Kabwe, Zambia	282	[28]
	Kabwe, Zambia	379	[23]
	Lake Victoria, Uganda	133	[26]
	Lusaka, Zambia	26.0	[28]
	Maromizaha, Madagascar	39.9	[24]
S	Maromizaha, Madagascar	279	[24]

Supplementary Table 4 mgkg⁻¹ and Standard error (SE) of analytes in plant samples collected in Maromozaha Forest, Mitsinjo Park, and Torotorofotsy Forest

Site		Maromizaha forest			Mitsinjo Park			Torotorofotsy forest		
Plant Species		<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>	<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>	<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>
Al	Mean	1210.0	38	127	173	190	26	275	88	32
	SE	0.6	2	4	7	10	2	3	2.5	3
As	Mean	<LOD	0.015	<LOD	<LOD	0.005	<LOD	<LOD	<LOD	0.0255
	SE	<LOD	0.001	<LOD	<LOD	0.003	<LOD	<LOD	<LOD	7×10 ⁻⁴
Ba	Mean	1130	21.50	14.2	22	16.9	48	20.3	32.9	18.4
	SE	6	0.08	0.4	6	0.5	3	0.2	0.4	0.5
Ca	Mean	2.29×10 ⁴	1×10 ⁴	4780	1.1×10 ⁴	3600	4070	9300	1.24×10 ⁴	2700
	SE	400	80	100	1×10 ³	800	100	40	30	100
Ce	Mean	1.230	0.34	0.048	0.1	0.090	0.14	0.088	0.18	0.178
	SE	0.007	0.06	0.004	0.7	0.003	0.01	0.004	0.02	4×10 ⁻⁴
Co	Mean	0.075	0.08	0.6	0.12	1.00	1.9	0.10	0.123	5.7
	SE	0.008	0.02	0.2	0.04	0.025	0.1	0.01	0.002	0.6
Cr	Mean	0.002	0.166	0.06	0.101	0.12	0.18	0.178	0.13	0.7
	SE	0.001	0.004	0.02	0.001	0.02	0.03	0.006	0.01	0.3
Cu	Mean	1.5	2.1	4.8	2.8	7.2	7.0	2.8	4.7	6.99
	SE	0.2	0.5	0.8	0.8	0.3	0.4	0.2	0.1	0.02
Dy	Mean	0.0184	0.09	0.0024	0.0016	0.0032	0.0022	0.00226	0.0033	0.0094
	SE	8×10 ⁻⁴	0.01	3×10 ⁻⁴	1×10 ⁻⁴	2×10 ⁻⁴	2.5×10 ⁻⁴	8×10 ⁻⁵	5×10 ⁻⁴	4×10 ⁻⁴
Er	Mean	0.0099	0.055	0.0016	<LOD	0.0020	0.0017	0.00136	0.0021	0.0091
	SE	1×10 ⁻⁴	0.008	3×10 ⁻⁴	<LOD	1×10 ⁻⁴	3×10 ⁻⁴	9×10 ⁻⁵	4×10 ⁻⁴	5×10 ⁻⁴
Eu	Mean	0.0189	0.017	0.0013	0.01	0.0022	0.0045	0.0033	0.0094	0.0045
	SE	7×10 ⁻⁴	0.003	8×10 ⁻⁴	0.01	3×10 ⁻⁴	2×10 ⁻⁴	2×10 ⁻⁴	2×10 ⁻⁴	1.5×10 ⁻⁴
Fe	Mean	1180	39	43.1	35	40.5	30	49	83	44
	SE	3	1	0.2	7	0.7	2	3	6	4
Gd	Mean	0.0405	0.08	0.0036	0.004	0.0044	0.0042	0.0040	0.0065	0.0076
	SE	9×10 ⁻⁴	0.01	3×10 ⁻⁴	0.002	4×10 ⁻⁴	2×10 ⁻⁴	2×10 ⁻⁴	3×10 ⁻⁴	2×10 ⁻⁴
Ho	Mean	0.0034	0.019	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0027
	SE	1×10 ⁻⁴	0.003	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2×10 ⁻⁴
K	Mean	1.2×10 ⁴	1.08×10 ⁴	4000	8900	4880	2620	1.08×10 ⁴	2.240×10 ⁴	3310
	SE	100	60	300	600	40	65	300	8	3820
La	Mean	0.531	0.15	0.019	0.025	0.027	0.021	0.045	0.051	0.0252
	SE	0.004	0.03	0.001	0.003	0.002	0.001	0.001	0.004	2.53×10 ⁻⁴
Lu	Mean	<LOD	0.003	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.00204
	SE	<LOD	0.0001	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	7.61×10 ⁻⁵
Mg	Mean	2740	3960	4200	3500	6500	6300	3280	5530	3100
	SE	25	30	200	100	250	350	20	6	150
Mn	Mean	1900	182	19.6	63	84	36	61	103	24
	SE	3.91	5	0.6	1	1	3	1.5	1	2
Na	Mean	3050	3980	1.93×10 ⁴	3270	3.88×10 ⁴	2.33×10 ⁴	9200	860	4.51×10 ⁴
	SE	235	150	400	200	600	600	400	20	600

Site		Maromizaha forest			Mitsinjo Park			Torotorofotsy forest		
<i>Plant Species</i>		<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>	<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>	<i>Ficus lutea</i>	<i>Ficus reflexa</i>	<i>Uapaca thouarsii</i>
Nd	Mean	0.248	0.41	0.013	0.011	0.016	0.0112	0.019	0.028	0.0213
	SE	0.003	0.07	0.001	0.001	0.001	6×10 ⁻⁴	0.001	0.003	7×10 ⁻⁴
Ni	Mean	<LOD	<LOD	<LOD	<LOD	15.1	15.1	<LOD	<LOD	19
	SE	<LOD	<LOD	<LOD	<LOD	0.477	0.418	<LOD	<LOD	0.454
P	Mean	550	904	880	720	760	760	542	1100	700
	SE	70	4	30	30	10	35	2	50	40
Pr	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE	4.17×10 ⁻⁴	0.0142	3.57×10 ⁻⁴	0.0404	2.08×10 ⁻⁴	4.95×10 ⁻⁴	1.78×10 ⁻⁴	7.44×10 ⁻⁴	2.3×10 ⁻⁴
Pb	Mean	0.07	0.0841	0.0043	0.0041	0.0049	0.0042	0.0063	0.0091	0.0061
	SE	0.01	1×10 ⁻⁴	2×10 ⁻⁴	6×10 ⁻⁴	6.5×10 ⁻⁴	3×10 ⁻⁴	2.5×10 ⁻⁴	1×10 ⁻⁴	6×10 ⁻⁴
Sc	Mean	0.115	0.167	0.656	0.259	0.454	0.439	0.32	0.381	0.9
	SE	0.031	0.0405	0.00167	0.0665	0.00746	0.0223	0.04	0.0095	0.1
Sm	Mean	0.036	0.10	0.0013	0.002	0.0022	0.0016	0.0019	0.0041	0.0051
	SE	0.002	0.015	4×10 ⁻⁴	0.002	4×10 ⁻⁴	3×10 ⁻⁴	1×10 ⁻⁴	4×10 ⁻⁴	6×10 ⁻⁴
Sr	Mean	1930	171	52.7	55	47	94	21.4	106	89.1
	SE	10	2	0.7	1	1	9	0.3	2	7.8
Tb	Mean	0.00498	0.016	<LOD	<LOD	<LOD	<LOD	<LOD	0.0012	0.00177
	SE	2×10 ⁻⁵	0.003	<LOD	<LOD	<LOD	<LOD	<LOD	1×10 ⁻⁴	1.2×10 ⁻⁴
Ti	Mean	1.4	2.3	2.1	2.6	3.1	2.6	3.0	5.65	3.9
	SE	0.2	0.4	0.2	0.8	0.3	0.2	0.4	0.05	0.2
Tm	Mean	0.00128	0.007	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.0023
	SE	3×10 ⁻⁵	0.001	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	2×10 ⁻⁴
V	Mean	0.00427	0.04	0.026	0.016	0.033	0.026	0.02250	0.057	0.0893
	SE	9×10 ⁻⁵	0.01	0.003	0.0025	0.002	0.009	1×10 ⁻⁵	0.008	0.00674
Y	Mean	0.121	0.5	0.012	0.017	0.0163	0.014	0.019	0.025	0.0492
	SE	0.007	0.1	0.002	0.007	7×10 ⁻⁴	0.001	0.002	0.0015	7.08×10 ⁻⁴
Yb	Mean	0.00409	0.034	0.0023	<LOD	0.0028	0.0024	0.0011	0.00213	0.0146
	SE	9×10 ⁻⁵	0.005	2×10 ⁻⁴	<LOD	2×10 ⁻⁴	2×10 ⁻⁴	3×10 ⁻⁴	9×10 ⁻⁵	3×10 ⁻⁴
Zn	Mean	5.6	2.9	7.6	14	12.9	16	5.6	15.1	8.3
	SE	0.4	0.4	0.5	3	0.2	2	0.3	0.4	0.8

Supplementary Table 5 Results of ANOVA and Tukey (HSD) tests for Zn concentrations in plant samples: evaluation of differences among sampling sites groups (95% confidence level) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	115	2	57.5	5.53	0.0435	5.14
One site	62.4	6	10.4			
Total	177	8				

Comparison	Difference	Standardized Difference	Critical value	Pr > Diff	Significance
Mitsinjo vs Maromizaha	8.76	3.33	3.07	0.0370	Yes
Mitsinjo vs Torotorofotsy	4.47	1.70	3.07	0.280	No
Torotorofotsy vs Maromizaha	4.28	1.63	3.07	0.306	No

Supplementary Table 6 ANOVA and Tukey (HSD) tests of Ti concentrations in plant samples among sampling sites groups (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	7.75	2	3.87	5.41	0.0454	5.14
One site	4.30	6	0.716			
Total	12.0	8				

Comparison	Difference	Standardize Difference	Critical value	Pr > Diff	Significance
Torotorofotsy vs Maromizaha	2.25		3.07	0.0400	Yes
Torotorofotsy vs Mitsinjo	1.42		3.07	0.180	No
Mitsinjo vs Maromizaha	0.83		3.07	0.497	No

Supplementary Table 7 ANOVA and Tukey (HSD) tests of Sc concentrations in plant samples among plant species (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	0.321	2	0.161	5.18	0.0496	5.14
One site	0.187	6	0.0311			
Total	0.508	8				

Comparison	Difference	Standardize Difference	Critical value	Pr > Diff	Significance
Uapaca thouarsii vs Ficus lutea	0.442		3.07	0.0500	Yes
Uapaca thouarsii vs Ficus reflexa	0.340		3.07	0.122	No
Ficus reflexa vs Ficus lutea	0.102		3.07	0.768	No

Supplementary Table 8 ANOVA and Tukey (HSD) tests of Ba concentrations in soil samples among plant species (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	2460	2	1230	24.1	0.001	5.14
One site	307	6	51.1			
Total	2770	8				

Comparison	Difference	standardize difference	Critical value	Pr > Diff	Significance
Mitsinjo vs Torotorifotsy	36.1		3.07	0.002	Yes
Mitsinjo vs Maromizaha	33.9		3.07	0.003	Yes
Maromizaha vs Torotorifotsy	2.25		3.07	0.922	No

Supplementary Table 9 ANOVA and Tukey (HSD) tests of Ni concentrations in soil samples among plant species (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	890	2	445	0.760	0.530	6.94
One site	2350	4	588			
Total	3240	6				

Comparison	Difference	standardize difference	Critical value	Pr > Diff	Significance
Mitsinjo vs Maromizaha	34.1	1.22	3.56	0.505	No
Mitsinjo vs Torotorifotsy	11.7	0.590	3.56	0.831	No
Torotorifotsy vs Maromizaha	22.3	0.800	3.56	0.724	No

Supplementary Table 10 ANOVA and Tukey (HSD) tests of Ni concentrations in soil samples among plant species (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	2980	2	1490	2.11	0.200	5.14
One site	4240	6	707			
Total	7220	8				

Comparison	Difference	standardize difference	Critical value	Pr > Diff	Significance
Mitsinjo vs Torotorifotsy	44.4	2.05	3.07	0.182	No
Mitsinjo vs Maromizaha	25.1	1.16	3.07	0.517	No
Maromizaha vs Torotorifotsy	19.3	0.890	3.07	0.666	No

Supplementary Table 11 ANOVA and Tukey (HSD) tests of Co concentrations in soil samples among plant species (95% interval confidence)) (Pr>Diff=Probability greater than Difference)

Variance origin	SQ	Fd	MQ	F	Significance value	F crit
Among-sites	291	2	145	3.89	0.0800	5.14
One site	224	6	37.4			
Total	515	8				

Comparison	Difference	standardize difference	Critical value	Pr > Diff	Significance
Torotorifotsy vs Maromizaha	13.9	2.79	3.07	0.071	No
Torotorifotsy vs Mitsinjo	6.53	1.31	3.07	0.441	No
Mitsinjo vs Maromizaha	7.39	1.48	3.07	0.364	No

Monitoring Essential and Non-Essential Elements in Lemurs Using Non-Invasive Sampling: A One Health Perspective

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Abstract

Monitoring essential and non-essential elements of endangered animal species represents a rising challenge in animal health conservation. This study employed a non-invasive biomonitoring approach to evaluate the elemental composition in fur, feces, and blood of four lemur species (*Lemur catta*, *Eulemur macaco*, *Eulemur mongoz*, *Eulemur rubriventer*) collected in two different zoological parks: Bioparco Zoom (Turin, Italy) and Parc Botanique et Zoologique de Tsimbazaza (PBZT, Antananarivo, Madagascar). Elemental analysis was performed via Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), and Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS) following standardized digestion protocols. Results highlight significant differences in element content among biological matrices, geographical origin, and seasons. The fur dataset is related to the long-term accumulation mechanism, reflecting the geochemical properties of the original habitat, while the feces dataset exhibits a sensitivity to environmental short-term changes as seasonal ones. The experimental results were processed with multivariate chemometric techniques. Pb, Al, Ba, and Fe were identified as the elements that describe Malagasy samples in alliance with the anthropogenic polluted origin area. Italian fur samples were characterized by As, Cr, Cu, and Ni, reflecting the element content of the soil in the area around Turin. Seasonal separation patterns observed in both fur and feces datasets suggest climatic influences on bioavailability and exposure pathways. This study demonstrates the

efficiency of non-invasive biomonitoring as a practical and ethical method for assessing ecosystem and animal health in conservational contests. The proposed approach is in line with the One Health principles, offering a replicable model for future applications in both natural and managed environments, contributing to the development of targeted strategies for the protection of biodiversity.

1 Introduction

Exposition to environmental contaminants poses an escalating risk for global biodiversity and the health of ecosystems. Among those, potentially toxic elements (PTEs) have the capability to accumulate in the trophic chain and constitute a threat even at low concentrations. [1,2] To have a thorough knowledge of animal health, it is not adequate to confine the research to the PTEs, but it is necessary to extend the investigation to the other macro- and micronutrients with a crucial role in the physiological, metabolic, and immunological functions; deficiency or surplus may influence the health status and the reaction to environmental stresses. [3–6] The availability in the environment of rare earth elements (REEs), now increasingly diffuse because of human intervention, should be considered for the still largely unexplored possible impact on animal health. [7,8] In this context, the One Health approach, which bonds ecosystem, animal, and human health, calls for innovative, sustainable, and non-invasive biomonitoring tools. [9] This study responds to One health principle, considering environment quality, animal health, anthropical influences, and underlying how lemur health is connected to the state of their habitat. The combined evaluation of different biological matrices, collected in non-invasive modes, offers a new perspective to monitor animal exposition and health, minimizing the impact on individuals. This paradigm shift in environmental monitoring offers the possibility to research endangered species with a low ethical impact. Despite the high vulnerability of lemurs and their conservation importance, data regarding the effect of contaminants on their organism are surprisingly limited. [10–12] The lack of evidence on this topic in the scientific literature highlights a potential underestimation of the risks associated with biodiversity loss, potentially spoiling the efforts on ecosystem conservation and wildlife protection. Amid these concerns, this study proposes an innovative approach that is non-invasive and supported by standardized protocols to monitor element content in fur, feces, and blood of four lemur species (*Lemur catta*, *Eulemur macaco*, *Eulemur mongoz*, *Eulemur rubriventer*) classified by the International Union for Conservation of Nature (IUCN) Red List as Endangered or Critically Endangered [13–16]. Sampling campaigns were conducted in two contrasting but representative parks: Bioparco Zoom,

an Italian zoological facility in Turin, Italy, and the Parc Botanique et Zoologique de Tsimbazaza (PBZT) in Antananarivo, Madagascar. This area of study allowed us to compare areas with different climate, geochemical, and pollution characteristics. The determination of the elements was performed using Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES), or Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS), depending on the analyte concentration level, following microwave-assisted acid digestion. Statistical data processing enabled the evaluation of differences in elemental composition and distinctive patterns among matrices, areas, and seasons.

The results show significant differences in the distribution of elements as a function of geographical origin and biological matrix, revealing the potential influence of environmental, management and seasonal factors. In addition, the analysis of soils and waters collected in the area where lemurs live, highlighted the consistency between the contaminants detected and the accumulation observed in animal tissues, suggesting a strong correlation between habitat and bioaccumulation. This study therefore provides an operational model useful both for health surveillance in zoological contexts and for ex-situ conservation, opening up prospects for monitoring in natural environments according to the One Health principles.

2 Materials and Methods

2.1 Sampling

The sampling campaign was conducted in two areas to compare environments with various characteristics, climate, and element exposition levels. The areas examined were Bioparco Zoom (in Cumiana, Turin, Italy) and Parc Botanique et Zoologique de Tsimbazaza (PBZT, in Antananarivo, Madagascar). As shown in Table 1, sampling campaigns were conducted with standardized - protocols to obtain comparable data between the two sites. In the Bioparco Zoom, the study involved twelve months divided into two sampling sessions, one in March 2023 and one in December 2023, to ensure a more complete characterization of the environment and biological matrices selected. It is important to underline some critical issues that emerged during the sampling phase, which influenced the size and completeness of the dataset, in particular about the Malagasy site. A single sampling campaign was conducted in Madagascar in November 2023 due to political and administrative challenges arising from a change in the park's leadership. The transition in

management led to delays in obtaining research permits and logistical complications, limiting the possibility of conducting additional fieldwork. These limitations have led to lower representativeness of the Malagasy site compared to the Italian one and an uneven coverage between species and biological matrices. Despite this, the data collected offer valuable information, paving the way for future more extensive and structured studies and underlining the urgency of strengthening local and international collaborations to facilitate ecotoxicological research in contexts of high conservation priority.

The biological matrices involved in the study were feces, blood, and fur, while the species of lemur involved were four: *Lemur catta*, *Eulemur macaco*, *Eulemur mongoz*, and *Eulemur rubriventer*. The fecal sampling encompassed all four species at both study sites. The collection protocol involved manually gathering samples from the ground in areas of the parks where the lemurs rest and are divided by species. In the Bioparco Zoom, the resting area is the indoor habitat of the so-called “Lemur Island”, while in PBZT, samples were taken inside the concrete enclosures. Samples were stored in clean plastic bags labeled and frozen at -20°C until pretreatment and analysis. fur gathering was performed by cutting hair with stainless steel sterile scissors from the leg of individuals and then stored in clean plastic bags and labeled. The procedure was conducted by veterinarian staff, ensuring minimal invasiveness. In Madagascar, the sampling campaign involved all four species, while in Italy, it was possible to collect fur samples only from **Lemur catta and Eulemur macaco because individuals of Eulemur mongoz and Eulemur rubriventer** housed in Bioparco Zoom were old and would have been too sensitive to be captured, even for non-invasive procedures. Blood samples were gathered only in Italy because, in Madagascar, it was not possible to guarantee a stress-free procedure to safeguard animal welfare. As for fur sampling, in Italy, blood samples were collected only from **Lemur catta and Eulemur macaco**. Sampling was conducted during occasional veterinary control planned by the park to guarantee animal welfare and reduce the induced stress. Blood collection was performed through venipuncture, with volumes kept below 1% of the animal's body weight, in accordance with veterinary guidelines. Samples were stored in EDTA blood collection tubes at -20°C until pretreatment and analysis.

Table 2 Sampling campaigns and details on collected matrices

Matrix	Place	Specie	Sampling date
Feces	Italy	<i>Lemur catta</i> , <i>Eulemur macaco</i> , <i>Eulemur mongoz</i> ed <i>Eulemur rubriventer</i> .	March 2023 and December 2023
	Madagascar	<i>Lemur catta</i> , <i>Eulemur macaco</i> , <i>Eulemur mongoz</i> ed <i>Eulemur rubriventer</i> .	November 2023
Fur	Italy	<i>Lemur catta</i> and <i>Eulemur macaco</i>	March 2023 and December 2023
	Madagascar	<i>Lemur catta</i> , <i>Eulemur macaco</i> , <i>Eulemur mongoz</i> ed <i>Eulemur rubriventer</i>	November 2023
Blood	Italy	<i>Lemur catta</i> , <i>Eulemur macaco</i>	December 2023

In parallel with the biological sampling campaign, environmental and feed samples were collected to compare the elemental concentrations of the biological matrices with those of animal habitats. Soil and water were collected from both sites to characterize the areas considered. Soil samples were stored in clean plastic bags, while water was acidified by adding 1% HNO₃ and stored in HDPE (High-Density Polyethylene) clean vessels until analysis. The locations of the sampling points were identified to ensure a homogeneous representation of the study area. In Italy, soil was collected in March 2023 from seven different points in the outdoor habitat of lemurs (“Lemur Island”), while water was collected in March, September, and November 2023 to observe possible differences in the concentrations. In Madagascar, soil and water samples were collected in November 2023. Three soil samples were collected from three different points within the study area. Similarly, three water samples were collected from distinct locations to account for potential variability in the water composition. Lemur feed could be collected only in Italy in December 2023, where both fruit and dry feed were sampled from three different batches obtained from the supplier regularly used by Zoom Biopark. Samples were stored in clean plastic containers until pretreatment.

2.2 Instruments and reagents

The study was conducted using highly accurate and sensitive analytical instrumentation and high-purity reagents to ensure accuracy and reliability in elemental analysis. The microwave-assisted digestions system used for sample mineralization were Milestone MLS-1200 Mega and Milestone

Ethos One, equipped with high-pressure TFM (modified PTFE) vessels, allowing for controlled temperature and pressure conditions to optimize the decomposition of organic matrices while preventing the loss of volatile elements. The "vessel into vessel" technique was applied to enhance digestion efficiency and minimize contamination for Fur and blood samples.

Elemental analysis was performed using three complementary techniques: ICP-OES (Thermo Scientific iCAP 7000), ICP-MS (Thermo Element XR), and GF-AAS (Perkin Elmer Analyst 600). The calibration of instruments was carried out using external calibration performed with multi-element standard solutions at different concentrations prepared from certified 1000 mg/L stock solutions (Sigma-Aldrich, USA). For the sample preparation process, all reagents were selected to minimize contamination risks. Analytical-purity nitric acid (HNO₃, 65%), hydrogen peroxide (H₂O₂, 30%), and hydrochloric acid (HCl, 37%) (all from Sigma-Aldrich, trace metal grade) were used for the mineralization of biological and environmental samples. Ultrapure water (HPW, 18.2 MΩ·cm) was obtained from a Millipore Milli-Q system and used for all dilution steps and sample rinsing.

2.3 Pretreatment procedures

2.3.1 Soil, water, and feed characterization

Water samples were vacuum-filtered with a 0,45 µm Whatman membrane filter to remove solid suspended particles. Soil samples were first sieved to divide the fraction of the solid particles having grain sizes larger than 2 mm and quantify the percentage of the latter in the total. Then, soils were ground in a mill and homogenized. 0,1 g of the resulting powder of each sample was mineralized in a microwave oven with 5 mL of *aqua regia* (the solution is comprised of a 3:1 mixture of hydrochloric acid and nitric acid, respectively). The program began with 250 W for 3 minutes, followed by 250 W for 5 minutes, and then 550 W for 3 minutes. A final ventilation phase of 90 minutes at 0 W was applied to allow controlled cooling and pressure release. The obtained solutions were then filtered through Whatman cellulose filters and diluted to 50 mL with HPW.

2.3.2 Fur samples

Before mineralization, fur samples were washed with 20 mL of HPW and stirred for 2 min to remove the contaminants deposited on the fur surface. Typically, similar studies use the International Atomic Energy Agency (IAEA) washing protocol, which includes a combination of HPW and CH₃COCH₃. In this study, only HPW was used to remove dust without risking the leaching of

elements bound to the keratin matrix of the samples. Research, as the one of Raposo et.al (2014), has shown that HPW is effective in removing surface contaminants; ultrapure water proved to be an effective cleaning agent for eliminating environmental trace metals like Al, Fe, Cu, Mn, Cr, and Pb, whereas the IAEA method can lead to a reduction in the concentration of elements fixed within the keratin structure.[17,18] After washing, the samples were air-dried for one day in a laminar flow hood. Then, the fur was cut into small sections of 0.1-0.5 cm with stainless-steel scissors, which were cleaned between each sample with HPW. This step was necessary to homogenize the samples and reduce the potential effect of intra-individual variance. The washed fur was then mineralized with the above-mentioned microwave-assisted digestion system (Milestone Ethos-One) employing the "vessel into vessel" technique. This method involves placing a smaller digestion vessel (30 mL capacity) into the primary one to enhance digestion efficiency by improving the heat and pressure distribution, minimizing cross-contamination between samples, and reducing the loss of volatile elements, which is particularly critical in trace metal analysis. 0,05 g of sample and 1,5 mL of HNO₃ were placed in smaller vessels, while the external vessels were filled with a solution of 1 mL of H₂O₂ and 10 mL of HPW to ensure uniform heat transfer. The digestion process followed a program consisting of a 20-minute power ramp-up to 1200 W, followed by a 15-minute stabilization period at 1200 W, and concluded with a 1-hour ventilation phase at 0 W to allow for gradual cooling and pressure release. The resulting solutions were filtered with Whatman cellulose filters and diluted to 12 mL with HPW. Mineralization efficiency and accuracy of the analytical method were verified by processing the Certified Reference Materials (CRM) ERM®-DB001, namely human hair (Supplementary Table 1).

2.3.3 *Feces samples*

Feces were weighed and dried to evaluate their water content. Dried samples were minced using a stainless knife and a blender to achieve a homogeneous and uniform powder; both tools were washed with HPW between samples. The mineralization was performed with a Milestone MLS-1200 Mega Microwave, weighing 0,5 g inside TFM vessels and adding 5 mL of HNO₃, 2 mL of H₂O₂, and 1 mL of HCl. After 1 hour of pre-mineralization under the fume hood, the mineralization process was carried out according to a five-step microwave digestion program, consisting of 250 W for 2 minutes, 400 W for 5 minutes, 550 W for 5 minutes, 300 W for 5 minutes, and concluding with a 30-minute ventilation phase to ensure controlled cooling and pressure release. The obtained solutions

were filtered with Whatman cellulose filters and diluted to 50 mL with HPW. Mineralization efficiency and accuracy of the analytical method were verified by processing the Certified Reference Material NIST® SRM® 1573a, namely Tomato Leaves (Supplementary Table 2).

2.3.4 *Blood samples*

Three different acid mixtures were tested for the mineralization of blood samples using bovine blood added with the elements of study. The conditions adopted for all tested mixtures can be found in Supplementary Table 3. Supplementary Table 4 reports the corresponding recoveries: method 3 gave the best results and was chosen for the analyses. The mineralization protocol used for blood samples involves the use of Milestone Ethos-One digestion system employing the "vessel into vessel" technique. 0,5 g of sample, 2,5 mL of HNO₃, and 0,5 mL of H₂O₂ were placed in smaller vessels, while the external vessels were filled with a solution of 1 mL of H₂O₂ and 10 mL of HPW to ensure uniform heat transfer. The digestion process followed a program consisting of an 8-minute power ramp up to 800W, followed by 3 minutes at 800 W, and concluded with a 1-hour ventilation phase. Mineralized samples were filtered through Whatman cellulose filters and diluted to 20 mL with HPW.

2.4 Statistical analysis

For elements with concentration below the limit of detection (LOD) in more than 50% of the measurements, the variables were not considered for the statistical treatment. In the case of a few missing data (concentration lower than the LOD), a random value was set under the detection limit. Statistical analysis of data was performed using Python. Data pretreatment was carried out by applying a base-10 logarithmic transformation followed by autoscaling to standardize the variables. [19] t-Test was performed among different groups of samples and obtained p-values were represented in Volcano plots. These graphs show statistical significance (-log₁₀p-value on y-axis) and the influence of the variable (log₂ fold change on x-axis). The concentration ranges of significant variables were represented in a Box plot to highlight their distribution. A chemometric multivariate treatment was performed to explore the correlation between all the variables and the similarity or difference between samples. Principal Component Analysis (PCA) was employed to reduce the dimensionality of the dataset and uncover correlations among chemical variables in different environmental compartments or seasonal differences. [19]

3 Results and Discussion

3.1 Soil, Water and Feed characterization

Element concentrations in the soil and water samples from Italy and Madagascar (Supplementary Tables 5) showed distinct elemental signatures that reflect the underlying geological and environmental conditions of the two regions. In Italy, the soil samples were characterized by higher levels of Cr and Ni, which are consistent with the geogenic origin of these elements in the alluvial soils of the Turin area. Cr and Ni are naturally abundant in serpentine-rich formations, which are common in this part of Piedmont region. The presence of these elements in soil, therefore, reflects natural background levels rather than anthropogenic contamination. [20,21] In contrast, the Malagasy soil samples contained higher levels of lead (Pb), which is more likely to originate from anthropogenic sources, including mining, vehicular traffic, agricultural runoff, and industrial emissions. The Pb levels in Malagasy soil, although elevated compared to the Italian samples, remained below the legal limits for soil quality established for public green areas in Italy (D.Lgs. 152/2006). [22] As there are no regulations regarding PTEs levels in Malagasy soils, the data obtained were compared to the Italian D.Lgs. 152/2006 threshold values. The higher Pb content in Malagasy soil could also reflect historical contamination from leaded gasoline, and industrial processes. PBZT is situated in an area historically characterized by heavy traffic, predominantly consisting of older vehicles associated with higher emission rates. Although vehicular traffic within the PBZT park was halted only in 2022, the longstanding exposure is significant. Furthermore, Madagascar currently lacks regulations regarding vehicle emissions and fuel composition.

Water samples from the two sites similarly reflected these environmental differences. Italian water samples showed higher concentrations of Ca and Mg, consistent with the hard water profile typically associated with the dissolution of carbonate-rich rock. [23] Malagasy water samples, on the other hand, were richer in trace elements such as Pb as soil, reflecting both natural mineralization processes and potential anthropogenic contamination. (Supplementary Table 6 and 7)

The results of lemur feed analysis present considerable variability in the concentrations of elements among samples such as Fe, Cu, Zn, and Mn, which are crucial for the metabolism of animals, and the presence, in some cases, of trace elements such as Cd, Pb and As (Supplementary Table 18). Most

of the values fall within the limits established by Directive 2002/32/EC; the presence of heavy metals and other undesirable substances in animal feed is subject to strict limits.[24]

3.2 Element Distribution in the Italian dataset

Since the Italian dataset is the most complete, the distribution of elements across various biological matrices in samples collected from Italy is examined (Supplementary Tables 8-17). Figure 1 shows the volcano plot of the variable in fur and feces; the significant variables are green, and the grey ones are the variables without statistical significance. Supplementary Figure 1 shows the boxplot of the significant variables (pretreated) in the two matrices. The data show that feces generally contained the highest concentrations of most elements, including essential macronutrients such as Mg, Ca, Fe, and Mn, and PTEs like As and Cr. This pattern reflects the physiological role of the digestive and excretory systems in regulating metal homeostasis and detoxification. [25,26] The high levels of Mg and Ca in feces are consistent with their known involvement in skeletal and muscular functions, as well as their role in maintaining cellular osmotic balance. [5,27] Interestingly, it is known that Mn has a rapid turnover rate, with over 50% of the element being excreted within a few days, consistent with its known metabolic pathways involving biliary excretion; others, such as As, Cd, Fe and Ti, are eliminated after weeks. [28] This finding aligns with the role of Mn, which is an essential trace element involved in enzymatic reactions, including those related to antioxidant defence and neurotransmitter metabolism. [25,29] Gd, La, and Pb have higher concentrations in fur samples; among those, Pb is a PTE known for its capability of accumulating in keratin due to its affinity with sulphur proteins. [30,31]

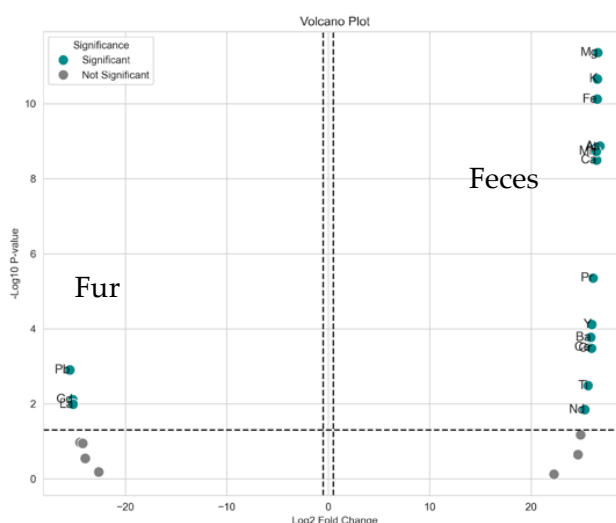


Figure 5 Volcano plot of significant variables that distinguish fur and feces dataset

Blood samples exhibited a distinct pattern of element accumulation, with significantly higher Na concentrations than the other matrices (Supplementary Table 16 and 17). The high levels of Na reflect its fundamental role in regulating osmotic balance, nerve transmission, and muscle contraction. The observed Na concentrations in lemur blood were comparable to physiological levels reported in other mammals, supporting the conclusion that the detected values fall within a normal biological range.[32] (Supplementary Figure 2)

3.3 Multivariate analysis of fur dataset

The PCA plot of the fur dataset shows an evident separation between samples collected in Italy and in Madagascar, enhanced in particular by observing PC1 vs PC3 (Figure 2). Malagasy samples result rich in most of the elements analyzed, while Italian ones are characterized mostly by As, Ca, Cr, Cu, K, and Yb. In order to determine more reliably which elements significantly contribute to differentiating the two classes, t-tests were conducted on the data obtained. The results, represented by a volcano plot (Figure 3), confirm that the elements that describe the Madagascar dataset are Al, Ba, Ca, Cd, Ce, Dy, Fe, Gd, K, La, Mg, Na, Pb, Pr, Sc, Ti, V, and Y, while the Italian one is represented by As, even though the concentrations are not alarming (supplementary Tables 12-15). These findings are particularly interesting if compared with the soil PCA and volcano plot (Figure 2 and 3). Most of the elements that differentiate fur samples by origin (i.e. Italy or Madagascar) play a dominant role in discriminating soil ones collected in the two locations. Madagascar soil is characterized by Al, Ba, Fe, Pb, and V as fur samples, while As, Cr, Cu, Mg, and Yb are more present both in Italian soil and fur (as is shown in Supplementary Figure 3). These results are consistent with the characteristics of the environments of the areas studied. PBZT is situated in an area historically characterized by heavy traffic, predominantly consisting of older vehicles associated with higher emission rates. Although vehicular traffic within the PBZT park was halted only in 2022, the longstanding exposure is significant. Furthermore, Madagascar currently lacks regulations regarding vehicle emissions and fuel composition, The higher Pb content in Malagasy soil reflects, as already discussed in section 2.3.1, historical contamination from leaded gasoline and industrial processes, thus clearly explaining the higher levels of lead detected in the Malagasy samples. On the other hand, the elevated Cr and Ni levels in Turin soil reflect its natural geogenic origin, which contributes to elevated baseline levels of these elements in the local environment and influences their concentrations in local flora and fauna, as demonstrated by the results obtained for fur in the present study.[20,21] These observations suggest a direct relationship between the elemental composition of

biological samples and soil geochemical characteristics, indicating how organisms can reflect the environmental peculiarities of their habitat of origin.

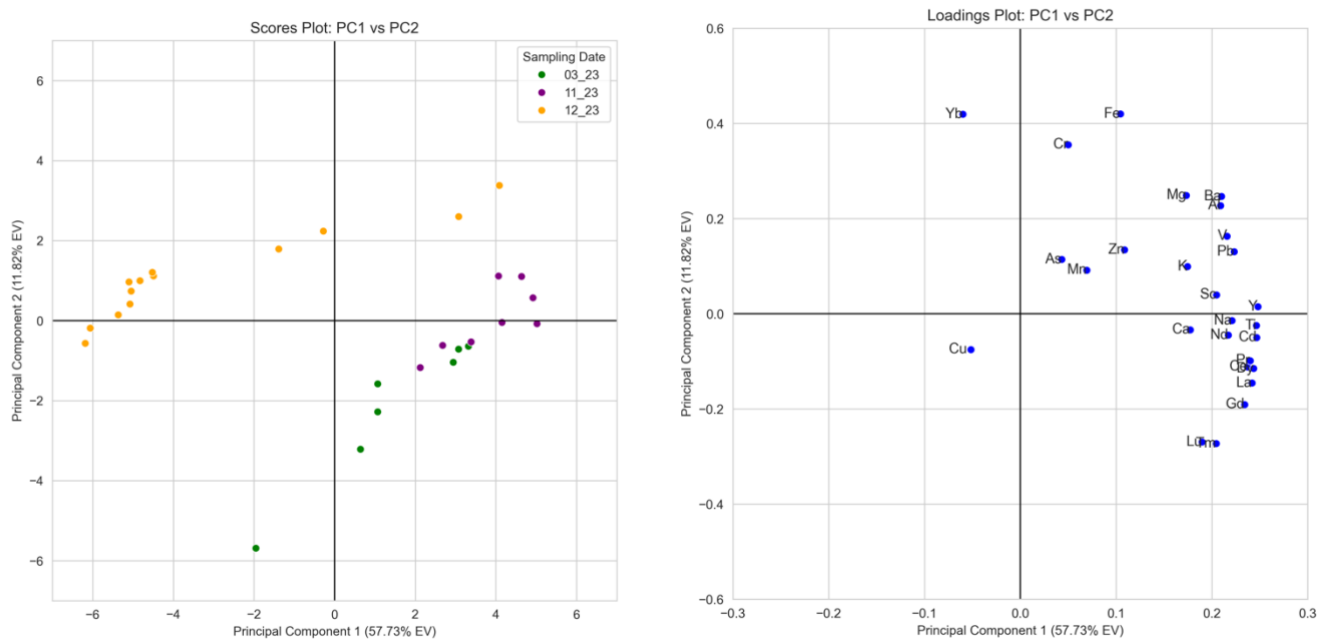


Figure 6 Score plot and Loading plot of PCA performed on Fur dataset, displacing PC1 vs PC3

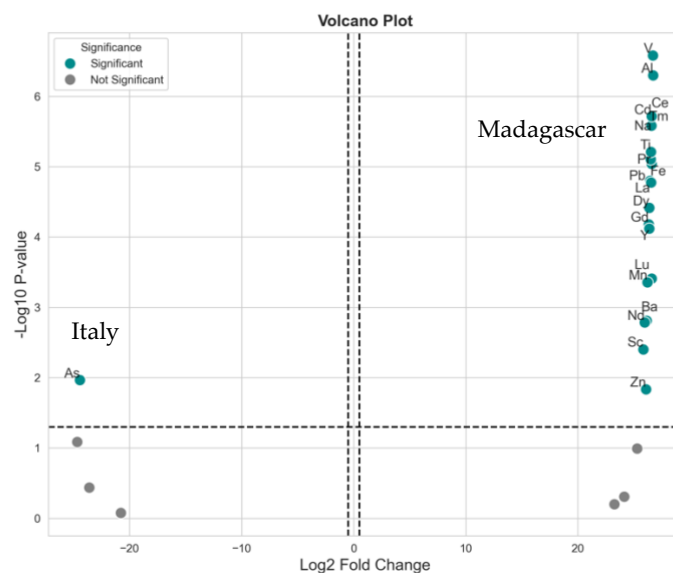


Figure 7 Volcano plot of significant variables that distinguish Italian and Malagasy samples in fur dataset

PCA of the fur dataset does not differentiate between gender or species (Supplementary figure 5); however, the results do indicate a separation based on the sampling season, as illustrated in Figure 4. Samples were collected in Italy in March 2023 and December 2023, while those from Madagascar

were collected in November 2023. Given that the period from October to February corresponds to the hot season in Madagascar, it is noteworthy that the March samples from Italy and the November samples from Madagascar are grouped, distinct from the winter samples collected in Italy in December 2023. The loading plot shows that the cold season samples (Italy, 12_2023) are characterized mostly by Fe, Cr, Yb, Ba, Al, Mg, and K, while samples taken in spring (Italy, 03_2023 and Madagascar 11_2023) are associated with Lu, Gd, Pr, Ce, Nd, Ca e Cu. Those differences could be determined by specific physiological adaptations to climatic conditions or seasonal changes in the availability of elements in the environment due to temperature, humidity, and pH

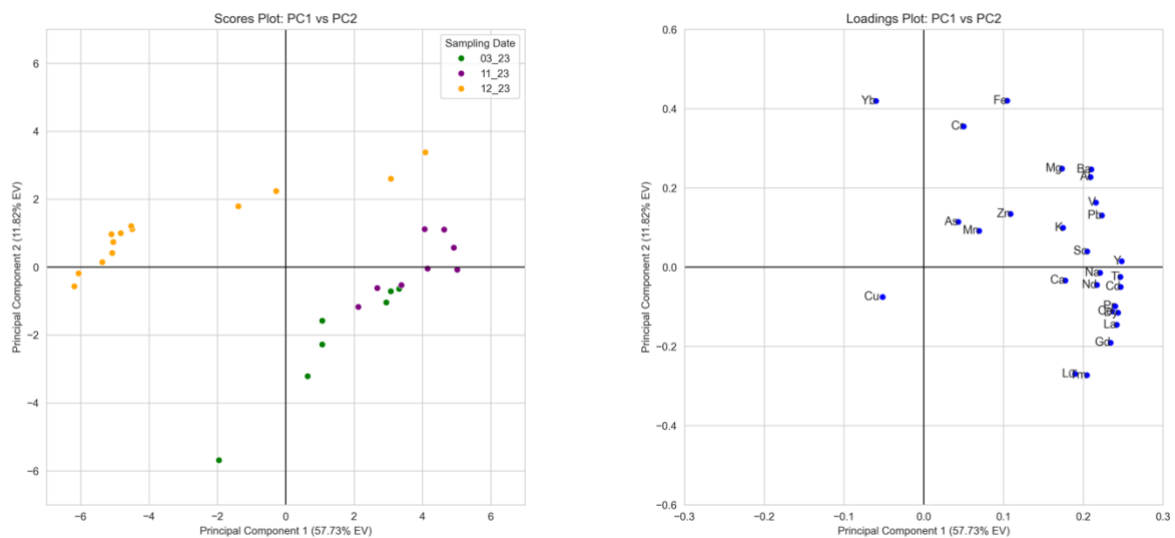


Figure 8 Score plot and Loading plot of PCA performed on Fur dataset, displacing PC1 vs PC2

3.4 Multivariate analysis of feces dataset

By analyzing the feces dataset, similar conclusions can be drawn: PCA shows a similar clusterization by season (Figure 5). The loading plot indicates that rare earth elements (REEs), such as Ce, Nd, Pr, Sm, Gd, Tb, and Yb, are variable that mainly describe Italian winter samples. Spring Italian and Malagasy group is represented mostly by Fe, Al, Mg, Mn, Zn, Pb, V, Na and K. The significant difference in these elements in the different seasons could be linked to a different mineral composition of the feed or water resources administered at these specific times of the year.

The season separation occurring in both the fur and feces datasets is particularly interesting; it suggests that there are common environmental or management factors that influence both matrices.

However, it should be noted that the variables that influence the two seasons are different from those highlighted for fur. This may be because the differences observed in fecal samples reflect more

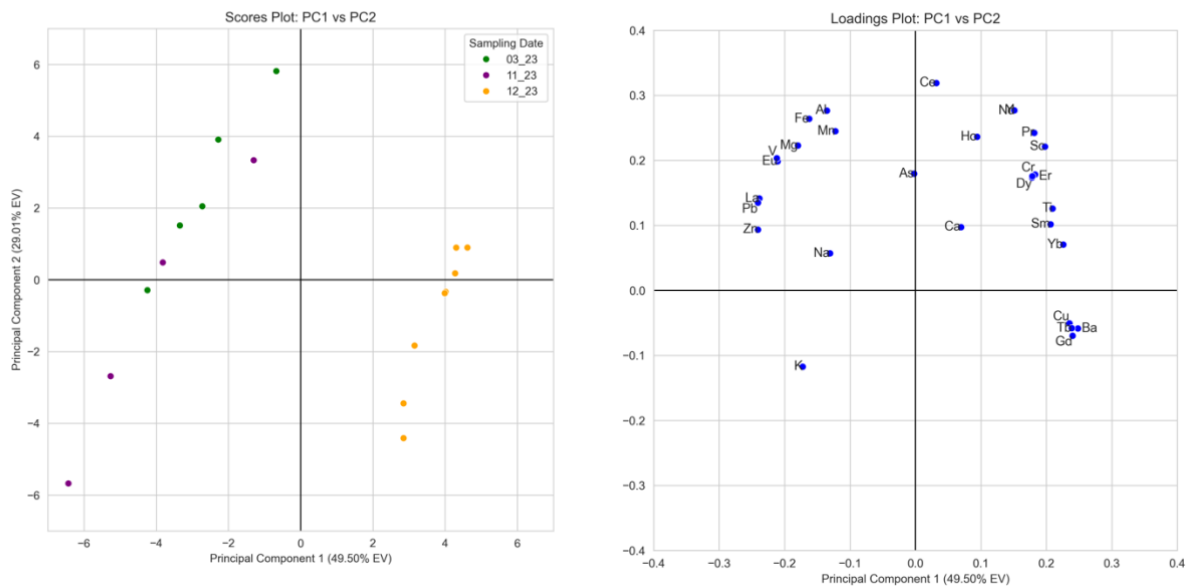


Figure 5 Score plot and Loading plot of PCA performed on Feces dataset, displacing PC1 vs PC2

immediate changes in diet or environment than in fur samples, which are affected by more prolonged exposures over time. While the current dataset is not sufficiently comprehensive to thoroughly examine the seasonal variations in fur element composition, it offers valuable insights and serves as a foundation for future research. In particular, the findings of this study indicate the importance of investigating seasonal variations and understanding how they are influenced by environmental factors or dietary habits. Such insights could enhance animal welfare through the implementation of targeted management strategies by bio parks.

The separation of the feces dataset by sampling locations was not visible in PCA plots; the samples were well differentiated by sampling period, while regional clusters were not visible, as shown in Supplementary Figure 6. This is probably because feces are involved in the metabolic excretion mechanism, which has a more rapid turnover compared to the accumulation mechanism within the keratin matrix of fur. This demonstrates the different potentialities of the two biological matrices; while fur could be useful to discriminate between different locations or in a long-period monitoring study, feces can be useful to individuate problems or changes in a shorter period helping zoos to address specific situations (for example in case of environmental hazards) or to develop management strategies for animal welfare.

3.5 Biological and Ecological Implications

From an ecological perspective, the observed differences in metal accumulation patterns reflect both environmental availability and species-specific metabolic processing. The levels of some PTEs in the soil samples (Pb for Malagasy and Cr and Ni for the Italian one) can pose a greater risk to lemur health through the toxicological impact of these elements, including neurological, hematological, and reproductive toxicity. Moreover, given that geophagy was a repeated behavior observed in lemurs, it would be desirable to maintain the level of PTEs in the soil under control to prevent the risk of chronic toxicity and safeguard the health of lemur populations in the long term. The ingestion of Malagasy soil, rich in Pb, could have profound influences on the intestinal health of lemurs, mainly by altering the composition and functionality of their gut microbiome. Pb pollution of the gut environment could promote the selection of microbial species resistant to metals, thereby reducing global microbial diversity and impairing symbiotic functions involved in nutrient absorption and metabolic homeostasis. [12] Meanwhile, the high levels of Cr and Ni in Italian samples, even if reflecting the natural background levels, could still pose a long-term health risk through bioaccumulation and putative oxidative stress. The selective pressure of the two heavy metals could trigger a shift in the composition of the microbiome, promoting the development of resistant microorganisms at the expense of beneficial symbionts and potentially compromising digestive efficiency and immune regulation. Furthermore, the daily habit of geophagy can aggravate the exposure to elevated levels of PTEs, thereby constituting an additional pressure on the gut microbiome. The greater exposure could enhance the selection of resistant microbial species, leading to a more specialized but potentially less functionally diverse microbial community. The synergistic effect of metal-induced microbial imbalances and elevated fungal activity underscores the complex interplay between environmental pollution, gut microbial ecology, and gastrointestinal health in lemurs and suggests the adaptive trade-offs of geophagy behavior in polluted habitats. While such a shift may increase a PTEs tolerance, it may be at the cost of reduced digestive efficiency and metabolic versatility. This warrants monitoring of PTEs in the soil of zoological environments to exclude future chronic health effects of metal pollution.[10–12]

4 Conclusion

This study provides a significant contribution to the field of ecotoxicological biomonitoring, particularly in the context of non-invasive methodologies applied to endangered species. The

methodology used allowed us to characterize the mineral element content of lemur in the two habitats. Results highlight how different matrices reflect different exposure and accumulation dynamics: fur showed a more stable pattern and representative of chronic accumulation, revealing a significant correlation with the elemental composition of the soils; feces, instead, exhibit a particular sensitivity to seasonal and environmental variations providing useful information on the physiological state and recent exposure to the elements. Multivariate analysis of the fur dataset made it possible to distinguish samples by geographical origin. Pb, Al, Ba, and Fe emerged as key differentiating elements in the Malagasy dataset, aligned with the characteristics of the PBZT environment historically exposed to contamination by vehicular traffic and without regulation on emissions. On the other hand, the Italian dataset was described by As, Cr, Cu, and Ni, in line with the geochemical nature of soils around Turin. This similar pattern among elemental content in environmental and biological samples strongly supports the hypothesis of a direct relationship between habitat and bioaccumulation. Another significant element that emerged in the study was the seasonal separation observed in both fur and feces datasets, where samples were divided into groups associated with the hot and cold seasons, suggesting that climate factors could influence element content in lemur organisms, determining differences in diet or element bioavailability in the environment. This seasonal variability, if further confirmed by future longitudinal studies, may offer relevant insights for the optimization of nutritional and environmental management in zoological and conservation contexts.

Evidence confirms the validity and usefulness of a non-invasive approach to environment biomonitoring, which allows the acquisition of data minimizing animal stress, a particularly critical aspect in case of vulnerable or endangered species. The comparison among environmental and biological data provides a more complete picture of the interactions in the ecosystem compartments, allowing to identify early signs of exposure to contaminants and potential health risks. In light of the findings, this work offers a solid methodological basis for the application of the One Health paradigm in zoology and conservation, underlining the need to strengthen international and local collaborations to overcome logistical and regulatory barriers, especially in countries with high biodiversity and ecological vulnerability. The approach proposed here can be extended to future studies on other species and in different contexts, concretely contributing to the protection of biodiversity through effective, ethical and scientifically based monitoring.

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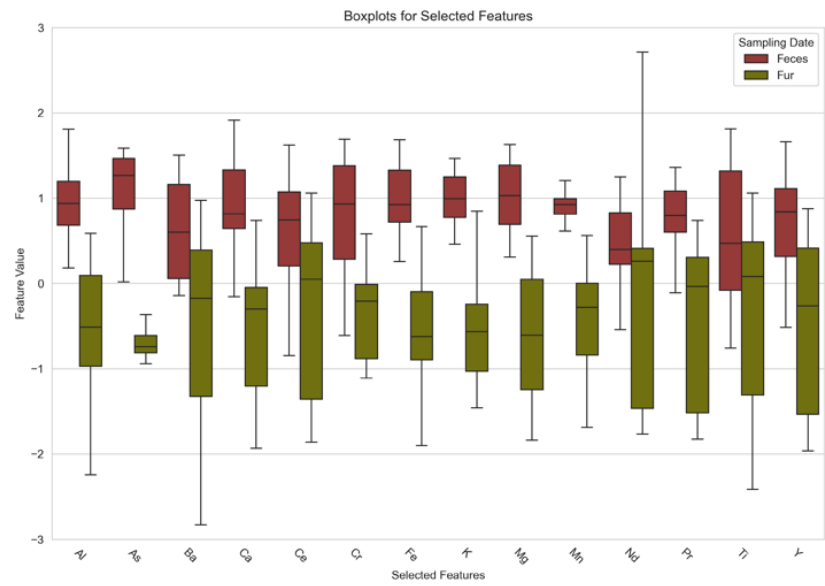
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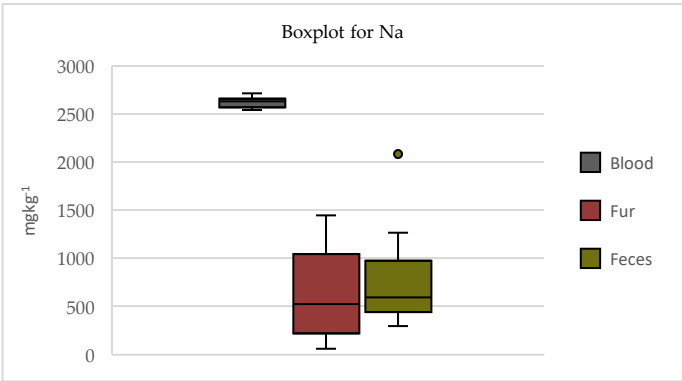
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Supplementary Material

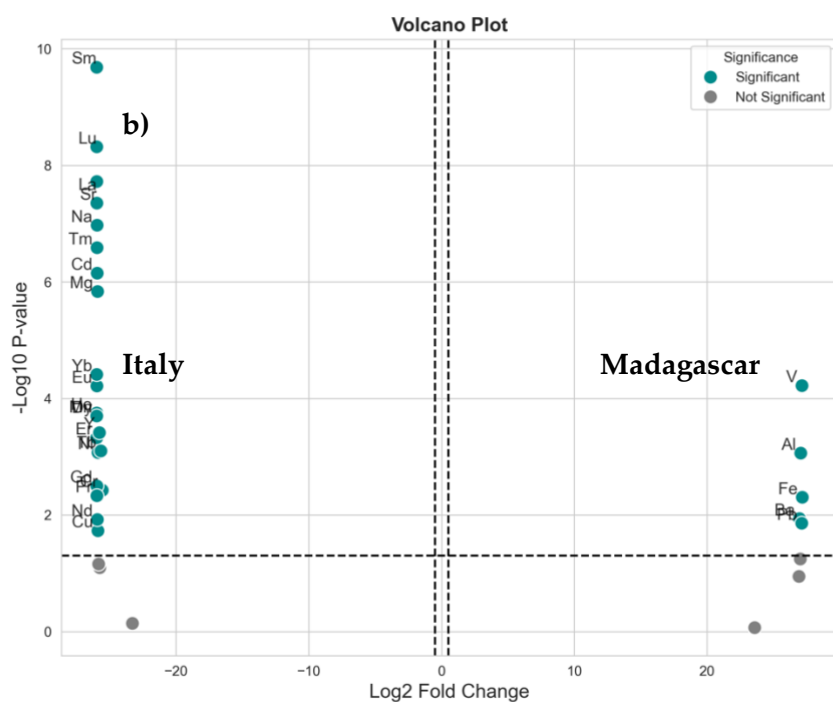
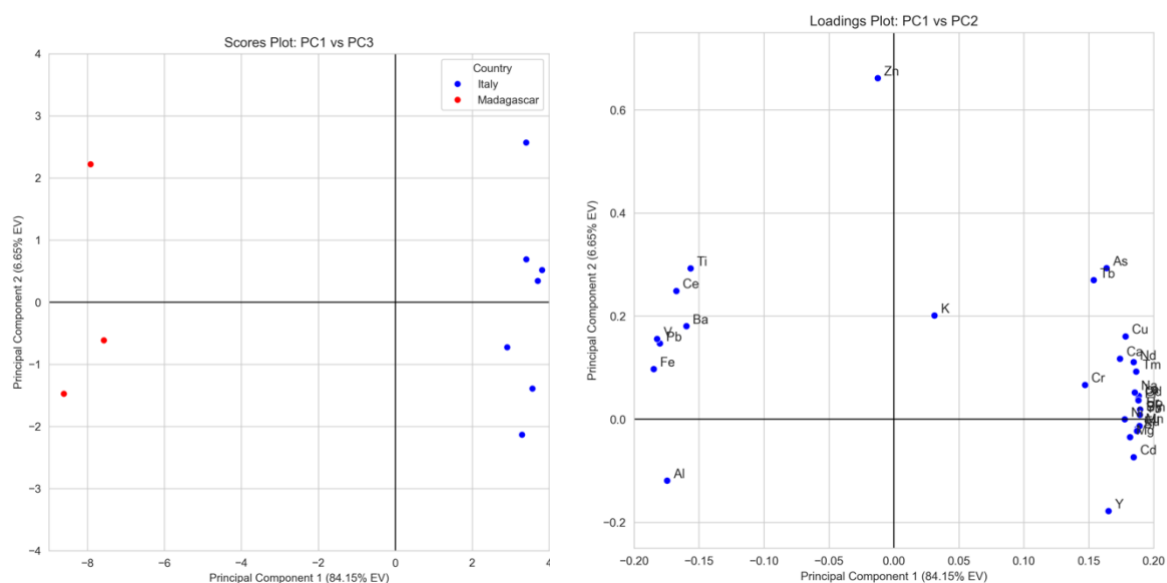


Supplementary Figure 1 Boxplot of variables showing statistically significantly higher concentrations in feces compared to fur for Italian samples

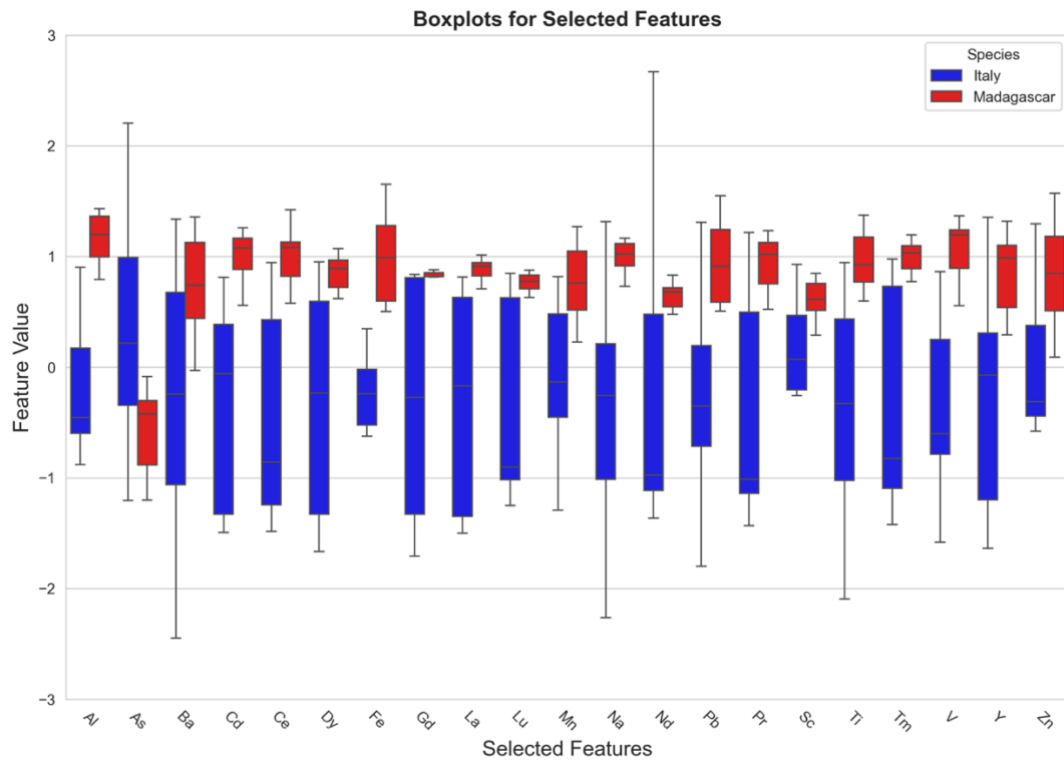


Supplementary Figure 2 Boxplot of variables showing statistically significantly higher concentrations in Blood compared to fur and feces for Italian samples

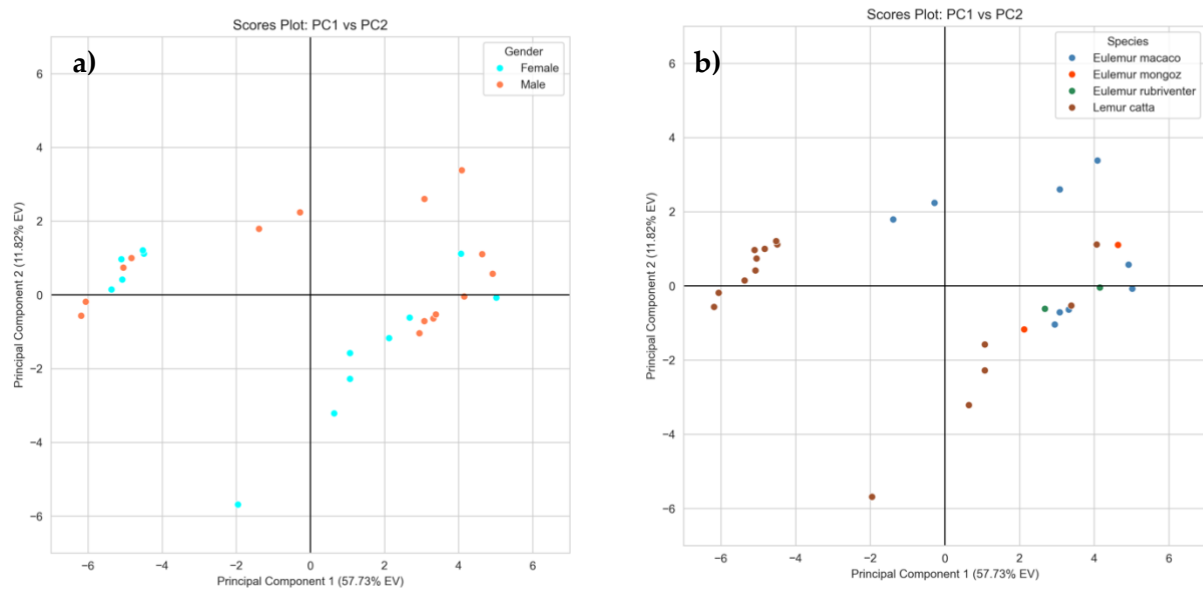
a)



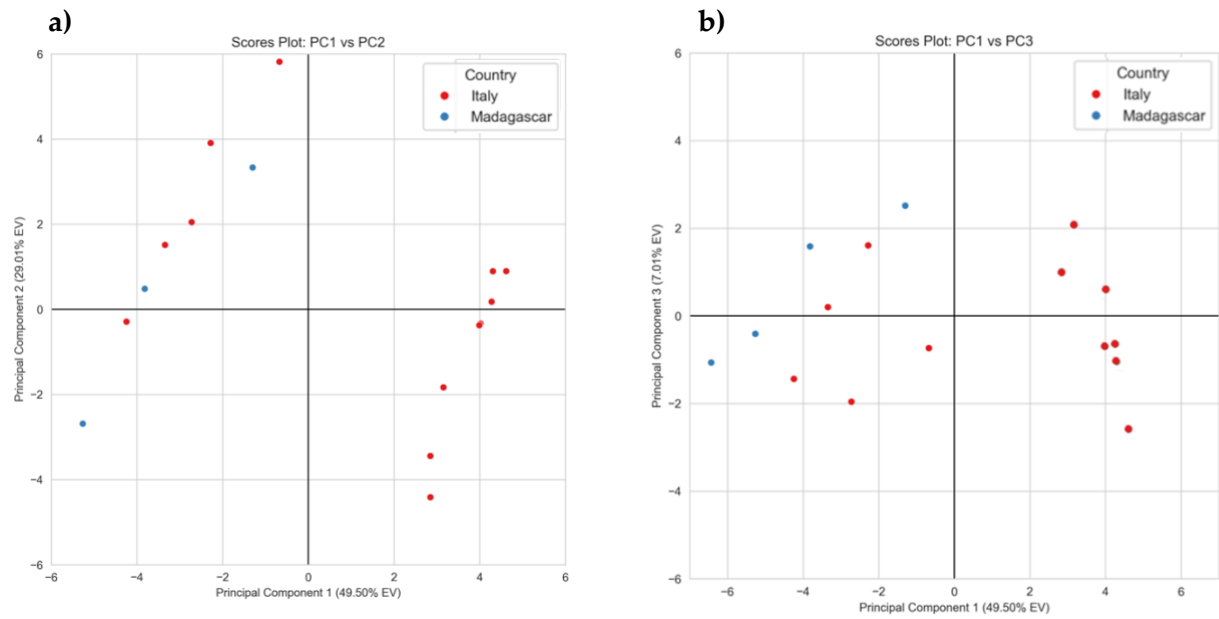
Supplementary Figure 3 a) PCA Score and loadings plot of soil dataset b) volcano plot of significant variables that distinguish Italian and Malagasy soil samples



Supplementary Figure 4 Boxplot of variables showing statistically significantly different Italian and Malagasy samples in fur dataset



Supplementary Figure 5 PCA Scores plot of fur dataset colored by gender (a) and species (b) of samples



Supplementary Figure 6 PCA Scores plot of feces dataset: a) PC1 vs PC2 and b) PC2 vs PC3

Supplementary Table 1 Recovery , certified values and obtained concentration obtained for Human hair certified reference material (mgkg⁻¹ ± Standard Error (SE))

Element	Certified value (mgkg ⁻¹ ±SE)	Obtained concentration (mgkg ⁻¹ ±SE)	% Recovery
Cu	33± 4	39 ± 2	118.0
As	0.044 ± 0.006	0.059 ± 0.007	134.1
Cd	0.125 ± 0.007	0.14 ± 0.01	112.8
Pb	2.1 ± 0.2	2.2 ± 0.2	102.3
Zn	210 ± 10	196 ± 6	93.8

Supplementary Table 2 Recovery , certified values and obtained concentration obtained for tomato leaves certified reference material (mgkg⁻¹ ± Standard Error (SE))

Element	Certified value (mgkg ⁻¹ ± SE)	Obtained concentration (mgkg ⁻¹ ± SE)	% Recovery
Al	598 ± 7	521 ± 20	87.12
Cd	1.52 ± 0.03	1.18 ± 0.05	77.63
Cr	2.00 ± 0.03	1.47 ± 0.01	73.5
Cu	4.7 ± 0.1	4,0 ± 0.2	84.47
Fe	368 ± 4	303 ± 3	82.34
Mn	246 ± 7	202 ± 3	82.11
Na	220 ± 4	134 ± 1	60.91
Ni	1.58 ± 0.04	2.2 ± 0.1	136.71
V	0.83 ± 0.03	0.560 ± 0.005	67.07
Zn	30.9 ± 0.6	24.2 ± 0.4	78.32

Supplementary Table 3 Methods tested for blood samples pretreatment

	Method 1	Method 2	Method 3 (chosen one)
Sample weight	0.5g	0.5g	0.5g
Internal vessel reagents	3 mL HNO₃/H₂O₂ ratio 2:1	1.5 mL of HNO₃	2.5 mL HNO₃ and 0.5 mL H₂O₂
External vessel Reagents	1mL of H ₂ O ₂ and 10ml of H ₂ O	1mL of H ₂ O ₂ and 10ml of H ₂ O	1mL of H ₂ O ₂ and 10ml of H ₂ O
MW program	8 min power ramp-up to 800W 3 min period at 800 W 1-hour ventilation phase	8 min power ramp-up to 800W 3 min period at 800 W 1-hour ventilation phase	8 min power ramp-up to 800W 3 min period at 800 W 1-hour ventilation phase

Supplementary Table 4 Recoveries obtained for the methods tested to pretreat blood samples. Some values in method 1 and 2 results under LOD due to a low recovery rate

Element	Method 1	Method 2	Method 3 (chosen on)
Al	57.8	<LOD	81.4
As	<LOD	<L.D	99.0
Ba	44.5	93.6	69.2
Ca	98.8	96.1	95.7
Cd	15.0	94.2	65.8
Fe	86.0	95.6	96.5
K	89.1	96.8	87.2
Mg	96.4	98.2	89,6
Mn	49.3	72.4	91.2
Na	96.2	93.6	87.3
Ni	59.3	89.9	67.1
Pb	51.4	<LOD	74.8
REE	22.0	91.3	91.3
Ti	<LOD	47.8	90.8
V	61.0	94.1	96.2
Zn	62.3	<LOD	79.9

Supplementary Table 5 Concentrations (mgkg⁻¹) of the analytes in soil samples collected both in Italy and in Madagascar

Sampling location	Soil Sample	Al	As	Ba	Ca	Cd	Ce	Cr	Cu	Dy	Er	Eu	Fe	Gd	Ho	K	La	Lu
Italy	1	23410	11.2	138	9100	1.78	53.7	251	34.8	30.5	17.2	9.36	34071	34.5	6.27	6107	311	2.33
Italy	2	19592	10.1	112	9088	2.62	50	255	24.9	33.2	18.3	12.1	28852	37.9	6.6	5169	212	2.6
Italy	3	22110	10.2	118	9784	3.23	54.7	231	26.7	38.4	20.6	15.8	29744	44.1	7.27	5590	155	2.97
Italy	4	26706	9.53	113	10586	2.97	48.4	131	18.1	32.7	17.3	13.4	26556	38.6	6.07	5958	152	2.49
Italy	5	36775	9.23	126	12602	3.47	60.7	218	28.6	37.6	20.3	15.5	32714	43.1	7.06	6787	159	3.01
Italy	6	31456	7.45	91.5	9721	3.83	45.4	278	27	33.1	17.8	13.6	31492	37.4	6.31	3952	118	2.57
Italy	7	31710	7.32	91.3	9598	2.4	43.5	240	23.7	30.4	16.8	12.6	29740	34.2	5.97	4435	113	2.48
Madagascar	8	58266	3.06	214	980	0.046	78.7	152	4.19	1.42	1.18	0.81	111853	5.59	0.464	6723	<LOD	<LOD
Madagascar	9	61351	6.4	158	1499	<LOD	147	142	9.94	2	1.46	1.06	131707	7.28	0.505	4202	<LOD	<LOD
Madagascar	10	80132	2.14	176	108	<LOD	93.5	120	5.42	1.28	0.84	0.49	88871	4.26	0.256	4472	<LOD	<LOD

Sampling location	Soil Sample	Mg	Mn	Na	Nd	Ni	Pb	Pr	Sc	Sm	Sr	Tb	Ti	Tm	V	Y	Yb	Zn
Italy	1	12429	785	1.124	124	157	10.9	52.1	<LOD	85	43.7	50.5	1.473	5.05	58.8	3.38	14.46	67.1
Italy	2	10573	724	953	118	150	10.6	57.1	<LOD	79.2	54	23.6	1.349	3.93	50.8	35.1	15.84	53.1
Italy	3	10350	821	1.005	124	130	13	64	<LOD	87.4	65.9	6.87	1.348	3.36	50.7	65	17.98	52.7
Italy	4	8688	595	660	124	84.3	9.82	62.2	<LOD	60.8	104	5.77	1.178	2.84	50.2	55.8	15	47
Italy	5	10655	706	1.487	128	124	11.6	65.3	<LOD	80.5	93	6.55	1.418	3.4	60.7	63.4	18.04	54.3
Italy	6	14044	706	398	93	144	10.7	51.5	<LOD	71.6	48.2	5.87	1.189	2.95	45.4	56.6	15.65	46.9
Italy	7	11753	619	618	86.7	132	9.29	48.4	<LOD	64	54.5	5.47	1.134	2.77	43.5	54.4	14.9	39.5
Madagascar	8	5028	22.9	22.9	25	43.5	23.1	4.28	4.82	<LOD	<LOD	0.69	1.766	0.068	124	1.98	0.863	46.5
Madagascar	9	4600	17.4	17.4	25.5	41.1	35.8	4.47	6.12	<LOD	<LOD	0.95	4.318	0.092	127	1.2	1.11	68.8
Madagascar	10	4845	25.6	25.6	12.6	57.5	25.8	1.9	1.99	<LOD	<LOD	0.59	2.347	0.045	107	0.073	0.716	45.2

Supplementary Table 6 mgkg⁻¹ and standard error (SE) of the analyte in water samples collected both in Italy and in Madagascar

Location	Water Sample	Al		As		Ba		Ca		Cd		Ce		Cr		Cu		Dy		Er	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Italy	W_1	0.0026	4×10 ⁻⁴	7.5×10 ⁻⁴	8×10 ⁻⁵	0.0067	5×10 ⁻⁴	12	1	<LOD		<LOD		3.7×10 ⁻⁴	5×10 ⁻⁵	0.0051	4×10 ⁻⁴	<LOD		<LOD	
Italy	W_2	0.0017	2.5×10 ⁻⁴	2.1×10 ⁻⁴	1×10 ⁻⁵	0.0050	3×10 ⁻⁴	15.90	0.07	<LOD		<LOD		1.4×10 ⁻⁴	1×10 ⁻⁵	0.0021	3×10 ⁻⁴	<LOD		<LOD	
Italy	W_3	0.0021	2×10 ⁻⁴	4.3×10 ⁻⁴	1×10 ⁻⁵	0.0048	6×10 ⁻⁴	17	3	<LOD		<LOD		1.76×10 ⁻⁴	2×10 ⁻⁶	0.0022	3×10 ⁻⁴	<LOD		<LOD	
Italy	W_4	0.00134	7.5×10 ⁻⁵	2.3×10 ⁻⁴	1.5×10 ⁻⁵	0.0051	6×10 ⁻⁴	16	3	<LOD		<LOD		3.6×10 ⁻⁴	3×10 ⁻⁵	0.0022	1×10 ⁻⁴	<LOD		<LOD	
Italy	W_5	0.0084	2×10 ⁻⁴	1.50×10 ⁻⁴	1×10 ⁻⁶	0.0035	4×10 ⁻⁴	10	1	<LOD		<LOD		5.8×10 ⁻⁴	5×10 ⁻⁵	0.00617	8×10 ⁻⁵	<LOD		<LOD	
Italy	W_6	0.0054	8×10 ⁻⁴	1.220×10 ⁻⁴	2×10 ⁻⁷	0.00285	4×10 ⁻⁵	12	1	<LOD		<LOD		5.2×10 ⁻⁴	1×10 ⁻⁵	0.0044	2×10 ⁻⁴	<LOD		<LOD	
Italy	W_7	0.019	0.001	1.3×10 ⁻⁴	1×10 ⁻⁵	0.0028	4×10 ⁻⁴	10	1	<LOD		<LOD		4.9×10 ⁻⁴	2×10 ⁻⁵	0.017	0.003	<LOD		<LOD	
Italy	W_8	0.0046	1×10 ⁻⁴	1.02×10 ⁻⁴	7×10 ⁻⁶	0.0020	1×10 ⁻⁴	8.8	0.3	<LOD		<LOD		2.77×10 ⁻⁴	7×10 ⁻⁶	0.0022	2×10 ⁻⁴	<LOD		<LOD	
Madagascar	W_9	0.21	0.03	4.6×10 ⁻⁴	4×10 ⁻⁵	0.132	0.007	18	2	<LOD		0.0151	3×10 ⁻⁴	<LOD		0.0059	7×10 ⁻⁴	5.0×10 ⁻⁴	4×10 ⁻⁵	1.7×10 ⁻⁴	1×10 ⁻⁵

Location	Water Sample	Eu		Fe		Gd		Ho		K		La		Lu		Mg		Mn		Na		Nd	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Italy	W_1	<LOD		0.18	0.02	<LOD		<LOD		3.04	0.04	<LOD		<LOD		5.5	0.9	0.0059	2×10 ⁻⁴	13		<LOD	
Italy	W_2	<LOD		0.17	0.02	<LOD		<LOD		3.6	0.2	<LOD		<LOD		7	0.7	0.0024	3.5×10 ⁻⁴	17	2	<LOD	
Italy	W_3	<LOD		0.18	0.03	<LOD		<LOD		3.4	0.5	<LOD		<LOD		6.5	0.2	0.00212	6.5×10 ⁻⁵	14.8	0.7	<LOD	
Italy	W_4	<LOD		0.22	0.02	<LOD		<LOD		3.1	0.15	<LOD		<LOD		6.5	0.9	0.0047	1×10 ⁻⁴	15	2	<LOD	
Italy	W_5	<LOD		0.23	0.03	<LOD		<LOD		2.55	0.01	<LOD		<LOD		6.4	0.3	0.00189	3×10 ⁻⁵	12.8	0.7	<LOD	
Italy	W_6	<LOD		0.22	0.02	<LOD		<LOD		2.9	0.2	<LOD		<LOD		7.4	0.2	0.0017	1×10 ⁻⁴	15	1	<LOD	
Italy	W_7	<LOD		0.29	0.04	<LOD		<LOD		27	0.35	<LOD		<LOD		7	1	0.0027	4×10 ⁻⁴	14.10	0.03	<LOD	
Italy	W_8	<LOD		0.28	0.04	<LOD		<LOD		2.7	0.4	<LOD		<LOD		6.8	0.3	8.7×10 ⁻⁴	6×10 ⁻⁵	12.6	0.3	<LOD	
Madagascar	W_9	0.0021	3×10 ⁻⁴	9	1	0.044	0.006	<LOD		2.69	0.3	0.0062	3×10 ⁻⁴	<LOD		6.9	0.5	1.15	0.05	4	0.2	0.00538	5×10 ⁻⁶

Supplementary Table 7 mg kg^{-1} and standard error (SE) of the analyte in water samples collected both in Italy and in Madagascar

Location	Water Sample	Ni		Pb		Pr		Sc		Sm		Sr		Tb		Ti		Tm	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Italy	W_1	0.0024	2×10^{-4}	3.5×10^{-4}	5×10^{-5}	<LOD		5.2×10^{-4}	9×10^{-5}	<LOD		<LOD		<LOD		7.6×10^{-4}	7×10^{-5}	<LOD	
Italy	W_2	0.0040	7×10^{-4}	1.58×10^{-4}	2.5×10^{-6}	<LOD		5.8×10^{-4}	2×10^{-5}	<LOD		<LOD		<LOD		5.7×10^{-4}	5×10^{-5}	<LOD	
Italy	W_3	0.0026	2×10^{-4}	1.80×10^{-4}	2×10^{-6}	<LOD		7.1×10^{-4}	9×10^{-5}	<LOD		<LOD		<LOD		8.3×10^{-4}	5×10^{-5}	<LOD	
Italy	W_4	0.0024	2×10^{-4}	3.0×10^{-4}	4×10^{-5}	<LOD		6.0×10^{-4}	8×10^{-5}	<LOD		<LOD		<LOD		0.0012	1.5×10^{-4}	<LOD	
Italy	W_5	0.0070	1.5×10^{-4}	0.00213	7×10^{-5}	<LOD		0.00145	3.5×10^{-5}	<LOD		<LOD		<LOD		9.0×10^{-4}	7×10^{-5}	<LOD	
Italy	W_6	0.0042	7×10^{-4}	1.94×10^{-4}	3×10^{-6}	<LOD		0.0014	2×10^{-4}	<LOD		<LOD		<LOD		0.0010	1×10^{-4}	<LOD	
Italy	W_7	0.033	0.001	4.3×10^{-4}	1×10^{-5}	<LOD		0.0013	1×10^{-4}	<LOD		<LOD		<LOD		0.00160	5×10^{-5}	<LOD	
Italy	W_8	0.008	0.001	9.0×10^{-5}	1×10^{-5}	<LOD		0.0025	3×10^{-4}	<LOD		<LOD		<LOD		0.00161	8×10^{-5}	<LOD	
Madagascar	W_9	0.0034	3×10^{-4}	0.0048	3×10^{-4}	0.0012	2×10^{-4}	8.0×10^{-4}	8×10^{-5}	0.0010	2×10^{-4}	<LOD		<LOD		0.0024	2×10^{-4}	<LOD	

Location	Water Sample	V		Y		Yb		Zn	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Italy	W_1	0.0013	1.5×10^{-4}	<LOD		<LOD		0.024	0.004
Italy	W_2	0.001010	3.5×10^{-6}	<LOD		<LOD		0.0068	7×10^{-4}
Italy	W_3	0.0010	1×10^{-4}	<LOD		<LOD		0.0108	5×10^{-4}
Italy	W_4	7.7×10^{-4}	2×10^{-5}	<LOD		<LOD		0.0041	4×10^{-4}
Italy	W_5	6.8×10^{-4}	4×10^{-5}	<LOD		<LOD		0.023	0.001
Italy	W_6	4.8×10^{-4}	5×10^{-5}	<LOD		<LOD		0.0108	9×10^{-4}
Italy	W_7	5.6×10^{-4}	2×10^{-5}	<LOD		<LOD		0.0630	1×10^{-4}
Italy	W_8	3.3×10^{-4}	2×10^{-5}	<LOD		<LOD		0.0063	2×10^{-4}
Madagascar	W_9			4.0×10^{-4}	3×10^{-5}	<LOD		0.049	0.003

Supplementary Table 8 Sample Labels of biological samples included in the study

Sample ID	Matrix	Location	Specie	Genre
20	Feces	Italy	Eulemur macaco	
21	Feces	Italy	Lemur catta	Male
22	Feces	Italy	Eulemur rubriventer	
23	Feces	Italy	Eulemur mongoz	
24	Feces	Italy	Lemur catta	Female
25	Feces	Italy	Eulemur macaco	
26	Feces	Italy	Eulemur macaco	
27	Feces	Italy	Eulemur rubriventer	
28	Feces	Italy	Eulemur rubriventer	
29	Feces	Italy	Lemur catta	
30	Feces	Italy	Lemur catta	Female
31	Feces	Italy	Lemur catta	Male
32	Feces	Italy	Lemur catta	Male
33	Feces	Madagascar	Eulemur macaco	
34	Feces	Madagascar	Lemur catta	
35	Feces	Madagascar	Eulemur rubriventer	
36	Feces	Madagascar	Eulemur mongoz	
37	Fur	Italy	Eulemur macaco	Male
38	Fur	Italy	Eulemur macaco	Male
39	Fur	Italy	Lemur catta	Female
40	Fur	Italy	Lemur catta	Female
41	Fur	Italy	Eulemur macaco	Male
42	Fur	Italy	Lemur catta	Female
43	Fur	Italy	Lemur catta	Female
44	Fur	Italy	Lemur catta	Female
45	Fur	Italy	Eulemur macaco	Male
46	Fur	Italy	Eulemur macaco	Male
47	Fur	Italy	Eulemur macaco	Male
48	Fur	Italy	Eulemur macaco	Male
49	Fur	Italy	Lemur catta	Female
50	Fur	Italy	Lemur catta	Male
51	Fur	Italy	Lemur catta	Female
52	Fur	Italy	Lemur catta	Female
53	Fur	Italy	Lemur catta	Male
54	Fur	Italy	Lemur catta	Female
55	Fur	Italy	Lemur catta	Male
56	Fur	Italy	Lemur catta	Male
57	Fur	Madagascar	Eulemur macaco	Male
58	Fur	Madagascar	Lemur catta	Male
59	Fur	Madagascar	Eulemur rubriventer	Male
60	Fur	Madagascar	Eulemur mongoz	Male
61	Fur	Madagascar	Eulemur macaco	Female
62	Fur	Madagascar	Lemur catta	Female
63	Fur	Madagascar	Eulemur rubriventer	Female
64	Fur	Madagascar	Eulemur mongoz	Female
65	Blood	Italy	Lemur catta	Female
66	Blood	Italy	Lemur catta	Female
67	Blood	Italy	Lemur catta	Male
68	Blood	Italy	Lemur catta	Female
69	Blood	Italy	Lemur catta	Female
70	Blood	Italy	Lemur catta	Male
71	Blood	Italy	Lemur catta	Female

Sample ID	Matrix	Location	Type
73	Food	Italy	Dry feed
74	Food	Italy	Dry feed
75	Food	Italy	Fruit
76	Food	Italy	Fruit
77	Food	Italy	Fruit

Supplementary Table 9 mgkg⁻¹ and standard error (SE) of the analyte in feces samples collected both in Italy and in Madagascar part 1

	Sample ID	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Al	Mean	2.5×10 ³	9×10 ³	6.1×10 ³	1.9×10 ³	2.8×10 ³	230	210	350	800	450	760	826	1.581×10 ³	1.366×10 ⁴	4.253×10 ³	1.464×10 ³	793
	SE	150	1×10 ³	200	100	100	20	20	40	90	65	80	103	188	923	331	136	28.7
As	Mean	1.96	6.5	5.2	1.48	1.9	1.0	1.6	3.6	5.9	2.71	5.5	4.19	0.22	108	7.14	4.52	0.256
	SE	9×10 ⁻²	0.9	0.7	6×10 ⁻²	0.2	0.1	0.1	0.2	0.4	2×10 ⁻²	0.7	0.328	3.789×10 ⁻²	8.89	1.17	0.319	2.375×10 ⁻³
Ba	Mean	1.8	4.7	2.7	1.9	2.4	9.8	8.2	9.2	8.2	14.2	9.74	13.9	15.1	2.14	2.5	2.14	2.5
	SE	0.3	0.5	0.1	0.1	0.4	0.9	0.6	0.5	1.39	0.87	0.198	1.58	0.701	3.052×10 ⁻²	0.193	0.352	7.777×10 ⁻²
Ca	Mean	2.6×10 ³	3.9×10 ³	2.2×10 ³	2.1×10 ³	4.1×10 ³	2.0×10 ³	2.7×10 ³	1.45×10 ³	2.599×10 ³	5.375×10 ³	4.904×10 ³	5.136×10 ³	6.572×10 ³	3.318×10 ³	2.947×10 ³	3.975×10 ³	2.681×10 ³
	SE	400	100	300	300	100	100	300	90	177	132	266	596	1.046×10 ³	60.9	177	409	28.5
Cd	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6.3×10 ⁻³	1.2×10 ⁻²	<LOD	3.54×10 ⁻³
	SE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	8.358×10 ⁻⁴	1.089×10 ⁻⁴	<LOD	4.921×10 ⁻⁴
Ce	Mean	0.43	1.7	0.82	0.41	0.52	0.10	3.8×10 ⁻²	0.190	0.53	0.42	0.53	0.72	0.798	0.81	0.34	0.13	7.9×10 ⁻²
	SE	7×10 ⁻²	0.1	7×10 ⁻²	6×10 ⁻²	4.5×10 ⁻²	1.5×10 ⁻²	3×10 ⁻³	5×10 ⁻³	3×10 ⁻²	6×10 ⁻²	8×10 ⁻²	3×10 ⁻²	4×10 ⁻³	6×10 ⁻²	5×10 ⁻²	2×10 ⁻²	2×10 ⁻³
Cr	Mean	3.08	4.8	3.1	1.51	1.87	1.1	1.000	3.5	5.8	2.5	5.2	4.8	7.4	0.640	0.28	0.42	0.09
	SE	0.335	0.41	0.313	7.543×10 ⁻²	0.15	0.1	6×10 ⁻³	0.35	0.1	0.3	0.7	0.2	0.1	8×10 ⁻³	2×10 ⁻²	6.5×10 ⁻²	1×10 ⁻²
Cu	Mean	1.76	2.18	1.9	1.76	1.98	25	6.5	22	7.4	23	6.4	20	11	0.55	0.73	0.59	0.54
	SE	0.166	0.36	0.27	0.269	0.0972	3	0.2	2	0.7	2	0.2	3	2	1×10 ⁻²	2×10 ⁻²	7×10 ⁻²	6×10 ⁻²
Dy	Mean	1.80×10 ⁻²	8×10 ⁻²	3.8×10 ⁻²	<LOD	2.11×10 ⁻²	1.3×10 ⁻²	9.7×10 ⁻³	2.40×10 ⁻²	5.1×10 ⁻²	3.0×10 ⁻²	5.1×10 ⁻²	5.8×10 ⁻²	7.2×10 ⁻²	2.99×10 ⁻²	9×10 ⁻³	<LOD	<LOD
	SE	2×10 ⁻⁴	1×10 ⁻²	5.5×10 ⁻³		9×10 ⁻⁴	2×10 ⁻³	8×10 ⁻⁴	1×10 ⁻⁴	2.5×10 ⁻³	4×10 ⁻³	7×10 ⁻³	4×10 ⁻³	4×10 ⁻³	6×10 ⁻⁴	1×10 ⁻³	<LOD	<LOD
Er	Mean	1.11×10 ⁻²	4.06×10 ⁻²	2.0×10 ⁻²	<LOD	1.15×10 ⁻²	6.2×10 ⁻³	5.1×10 ⁻³	1.2×10 ⁻²	2.56×10 ⁻²	1.43×10 ⁻²	2.6×10 ⁻²	2.9×10 ⁻²	3.6×10 ⁻²	1.20×10 ⁻²	4×10 ⁻³	<LOD	<LOD
	SE	8×10 ⁻⁴	4×10 ⁻³	3×10 ⁻³		2×10 ⁻⁴	7×10 ⁻⁴	7×10 ⁻⁴	1×10 ⁻³	6×10 ⁻⁴	1×10 ⁻⁴	3×10 ⁻³	1.5×10 ⁻³	2×10 ⁻³	8.5×10 ⁻⁴	3.512×10 ⁻⁴	<LOD	<LOD
Eu	Mean	0.204	0.23	0.22	0.20	0.21	6.1×10 ⁻³	5.3×10 ⁻³	8.0×10 ⁻³	1.46×10 ⁻²	1.23×10 ⁻²	1.5×10 ⁻²	1.9×10 ⁻²	2.2×10 ⁻²	0.117	0.096	7.8×10 ⁻²	8×10 ⁻²
	SE	9.5×10 ⁻³	2×10 ⁻²	2×10 ⁻²	3×10 ⁻²	2×10 ⁻²	6×10 ⁻⁴	7×10 ⁻⁴	6×10 ⁻⁴	3×10 ⁻⁴	9×10 ⁻⁴	2×10 ⁻³	1×10 ⁻³	2×10 ⁻³	4.5×10 ⁻³	9×10 ⁻³	5×10 ⁻³	1×10 ⁻²
Fe	Mean	3.4×10 ³	8×10 ³	7.6×10 ³	2.3×10 ³	3.5×10 ³	240	220	370	830	439	700	770	1.2×10 ³	3.6×10 ³	3.1×10 ³	1.8×10 ³	1.00×10 ³
	SE	600	1×10 ³	1×10 ³	200	200	6	20	20	60	7.5	100	70	200	300	400	300	100
Gd	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	0.0908	7.4×10 ⁻²	0.111	0.149	0.252	0.168	0.218	0.216	4.7×10 ⁻²	<LOD	<LOD	<LOD
	SE	<LOD	<LOD	<LOD	<LOD	<LOD	1.144×10 ⁻²	9.943×10 ⁻³	1.607×10 ⁻²	1.011×10 ⁻²	4.059×10 ⁻²	2.088×10 ⁻²	1.785×10 ⁻²	2.693×10 ⁻²	3.734×10 ⁻³	<LOD	<LOD	<LOD
Ho	Mean	2.33×10 ⁻²	8×10 ⁻²	4.4×10 ⁻²	1.7×10 ⁻²	2.52×10 ⁻²	4.4×10 ⁻³	3.6×10 ⁻³	8.4×10 ⁻³	1.23×10 ⁻²	1.13×10 ⁻²	1.3×10 ⁻²	1.5×10 ⁻²	1.53×10 ⁻²	3.17×10 ⁻²	1.16×10 ⁻²	3.7×10 ⁻³	<LOD
	SE	3×10 ⁻⁴	1×10 ⁻²	3×10 ⁻³	3×10 ⁻³	4×10 ⁻⁴	7.5×10 ⁻⁴	2×10 ⁻⁴	2.5×10 ⁻⁴	3×10 ⁻⁴	6×10 ⁻⁴	2×10 ⁻³	2×10 ⁻³	6×10 ⁻⁴	3×10 ⁻⁴	8×10 ⁻⁴	1×10 ⁻⁴	<LOD

Supplementary Table10 Concentrations (mgkg⁻¹) and standard error (SE) of the analytes in feces samples collected both in Italy and in Madagascar part 2

	Sample ID	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
K	Mean	1.7×10 ⁴	6.084×10 ³	1.52×10 ⁴	1.3×10 ⁴	9.1×10 ³	1.13×10 ⁴	9.9×10 ³	1.24×10 ⁴	1.20×10 ⁴	7.4×10 ³	7×10 ³	7.40×10 ³	9×10 ³	1×10 ⁴	1.9×10 ⁴	1.9×10 ⁴	1×10 ⁴
	SE	600	1×10 ³	300	2×10 ³	200	400	300	700	100	300	1×10 ³	30	1×10 ³	1×10 ³	1×10 ³	2×10 ³	2×10 ³
La	Mean	0.188	0.77	0.38	0.17	0.204	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.43	0.19	6 ×10 ⁻²	3.5×10 ⁻²
	SE	2×10 ⁻³	8×10 ⁻²	2×10 ⁻²	3×10 ⁻²	8.5×10 ⁻³	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	6×10 ⁻²	3×10 ⁻²	1×10 ⁻²	6×10 ⁻³
Lu	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	1.9×10 ⁻³	2.0×10 ⁻³	2.77×10 ⁻³	4.5×10 ⁻³	3.40×10 ⁻³	4.4×10 ⁻³	5.0×10 ⁻³	5.6×10 ⁻³	<LOD	<LOD	<LOD	<LOD
	SE	<LOD	<LOD	<LOD	<LOD	<LOD	3×10 ⁻⁴	3×10 ⁻⁴	6×10 ⁻⁵	6×10 ⁻⁴	2×10 ⁻⁵	7×10 ⁻⁴	6×10 ⁻⁴	1.5×10 ⁻⁴	<LOD	<LOD	<LOD	<LOD
Mg	Mean	4.1×10 ³	1.5×10 ³	1.29×10 ³	1.7×10 ³	1.77×10 ³	5.2×10 ³	4.8×10 ³	3.6×10 ³	4.6×10 ³	960	730	1.110×10 ³	1.0×10 ³	2.4×10 ³	2.3×10 ³	2.1×10 ³	2.4×10 ³
	SE	200	120	40	200	80	400	600	600	500	60	60	10	200	300	300	350	400
Mn	Mean	122	104	62	125	82	267	218	120	190	76	28	78	50	130	110	90	104
	SE	5	9	7	7	8	8.5	9	9	20	7	4	7	1	20	20	10	5
Na	Mean	1.27×10 ³	560	430	410	290	830	2.08×10 ³	450	360	600	500	1.0×10 ³	444	980	840	800	960
	SE	50	80	20	20	30	50.5	85	40	3	10	10	200	3	80	20	20	10
Nd	Mean	0.144	0.258	0.333	0.39	0.50	0.67	0.31	0.13	0.16	0.092	7.33×10 ⁻²	0.1440	0.33	0.32	0.123	3.4×10 ⁻²	1.3×10 ⁻²
	SE	1.281×10 ⁻²	3.712×10 ⁻²	1.697×10 ⁻²	2 ×10 ⁻²	7.5×10 ⁻²	0.01	4×10 ⁻²	2×10 ⁻²	2×10 ⁻²	2×10 ⁻³	1×10 ⁻³	8.5×10 ⁻⁴	5.5×10 ⁻²	4×10 ⁻²	3×10 ⁻³	2×10 ⁻³	1×10 ⁻³
Ni	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.97	0.38	0.18	0.126
	SE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.01	4.5×10 ⁻²	1×10 ⁻²	9×10 ⁻³
Pb	Mean	0.20	<LOD	<LOD	<LOD	<LOD	0.554	0.29	0.20	0.24	<LOD	<LOD	<LOD	<LOD	0.39	0.25	6.9×10 ⁻²	8.8×10 ⁻²
	SE	2×10 ⁻²	<LOD	<LOD	<LOD	<LOD	5×10 ⁻³	3×10 ⁻²	2×10 ⁻²	2×10 ⁻²	<LOD	<LOD	<LOD	<LOD	3×10 ⁻²	4×10 ⁻²	6×10 ⁻³	1×10 ⁻³
Pr	Mean	2.86×10 ⁻²	8×10 ⁻²	8.6×10 ⁻²	0.0995	0.12	0.161	6.9×10 ⁻²	2.0×10 ⁻²	2.8×10 ⁻²	3.0×10 ⁻²	2.5×10 ⁻²	4.3×10 ⁻²	8.4×10 ⁻²	8.2×10 ⁻²	2.7×10 ⁻²	5.26×10 ⁻³	1.16×10 ⁻³
	SE	2×10 ⁻⁴	1×10 ⁻²	4.5×10 ⁻³	2×10 ⁻⁴	2×10 ⁻²	5×10 ⁻³	1×10 ⁻³	2×10 ⁻³	2×10 ⁻³	2×10 ⁻³	4×10 ⁻³	3×10 ⁻³	2×10 ⁻³	9 ×10 ⁻³	1×10 ⁻³	7.5×10 ⁻⁵	9×10 ⁻⁵
Sc	Mean	6.75×10 ⁻²	0.17	0.172	0.2110	0.22	0.200	0.12	4.9×10 ⁻²	7.4×10 ⁻²	7.1×10 ⁻²	6.0×10 ⁻²	0.136	0.167	0.175	5.0×10 ⁻²	1.748×10 ⁻²	6.56×10 ⁻³
	SE	2×10 ⁻⁴	1×10 ⁻²	4.5×10 ⁻³	2×10 ⁻⁴	2×10 ⁻²	5×10 ⁻³	1 ×10 ⁻²	2×10 ⁻³	2×10 ⁻³	2×10 ⁻³	4×10 ⁻³	3×10 ⁻³	2×10 ⁻³	9×10 ⁻³	1×10 ⁻³	7.5×10 ⁻⁵	9×10 ⁻⁵
Sm	Mean	<LOD	3.3×10 ⁻²	5.5×10 ⁻²	6.5×10 ⁻²	0.09	0.09	2.2×10 ⁻²	<LOD	<LOD	1.1×10 ⁻²	8×10 ⁻³	1.75×10 ⁻²	5.8×10 ⁻²	4.5×10 ⁻²	3.9×10 ⁻³	<LOD	<LOD
	SE	<LOD	2×10 ⁻³	3.5×10 ⁻³	7×10 ⁻³	1×10 ⁻²	1×10 ⁻²	2×10 ⁻³	<LOD	<LOD	1×10 ⁻³	1×10 ⁻³	6×10 ⁻⁴	5×10 ⁻³	1×10 ⁻³	3×10 ⁻⁴	<LOD	<LOD

Supplementary Table 11 mgkg⁻¹ and standard error (SE) of the analyte in feces samples collected both in Italy and in Madagascar part 3

	Sample ID	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Sr	Mean	<LOD	19	17	17	22	<LOD	<LOD	<LOD	<LOD	7.3	10.5	6.8	9	<LOD	<LOD	<LOD	<LOD
	SE	<LOD	1	2	1	3	<LOD	<LOD	<LOD	<LOD	0.2	0.6	0.9	1	<LOD	<LOD	<LOD	<LOD
Tb	Mean	<LOD	5.1×10 ⁻³	8.5×10 ⁻³	9.9×10 ⁻³	1.3×10 ⁻²	<LOD	<LOD	<LOD	<LOD	2.1×10 ⁻³	1.39×10 ⁻³	3.6×10 ⁻³	9 ×10 ⁻³	4.4×10 ⁻³	<LOD	<LOD	<LOD
	SE	<LOD	7×10 ⁻⁴	7×10 ⁻⁴	7×10 ⁻⁴	2×10 ⁻³	<LOD	<LOD	<LOD	<LOD	2×10 ⁻⁴	3×10 ⁻⁵	4×10 ⁻⁴	1×10 ⁻³	2×10 ⁻⁴	<LOD	<LOD	<LOD
Ti	Mean	7.9	13.6	24	22	40	22	11	4.4	6.6	6.7	8.4	10.7	24.2	3.7	2.0	3.73	2.02
	SE	0.2	0.5	2	3	5	2.5	0.55	0.5	0.2	0.6	0.8	0.7	0.7	0.6	0.3	9×10 ⁻²	6×10 ⁻²
Tm	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
V	Mean	0.41	3.6×10 ⁻³	4.2×10 ⁻³	5.10×10 ⁻³	5.2×10 ⁻³	1.450	0.75	0.27	0.43	1.3×10 ⁻³	1.04×10 ⁻³	2.81×10 ⁻³	4.3×10 ⁻³	0.59	0.23	0.18	7.9×10 ⁻²
	SE	6×10 ⁻²	2×10 ⁻⁴	4×10 ⁻⁴	2×10 ⁻⁵	3×10 ⁻⁴	3×10 ⁻³	1×10 ⁻²	3×10 ⁻²	6×10 ⁻²	1.5×10 ⁻⁴	2.5×10 ⁻⁵	2×10 ⁻⁵	4×10 ⁻⁴	3×10 ⁻²	1×10 ⁻²	1×10 ⁻²	7×10 ⁻³
Y	Mean	0.104	0.133	0.18	0.22	0.23	0.41	0.20	5.9×10 ⁻²	0.12	5.4×10 ⁻²	3.71×10 ⁻²	0.118	0.17	0.138	5.0×10 ⁻²	1.54×10 ⁻²	7.6×10 ⁻³
	SE	4×10 ⁻³	9×10 ⁻³	1.5×10 ⁻²	3×10 ⁻²	1×10 ⁻²	7×10 ⁻²	2×10 ⁻²	6×10 ⁻³	1.5×10 ⁻²	4×10 ⁻³	7×10 ⁻⁴	8×10 ⁻³	2×10 ⁻²	8×10 ⁻³	5×10 ⁻³	6×10 ⁻⁴	8×10 ⁻⁴
Yb	Mean	<LOD	2.0×10 ⁻²	2.7×10 ⁻²	3.1×10 ⁻²	3.4×10 ⁻²	3.0×10 ⁻²	1.31×10 ⁻²	<LOD	<LOD	1.07×10 ⁻²	1.10×10 ⁻²	1.64×10 ⁻²	2.6×10 ⁻²	6.0×10 ⁻³	<LOD	<LOD	<LOD
	SE	<LOD	3×10 ⁻³	2×10 ⁻³	4×10 ⁻³	5×10 ⁻³	4×10 ⁻³	9×10 ⁻⁴	<LOD	<LOD	5×10 ⁻⁴	8×10 ⁻⁴	3×10 ⁻⁴	4×10 ⁻³	6×10 ⁻⁴	<LOD	<LOD	<LOD
Zn	Mean	600	77	29	62	39.5	760	720	890	840	80	112	76	31	1.16×10 ³	1.8×10 ³	950	610.0
	SE	90	8	4	3	6	80	90	10	10	10	8	12	4	95	200	75	0.7

Supplementary Table 12 mgkg⁻¹ and standard error (SE) of the analyte in fur samples collected both in Italy and in Madagascar part 1

	Al		As		Ba		Ca		Cd		Ce		Cr		Cu		Dy	
Sample ID	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
37	80	5	5.9×10 ⁻²	4×10 ⁻³	3.1	0.5	1.52×10 ³	90	0.1330	3×10 ⁻⁴	0.284	6×10 ⁻³	1.00	3×10 ⁻²	7	1	1.9×10 ⁻²	1×10 ⁻³
38	67	3	6.6×10 ⁻²	3×10 ⁻³	3.7	0.2	1.7×10 ³	200	0.100	6×10 ⁻³	0.24	2×10 ⁻²	0.605	4×10 ⁻³	6.4	0.6	1.87×10 ⁻²	5×10 ⁻⁴
39	<LOD	<LOD	3.8×10 ⁻²	3×10 ⁻³	5.8×10 ⁻²	9×10 ⁻³	1.6×10 ³	200	1.9×10 ⁻³	1×10 ⁻⁴	0.10	1.5×10 ⁻²	<LOD	<LOD	4.6	0.7	9.6×10 ⁻³	6×10 ⁻⁴
40	8.6	0.3	0.12	1×10 ⁻²	0.90	4×10 ⁻²	1.57×10 ³	80	5.151×10 ⁻²	9×10 ⁻⁵	0.16	1×10 ⁻²	0.183	9.5×10 ⁻³	11	2	1.19×10 ⁻²	8×10 ⁻⁴
41	60	7	5.1×10 ⁻²	3×10 ⁻³	3.6	0.2	1.7×10 ³	100	0.134	7×10 ⁻³	0.22	4×10 ⁻²	0.896	1.5×10 ⁻³	7.9	0.7	1.7×10 ⁻²	2×10 ⁻³
42	12.9	0.2	6.8×10 ⁻²	3×10 ⁻³	1.27	4×10 ⁻²	1.3×10 ³	100	1.3×10 ⁻²	2×10 ⁻³	0.14	2×10 ⁻²	7.73	1.19	16	2	1.27×10 ⁻²	7.5×10 ⁻⁴
43	11	1	8.9×10 ⁻²	7×10 ⁻³	1.07	2×10 ⁻²	1.3×10 ³	200	1.51×10 ⁻²	9×10 ⁻⁴	0.15	2×10 ⁻²	<LOD	<LOD	15	1	1.291×10 ⁻²	6×10 ⁻⁵
44	10	1.5	5.1×10 ⁻²	3×10 ⁻³	1.77	7×10 ⁻²	711	5.5	<LOD	<LOD	<LOD	<LOD	0.53	4.5×10 ⁻²	8.5	0.25	<LOD	<LOD
45	40	5	0.114	3×10 ⁻³	3.4	0.3	1.6×10 ³	200	2×10 ⁻²	2×10 ⁻³	<LOD	<LOD	0.432	9×10 ⁻³	4.5	0.7	3.2×10 ⁻³	4×10 ⁻⁴
46	200	10	0.137	3×10 ⁻³	7.5	0.7	2.75×10 ³	60	2.7×10 ⁻²	1×10 ⁻³	0.337	9×10 ⁻³	1.53	0.09	5.3	0.6	2.4×10 ⁻²	1×10 ⁻³
47	98	5	0.096	8×10 ⁻³	6.2	0.25	2.8×10 ³	200	2.7×10 ⁻²	2.5×10 ⁻³	0.15	2×10 ⁻²	0.89	0.015	5.9	0.1	1.0×10 ⁻²	2×10 ⁻³
48	32	4	3.4×10 ⁻²	2×10 ⁻³	2.13	2×10 ⁻²	1.08×10 ³	20	1.3×10 ⁻²	2×10 ⁻³	<LOD	<LOD	0.59	8×10 ⁻²	3.98	5×10 ⁻²	2.9×10 ⁻³	2.5×10 ⁻⁴
49	15	2	8.1×10 ⁻²	5×10 ⁻³	0.35	2×10 ⁻²	390	60	<LOD	<LOD	<LOD	<LOD	0.53	2×10 ⁻²	6	1	<LOD	<LOD
50	11.4	0.8	5.8×10 ⁻²	3×10 ⁻³	0.409	9×10 ⁻³	530	90	<LOD	<LOD	<LOD	<LOD	0.52	3×10 ⁻²	9.3	0.7	<LOD	<LOD
51	14	2	6.9×10 ⁻²	9×10 ⁻⁴	0.28	3×10 ⁻²	427	4	<LOD	<LOD	<LOD	<LOD	0.86	0.01	7.3	0.8	<LOD	<LOD
52	9.3	0.6	4.7×10 ⁻²	2×10 ⁻³	0.31	5×10 ⁻²	480	40	<LOD	<LOD	<LOD	<LOD	0.48	6.773×10 ⁻²	8	1	<LOD	<LOD
53	12	1	4.8×10 ⁻²	1×10 ⁻³	0.38	5.5×10 ⁻²	420	10.7	<LOD	<LOD	<LOD	<LOD	0.55	8×10 ⁻²	7.8	0.5	<LOD	<LOD
54	5	0.3	3.7×10 ⁻²	5×10 ⁻³	0.26	1×10 ⁻²	576	53	<LOD	<LOD	<LOD	<LOD	0.19	2×10 ⁻²	8.3	0.9	<LOD	<LOD
55	<LOD	<LOD	2×10 ⁻²	2×10 ⁻³	0.43	2×10 ⁻²	1.1×10 ³	100	<LOD	<LOD	<LOD	<LOD	0.1380	4×10 ⁻⁴	7.50	7×10 ⁻²	<LOD	<LOD
56	<LOD	<LOD	6.1×10 ⁻²	7×10 ⁻³	0.20	2×10 ⁻²	900	40	<LOD	<LOD	<LOD	<LOD	0.151	6×10 ⁻³	5.5	0.85	<LOD	<LOD
57	400	40	5.3×10 ⁻²	7.5×10 ⁻³	7.7	0.7	1.4×10 ³	100	0.74	1×10 ⁻²	0.70	4.5×10 ⁻²	0.42	3×10 ⁻²	5.6	0.65	2.3×10 ⁻²	2×10 ⁻³
58	340	40	4.7×10 ⁻²	6×10 ⁻³	2.6	0.4	1.1×10 ³	200	0.31	2×10 ⁻²	0.42	6.5×10 ⁻²	0.31	1×10 ⁻²	7.2	0.5	1.6×10 ⁻²	3×10 ⁻³
59	510	60	3.9×10 ⁻²	5×10 ⁻³	3.3	0.1	1.45×10 ³	10	0.50	6×10 ⁻²	0.30	2×10 ⁻²	0.37	4×10 ⁻²	5.3	0.1	1.99×10 ⁻²	4×10 ⁻⁴
60	595	3	3.4×10 ⁻²	4×10 ⁻³	5.37	8.5×10 ⁻²	1.48×10 ³	60	0.45	7×10 ⁻²	0.42	6×10 ⁻²	0.84	0.01	4.7	0.4	2.9×10 ⁻²	3×10 ⁻³
61	280	30	4.81×10 ⁻²	3×10 ⁻⁴	6.8	0.9	1.50×10 ³	45	0.56	2×10 ⁻²	0.41	6.5×10 ⁻²	0.39	5×10 ⁻²	4.9	0.3	2.3×10 ⁻²	1×10 ⁻³
62	530	80	4.606×10 ⁻²	3×10 ⁻³	3.62	2×10 ⁻²	1.2×10 ³	100	0.21	3×10 ⁻²	0.54	4×10 ⁻²	0.51	4×10 ⁻²	11.2	0.6	2.8×10 ⁻²	3×10 ⁻³
63	60	8	5.1×10 ⁻²	5×10 ⁻³	1.74	6×10 ⁻²	1.57×10 ³	20	0.102	5×10 ⁻³	0.192	4×10 ⁻³	0.17	1×10 ⁻²	3.0	0.2	1.34×10 ⁻²	8×10 ⁻⁴
64	159	1	3.7×10 ⁻²	5×10 ⁻³	1.29	5×10 ⁻²	400	60	5.1×10 ⁻²	7×10 ⁻³	0.2200	8×10 ⁻⁴	0.19	1×10 ⁻²	1.7	0.2	1.6×10 ⁻²	1×10 ⁻³

Supplementary Table 13 mgkg⁻¹ and standard error (SE) of the analyte in fur samples collected both in Italy and in Madagascar part 2

	Eu		Fe		Gd		Ho		K		La		Lu		Mg	
Sample ID	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
37	<LOD	<LOD	8	1	0.223	7×10 ⁻³	<LOD	<LOD	4.27×10 ³	30	0.22	3×10 ⁻²	1.65×10 ⁻³	5×10 ⁻⁵	1.06×10 ³	30
38	<LOD	<LOD	10	1	0.218	7×10 ⁻³	<LOD	<LOD	5.5×10 ³	800	0.19	8. ×10 ⁻³	1.6×10 ⁻³	2×10 ⁻⁴	1.0×10 ³	200
39	<LOD	<LOD	0.10	1×10 ⁻²	0.173	7×10 ⁻³	<LOD	<LOD	1.87×10 ³	90	0.11	9×10 ⁻³	1.1×10 ⁻³	2×10 ⁻⁴	30	3
40	<LOD	<LOD	0.95	8×10 ⁻²	0.21	2×10 ⁻²	<LOD	<LOD	1.5×10 ³	100	0.16	1×10 ⁻²	1.3×10 ⁻³	1×10 ⁻⁴	80	10
41	<LOD	<LOD	12.2	0.9	0.22	3×10 ⁻²	<LOD	<LOD	4.56×10 ³	10	0.20	2×10 ⁻²	1.5×10 ⁻³	2×10 ⁻⁴	950	40
42	<LOD	<LOD	1.6	0.2	0.21	2×10 ⁻²	<LOD	<LOD	2.1×10 ³	200	0.15	2×10 ⁻²	1.8×10 ⁻³	2×10 ⁻⁴	93	6
43	<LOD	<LOD	1.66	4×10 ⁻²	0.218	8×10 ⁻³	<LOD	<LOD	1.3×10 ³	200	0.15	2×10 ⁻²	1.3×10 ⁻³	2×10 ⁻⁴	36	1
44	<LOD	<LOD	17.8	0.8	<LOD	<LOD	<LOD	<LOD	238	20	<LOD	<LOD	<LOD	<LOD	163	5
45	6.1×10 ⁻⁴	5×10 ⁻⁵	51	9	2.4×10 ⁻²	1.5×10 ⁻³	<LOD	<LOD	5.8×10 ³	100	3.3×10 ⁻²	2×10 ⁻³	<LOD	<LOD	470	20
46	1.13×10 ⁻²	1×10 ⁻⁴	240	30	0.09	1.5×10 ⁻²	4.3×10 ⁻³	6×10 ⁻⁴	1.2×10 ⁴	1×10 ³	0.15	1.5×10 ⁻²	<LOD	<LOD	640	90
47	3.5×10 ⁻³	3×10 ⁻⁴	120	20	6.04×10 ⁻²	4.5×10 ⁻³	1.7×10 ⁻³	2×10 ⁻⁴	9×10 ³	1.5×10 ³	7.0×10 ⁻²	1×10 ⁻³	<LOD	<LOD	750	90
48	<LOD	<LOD	47	1	1.8×10 ⁻²	1×10 ⁻³	<LOD	<LOD	3.1×10 ³	400	2.3×10 ⁻²	3 ×10 ⁻³	<LOD	<LOD	270	30
49	<LOD	<LOD	21.6	0.1	<LOD	<LOD	<LOD	<LOD	1.1×10 ³	100	<LOD	<LOD	<LOD	<LOD	60	6
50	<LOD	<LOD	25	3	<LOD	<LOD	<LOD	<LOD	270	15	<LOD	<LOD	<LOD	<LOD	70	10
51	<LOD	<LOD	27	3	<LOD	<LOD	<LOD	<LOD	910	20	<LOD	<LOD	<LOD	<LOD	63	9
52	<LOD	<LOD	19.9	0.6	<LOD	<LOD	<LOD	<LOD	660	30	<LOD	<LOD	<LOD	<LOD	63	9
53	<LOD	<LOD	24	3	<LOD	<LOD	<LOD	<LOD	710	55	<LOD	<LOD	<LOD	<LOD	80	10
54	<LOD	<LOD	15	1	<LOD	<LOD	<LOD	<LOD	1.0×10 ³	100	<LOD	<LOD	<LOD	<LOD	64	9
55	<LOD	<LOD	14.2	0.35	<LOD	<LOD	<LOD	<LOD	700	100	<LOD	<LOD	<LOD	<LOD	100	20
56	<LOD	<LOD	11.8	0.6	<LOD	<LOD	<LOD	<LOD	1.5×10 ³	200	<LOD	<LOD	<LOD	<LOD	95	10
57	<LOD	<LOD	630	80	0.24	3×10 ⁻²	<LOD	<LOD	2.5×10 ³	200	0.49	5×10 ⁻²	1.7×10 ⁻³	3×10 ⁻⁴	236	5
58	<LOD	<LOD	80	1	0.22	2×10 ⁻²	<LOD	<LOD	2.1×10 ³	200	0.27	4×10 ⁻²	1.46×10 ⁻³	3×10 ⁻⁵	210	30
59	<LOD	<LOD	160	10	0.21	2×10 ⁻²	<LOD	<LOD	1.45×10 ³	10	0.24	2×10 ⁻²	1.5×10 ⁻³	2×10 ⁻⁴	571	3
60	<LOD	<LOD	280	50	0.19	2. ×10 ⁻²	<LOD	<LOD	2.5×10 ³	400	0.274	6×10 ⁻³	1.93×10 ⁻³	1×10 ⁻⁵	620	100
61	<LOD	<LOD	420	60	0.23	3×10 ⁻²	<LOD	<LOD	2.3×10 ³	300	0.28	4×10 ⁻²	3.8×10 ⁻²	3×10 ⁻³	350	40
62	2.81×10 ⁻²	1×10 ⁻⁴	200	25	0.28	4.5×10 ⁻²	<LOD	<LOD	1.8×10 ³	200	0.34	5×10 ⁻²	1.74×10 ⁻³	5×10 ⁻⁵	420	10
63	<LOD	<LOD	84	5	0.21	2×10 ⁻²	<LOD	<LOD	2.1×10 ³	300	0.18	3×10 ⁻²	1.29×10 ⁻³	3×10 ⁻⁵	230	15
64	<LOD	<LOD	69	8	0.21	2×10 ⁻²	<LOD	<LOD	1.2×10 ³	200	0.193	9×10 ⁻³	1.4×10 ⁻³	2×10 ⁻⁴	74	8

Supplementary Table 14 mgkg⁻¹ and standard error (SE) of the analyte in fur samples collected both in Italy and in Madagascar part 3

	Mn		Na		Ni		Nd		Pb		Pr		Sc		Sm		Sr	
Sample ID	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
37	<LOD	<LOD	500	20	<LOD	<LOD	0.16	2.5×10 ⁻²	0.53	3×10 ⁻²	1.4×10 ⁻²	2×10 ⁻³	0.29	3×10 ⁻²	1.2×10 ⁻²	1×10 ⁻³	<LOD	<LOD
38	<LOD	<LOD	590	80	<LOD	<LOD	0.16	5×10 ⁻³	0.73	4×10 ⁻²	1.3×10 ⁻²	1×10 ⁻³	0.30	2.6×10 ⁻²	1.5×10 ⁻²	2.5×10 ⁻³	<LOD	<LOD
39	3.41	0.09	570	80	<LOD	<LOD	0.10	1×10 ⁻²	1.8×10 ⁻²	3×10 ⁻³	3.7×10 ⁻³	3×10 ⁻⁴	8.3×10 ⁻²	8×10 ⁻³	<LOD	<LOD	<LOD	<LOD
40	12.4	0.6	520	20	<LOD	<LOD	0.13	2×10 ⁻²	0.34	3×10 ⁻²	6.2×10 ⁻³	9×10 ⁻⁴	0.147	7×10 ⁻³	<LOD	<LOD	<LOD	<LOD
41	<LOD	<LOD	520	20	<LOD	<LOD	0.15	1.5×10 ⁻²	0.607	7×10 ⁻³	1.0×10 ⁻²	1×10 ⁻³	0.309	3×10 ⁻³	<LOD	<LOD	<LOD	<LOD
42	5.8	0.9	480	70	<LOD	<LOD	0.13	1×10 ⁻²	0.33	4×10 ⁻²	6.310×10 ⁻³	9×10 ⁻⁶	8.9×10 ⁻²	5×10 ⁻³	<LOD	<LOD	<LOD	<LOD
43	9	1.5	340	10	<LOD	<LOD	0.13	2×10 ⁻²	0.239	1×10 ⁻³	5.8×10 ⁻³	1×10 ⁻⁴	0.149	4×10 ⁻³	<LOD	<LOD	<LOD	<LOD
44	2.0	0.2	58	6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	5.3×10 ⁻²	7×10 ⁻³	<LOD	<LOD	1.57	4×10 ⁻²
45	6.5	0.7	620	90	<LOD	<LOD	<LOD	<LOD	0.31	4×10 ⁻²	<LOD	<LOD	0.15	2×10 ⁻²	<LOD	<LOD	3.0	0.3
46	13	2	1.5×10 ³	200	<LOD	<LOD	0.52	7×10 ⁻²	2.1	0.1	3.6×10 ⁻²	2×10 ⁻³	0.49	5×10 ⁻²	3.2×10 ⁻²	2×10 ⁻³	6	1
47	14	2	980	20	<LOD	<LOD	7.6	0.1	0.48	4×10 ⁻²	1.3×10 ⁻²	2×10 ⁻³	0.40	1×10 ⁻²	1.8×10 ⁻²	1×10 ⁻³	6.4	0.4
48	7.0	0.1	250	30	<LOD	<LOD	<LOD	<LOD	0.25	1.5×10 ⁻²	<LOD	<LOD	0.17	1×10 ⁻²	<LOD	<LOD	1.9	0.1
49	1.2	0.2	310	50	<LOD	<LOD	<LOD	<LOD	9×10 ⁻²	1×10 ⁻²	<LOD	<LOD	5.3×10 ⁻²	1×10 ⁻³	<LOD	<LOD	0.363	6×10 ⁻³
50	1.4	0.2	60	10	<LOD	<LOD	<LOD	<LOD	0.109	5×10 ⁻³	<LOD	<LOD	5.1×10 ⁻²	8×10 ⁻³	<LOD	<LOD	0.57	2×10 ⁻²
51	1.2	0.1	365	2	<LOD	<LOD	<LOD	<LOD	0.10	2×10 ⁻²	<LOD	<LOD	5.2×10 ⁻²	8×10 ⁻³	<LOD	<LOD	0.40	2×10 ⁻²
52	1.03	0.095	129	9	<LOD	<LOD	<LOD	<LOD	9×10 ⁻²	1×10 ⁻²	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.36	5×10 ⁻²
53	1.92	7×10 ⁻²	138	1	<LOD	<LOD	<LOD	<LOD	0.099	1×10 ⁻²	<LOD	<LOD	4.4×10 ⁻²	6×10 ⁻³	<LOD	<LOD	0.40	6×10 ⁻²
54	0.91	3×10 ⁻²	190	20	<LOD	<LOD	<LOD	<LOD	0.095	7×10 ⁻³	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.36	2×10 ⁻²
55	0.52	4×10 ⁻²	150	20	<LOD	<LOD	<LOD	<LOD	0.095	1×10 ⁻³	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.83	0.01
56	0.12	1×10 ⁻²	210	15	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.67	5.5×10 ⁻²
57	40	5	1.08×10 ³	70	<LOD	<LOD	0.20	1×10 ⁻²	3.0	0.3	2.82×10 ⁻²	8×10 ⁻⁴	0.36	4×10 ⁻²	2.1×10 ⁻²	2×10 ⁻³	<LOD	<LOD
58	5.6	0.6	1.26×10 ³	85	<LOD	<LOD	0.15	1×10 ⁻²	0.9	0.1	1.38×10 ⁻²	8×10 ⁻⁴	0.245	5×10 ⁻³	1.4×10 ⁻²	1×10 ⁻³	<LOD	<LOD
59	19	3	857	6	<LOD	<LOD	0.182	4×10 ⁻³	1.43	5×10 ⁻²	2.1×10 ⁻²	3×10 ⁻³	0.42	5×10 ⁻²	1.8×10 ⁻²	1×10 ⁻³	<LOD	<LOD
60	22	3	900	100	<LOD	<LOD	0.22	1×10 ⁻²	1.6	0.1	3.5×10 ⁻²	5×10 ⁻³	0.34	5×10 ⁻²	3.5×10 ⁻²	1×10 ⁻³	<LOD	<LOD
61	33	4	1.2×10 ³	90	<LOD	<LOD	0.20	2×10 ⁻²	2.83	0.09	2.6×10 ⁻²	1×10 ⁻³	0.28	1×10 ⁻²	1.7×10 ⁻²	3×10 ⁻³	<LOD	<LOD
62	7.8	0.9	1.1×10 ³	200	<LOD	<LOD	0.26	4×10 ⁻²	0.67	4×10 ⁻²	3.78×10 ⁻²	2×10 ⁻⁴	0.244	4×10 ⁻³	0.14	2×10 ⁻²	<LOD	<LOD
63	8.4	0.6	1.15×10 ³	90	<LOD	<LOD	0.133	2.5×10 ⁻³	0.68	0.09	7.68×10 ⁻³	5×10 ⁻⁴	0.141	2×10 ⁻³	<LOD	<LOD	<LOD	<LOD
64	3.7	0.3	1.2×10 ³	200	<LOD	<LOD	0.152	9×10 ⁻³	0.600	5.5×10 ⁻³	1.0×10 ⁻²	2×10 ⁻³	0.135	9×10 ⁻³	1.80×10 ⁻²	5 ×10 ⁻⁴	<LOD	<LOD

Supplementary Table 15 mgkg⁻¹ and standard error (SE) of the analyte in fur samples collected both in Italy and in Madagascar part 4

	Tb		Ti		Tm		V		Y		Yb		Zn	
Sample ID	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
37	<LOD	<LOD	8.5	0.4	1.1×10 ⁻³	2×10 ⁻⁴	0.92	1×10 ⁻²	6.77×10 ⁻²	8×10 ⁻⁴	<LOD	<LOD	19.1	0.2
38	<LOD	<LOD	7.5	0.5	1.1×10 ⁻³	2×10 ⁻⁴	0.93	2×10 ⁻²	6×10 ⁻²	1×10 ⁻²	<LOD	<LOD	230	20
39	<LOD	<LOD	2.4	0.2	6×10 ⁻⁴	1×10 ⁻⁴	<LOD	<LOD	1.40×10 ⁻²	9×10 ⁻⁴	<LOD	<LOD	25	1
40	<LOD	<LOD	5.4	0.8	7.3×10 ⁻⁴	7×10 ⁻⁵	2.5×10 ⁻²	3.5×10 ⁻³	2.1×10 ⁻²	3×10 ⁻³	<LOD	<LOD	450	80
41	<LOD	<LOD	7.8	0.9	1.01×10 ⁻³	6×10 ⁻⁵	0.79	5×10 ⁻²	5.38×10 ⁻²	7×10 ⁻³	<LOD	<LOD	210	20
42	<LOD	<LOD	5.1	0.7	8×10 ⁻⁴	1×10 ⁻⁴	0.46	5×10 ⁻²	2.17×10 ⁻²	9×10 ⁻⁴	<LOD	<LOD	17.1	0.65
43	<LOD	<LOD	5.32	2×10 ⁻²	8.0×10 ⁻⁴	3×10 ⁻⁵	4.0×10 ⁻²	7×10 ⁻³	2.25×10 ⁻²	8×10 ⁻⁴	<LOD	<LOD	500	20
44	<LOD	<LOD	1.23	8×10 ⁻²	<LOD	<LOD	2.2×10 ⁻²	2×10 ⁻³	<LOD	<LOD	1.40×10 ⁻³	8×10 ⁻⁵	90	10.5
45	<LOD	<LOD	2.5	0.4	<LOD	<LOD	7.7×10 ⁻²	9×10 ⁻³	2.25×10 ⁻²	4×10 ⁻⁴	3.3×10 ⁻³	6×10 ⁻⁴	119	3.5
46	4.0×10 ⁻³	3×10 ⁻⁴	10	1	<LOD	<LOD	0.40	4×10 ⁻²	0.125	3×10 ⁻³	1.2×10 ⁻²	2×10 ⁻³	300	20
47	1.97×10 ⁻³	5×10 ⁻⁵	6.3	0.7	<LOD	<LOD	0.190	8×10 ⁻³	6.0×10 ⁻²	9×10 ⁻³	5.8×10 ⁻³	2×10 ⁻⁴	257	5
48	<LOD	<LOD	2.16	7.672×10 ⁻²	<LOD	<LOD	7×10 ⁻²	1×10 ⁻²	1.9×10 ⁻²	2×10 ⁻³	2.8×10 ⁻³	4×10 ⁻⁴	90	15
49	<LOD	<LOD	1.07	6.376×10 ⁻²	<LOD	<LOD	2.7×10 ⁻²	6×10 ⁻⁵	<LOD	<LOD	1.689×10 ⁻³	8×10 ⁻⁶	110	10
50	<LOD	<LOD	1.040	8.15×10 ⁻³	<LOD	<LOD	2.8×10 ⁻²	2×10 ⁻³	<LOD	<LOD	1.6×10 ⁻³	2×10 ⁻⁴	110	7
51	<LOD	<LOD	1.05	3.385×10 ⁻²	<LOD	<LOD	3.3×10 ⁻²	3.5×10 ⁻³	<LOD	<LOD	1.8×10 ⁻³	2×10 ⁻⁴	108	3
52	<LOD	<LOD	0.90	5×10 ⁻²	<LOD	<LOD	2.0×10 ⁻²	3×10 ⁻³	<LOD	<LOD	1.6×10 ⁻³	1×10 ⁻⁴	110	20
53	<LOD	<LOD	1.0	0.2	<LOD	<LOD	3.3×10 ⁻²	4×10 ⁻³	<LOD	<LOD	1.74×10 ⁻³	2×10 ⁻⁴	100	20
54	<LOD	<LOD	0.66	7×10 ⁻²	<LOD	<LOD	1.2×10 ⁻²	2×10 ⁻³	<LOD	<LOD	1.64×10 ⁻³	3×10 ⁻⁵	113	5
55	<LOD	<LOD	0.32	3×10 ⁻²	<LOD	<LOD	5.1×10 ⁻³	6×10 ⁻⁴	<LOD	<LOD	1.61×10 ⁻³	6. ×10 ⁻⁵	119	4
56	<LOD	<LOD	0.29	4×10 ⁻²	<LOD	<LOD	3.5×10 ⁻³	5×10 ⁻⁴	<LOD	<LOD	1.36×10 ⁻³	4×10 ⁻⁵	110	15
57	<LOD	<LOD	13	1	1.28×10 ⁻³	6×10 ⁻⁵	2.0	0.3	8.4×10 ⁻²	2.5×10 ⁻³	<LOD	<LOD	650	60
58	<LOD	<LOD	6.9	0.1	9.6×10 ⁻⁴	7×10 ⁻⁵	1.00	7×10 ⁻²	3.93×10 ⁻²	9×10 ⁻⁴	<LOD	<LOD	409	7
59	<LOD	<LOD	14	1	1.12×10 ⁻³	2×10 ⁻⁵	2.1	0.3	6.5×10 ⁻²	3×10 ⁻³	<LOD	<LOD	320	20
60	<LOD	<LOD	17	2	1.5×10 ⁻³	3×10 ⁻⁴	2.7	0.3	0.12	2×10 ⁻²	2.7×10 ⁻³	4×10 ⁻⁴	160	20
61	<LOD	<LOD	9.4	0.6	1.3×10 ⁻³	1×10 ⁻⁴	2.0	0.2	8. ×10 ⁻²	1×10 ⁻²	<LOD	<LOD	610	40
62	<LOD	<LOD	10	1	1.3×10 ⁻³	2×10 ⁻⁴	2.9	0.4	0.095	3.5×10 ⁻³	1.1×10 ⁻²	2×10 ⁻³	340	40
63	<LOD	<LOD	6.7	0.3	7.9×10 ⁻⁴	6×10 ⁻⁵	0.46	4×10 ⁻²	2.73×10 ⁻²	2×10 ⁻⁴	<LOD	<LOD	280	40
64	<LOD	<LOD	8.7	0.9	9×10 ⁻⁴	1×10 ⁻⁴	0.990	1×10 ⁻³	3.80×10 ⁻²	3×10 ⁻³	<LOD	<LOD	62	9

Supplementary Table 16 mgkg⁻¹ and standard error
(SE) of the analyte in Blood samples collected in Italy

part 1

	Sample ID	65	66	67	68	69	70	71
Al	Mean	0.29	0.308	0.33	0.31	0.39	0.25	0.306
	SE	0.06	3×10 ⁻³	0.06	0.02	0.06	0.04	1×10 ⁻³
As	Mean	0.010	0.012	7×10 ⁻³	7×10 ⁻³	5×10 ⁻³	6×10 ⁻³	4×10 ⁻³
	SE	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³
Ba	Mean	0.0160	0.0210	<LOD	<LOD	<LOD	<LOD	<LOD
	SE	1×10 ⁻⁴	1×10 ⁻⁴					
Ca	Mean	52.1	57	51.2	55.6	55	49	49.7
	SE	0.2	1	0.6	0.8	4	2	0.1
Cd	Mean	2.5×10 ⁻³	3.4×10 ⁻³	2.1×10 ⁻³	2.3×10 ⁻³	2.0×10 ⁻³	2.4×10 ⁻³	2.0×10 ⁻³
	SE	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴
Ce	Mean	0.0140	0.0070	0.38	<LOD	0.03	0.06	<LOD
	SE	1×10 ⁻⁴	1×10 ⁻⁴	0.03		0.03	0.05	
Cr	Mean	0.198	0.22	0.16	0.136	0.12	0.10	0.075
	SE	5×10 ⁻³	0.01	0.025	4×10 ⁻³	0.01	0.02	9×10 ⁻³
Cu	Mean	0.80	0.82	0.61	0.50	0.59	0.52	0.29
	SE	0.05	0.04	0.06	0.02	0.05	0.13	0.03
Dy	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
Er	Mean	<LOD	<LOD	1.0×10 ⁻³	<LOD	<LOD	<LOD	<LOD
	SE			1×10 ⁻⁴				
Eu	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
Fe	Mean	549	530	562	529	510	554	519
	SE	1	6	4	6	30	6	8
Gd	Mean	<LOD	4.0×10 ⁻³	2.0×10 ⁻³	<LOD	<LOD	<LOD	<LOD
	SE		1×10 ⁻⁴	1×10 ⁻⁴				
Ho	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
K	Mean	1660	1800	1443	1740	1510	1100	1640
	SE	20	100	9	40	60	200	20
La	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
Lu	Mean	<LOD	4×10 ⁻⁴	<LOD	<LOD	<LOD	<LOD	<LOD
	SE		1×10 ⁻⁴					
Mg	Mean	40.3	36.5	36.7	39.5	32.9	34.7	35.1
	SE	0.2	0.8	0.7	0.1	0.2	0.4	0.5

Supplementary Table 17 mgkg⁻¹ and standard error
(SE) of the analyte in blood samples collected in Italy

part 2

	Sample ID	65	66	67	68	69	70	71
Mn	Mean	0.043	0.052	0.049	0.041	0.039	0.035	0.039
	SE	0.002	0.005	0.002	0.003	0.002	0.006	1×10 ⁻³
Na	Mean	2599	2700	2640	2633	2660	2570	2540
	SE	8	100	30	3	20	30	20
Nd	Mean	0.0050	0.005	0.005	0.0040	0.0030	<LOD	0.0030
	SE	1×10 ⁻⁴	1×10 ⁻³	1×10 ⁻³	1×10 ⁻⁴	1×10 ⁻⁴		1×10 ⁻⁴
Ni	Mean	0.108	0.14	0.1280	<LOD	0.06	0.04	0.05
	SE	7×10 ⁻³	0.04	1×10 ⁻⁴		0.08	0.06	0.03
Sc	Mean	0.28	0.280	0.23	0.18	0.153	0.167	0.120
	SE	0.02	2×10 ⁻³	0.03	0.01	5×10 ⁻³	8×10 ⁻³	9×10 ⁻³
Sm	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
Sr	Mean	0.045	0.053	0.053	0.054	0.060	0.048	0.044
	SE	1×10 ⁻³	3×10 ⁻³	3×10 ⁻³	2×10 ⁻³	4×10 ⁻³	3×10 ⁻³	1×10 ⁻³
Tb	Mean	<LOD	1.0×10 ⁻³	2.0×10 ⁻³	<LOD	<LOD	<LOD	<LOD
	SE		3×10 ⁻⁴	4×10 ⁻⁴				
Ti	Mean	0.024	0.025	0.018	0.017	0.015	0.009	0.011
	SE	3×10 ⁻³	2×10 ⁻³	2×10 ⁻³	2×10 ⁻³	3×10 ⁻³	1×10 ⁻³	1×10 ⁻³
Tm	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
V	Mean	0.045	0.051	0.04	0.034	0.028	0.026	0.021
	SE	4×10 ⁻³	2×10 ⁻³	4×10 ⁻³	2×10 ⁻³	3×10 ⁻³	2×10 ⁻³	1×10 ⁻³
Y	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE							
Yb	Mean	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	1×10 ⁻³	<LOD	<LOD	<LOD
	SE	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴	1×10 ⁻⁴			
Zn	Mean	2.39	3.08	3.22	2.05	3.0	2.90	2.44
	SE	0.01	0.08	0.3	0.05	0.3	0.02	0.02

Supplementary Table 19 mgkg⁻¹ and standard error (SE) of the analyte in feed samples collected in Italy

	Sample ID	72	73	74	75	76	77
Al	Mean	142	110	116	143	119	143
	SE	8.5	15	3	7	4	10
As	Mean	0.104	0.14	0.08	0.0226	0.057	0.09
	SE	5×10 ⁻³	0.02	0.01	9×10 ⁻⁴	7×10 ⁻³	0.01
Ba	Mean	2.1	1.4	2.04	<LOD	1.4	5.9
	SE	0.35	0.1	0.08		0.2	0.6
Ca	Mean	1.52×10 ⁴	1.31×10 ⁴	1.40×10 ⁴	3.50×10 ⁴	3700	2700
	SE	200	400	200	400	100	200
Cd	Mean	0.145	0.90	0.26	0.017	0.38	0.47
	SE	8×10 ⁻³	0.01	0.02	1×10 ⁻³	0.03	0.03
Ce	Mean	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	SE						
Cr	Mean	0.55	<LOD	<LOD	<LOD	<LOD	<LOD
	SE	0.06					
Cu	Mean	32	17	15	<LOD	<LOD	<LOD
	SE	3	3	2			
Dy	Mean	0.0235	0.19	0.066	1.38×10 ⁻³	4.36×10 ⁻³	0.08
	SE	3×10 ⁻⁴	0.02	9×10 ⁻³	5×10 ⁻⁵	2×10 ⁻⁴	0.01
Er	Mean	4.7×10 ⁻⁴	7.2×10 ⁻³	2.9×10 ⁻³	<LOD	<LOD	3.2×10 ⁻³
	SE	4×10 ⁻⁵	8×10 ⁻⁴	4.5×10 ⁻⁴			4×10 ⁻⁴
Eu	Mean	6×10 ⁻³	8.5×10 ⁻³	0.012	1.7×10 ⁻³	2.8×10 ⁻³	0.012
	SE	3×10 ⁻⁴	6×10 ⁻⁴	1×10 ⁻³	3×10 ⁻⁴	3×10 ⁻⁴	1×10 ⁻³
Fe	Mean	340	300	250	130	81	133
	SE	60	45	40	10	5	3
Gd	Mean	0.06	0.100	0.076	<LOD	0.054	0.11
	SE	7×10 ⁻⁴	2×10 ⁻³	7×10 ⁻³		0.0075	0.01
Ho	Mean	6.44×10 ⁻⁴	6.7×10 ⁻³	3.5×10 ⁻³	1.8×10 ⁻⁴	1.01×10 ⁻⁴	4.1×10 ⁻³
	SE	7×10 ⁻⁶	1×10 ⁻⁴	2.5×10 ⁻⁴	3×10 ⁻⁵	2×10 ⁻⁶	7×10 ⁻⁴
K	Mean	7800	7400	7700	1.1×10 ⁴	1.74×10 ⁴	1.29×10 ⁴
	SE	300	200	100	1500	300	500
La	Mean	0.098	0.27	0.24	0.017	0.059	0.28
	SE	1×10 ⁻³	0.03	0.01	3×10 ⁻³	2×10 ⁻³	0.01
Lu	Mean	<LOD	5.9×10 ⁻³	1.9×10 ⁻³	<LOD	<LOD	1.7×10 ⁻³
	SE		9×10 ⁻⁴	2×10 ⁻⁴			3×10 ⁻⁴
Mg	Mean	3200	2600	2620	820	2030	1500
	SE	100	300	80	90	90	100
Mn	Mean	111	100	120	2.0	33	42
	SE	5	9	10	0.1	3	1
Na	Mean	5500	5100	5600	1110	3800	3100
	SE	200	800	300	60	450	200
Nd	Mean	0.082	0.22	0.21	0.0149	0.049	0.25
	SE	7×10 ⁻³	0.03	0.01	9×10 ⁻⁴	7×10 ⁻³	0.04
Ni	Mean	2.07	1.7	1.850	1.30	0.95	1.54
	SE	0.01	0.1	3×10 ⁻³	0.07	0.09	0.06
Pb	Mean	0.17	0.34	0.30	0.024	0.058	0.331
	SE	0.01	0.05	0.03	4×10 ⁻³	4×10 ⁻³	3×10 ⁻³

Mineral element profile in African penguin feathers and its possible relationship with molting

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Abstract

Molting is an important biological and physiological stage in penguins, influenced by environmental and nutritional factors. Feather composition analysis before and after molting can consequently place boundaries on element bioaccumulation and excretion. This study assesses mineral deposition during pre- and post-molting of penguin feathers from three zoological institutions to understand the factors that govern molting at the environmental and physiological levels. Feathers were retrieved from individuals at Zoom Torino (Italy), ZooParc Overloon(Netherlands), and Magdeburg Zoo (Germany). Quantification of elemental concentrations were performed by analytical methods, with both ICP-OES and HR-ICP-MS techniques. A statistical approach involving MANOVA, and factorial analysis helped identify important trends. Pre-molt features exhibited greater variability than post-molt features, both of which showed significant differences in elemental concentrations. Factorial analysis showed geogenic trends in Mg, Sr, and Ni as well as anthropogenic trends in Pb. While Na and K differed among all treatment groups, this likely points to physiological adaptations in response to increased demand during feather regrowth. Additionally, inter-zoo comparisons highlighted distinct elemental profiles linked to local environmental and dietary conditions, particularly in Zoo Magdeburg, where Na levels were markedly elevated. The study highlights the influence of environmental and dietary conditions on feather composition during molt, offering insights for improving captive penguin welfare and broader ecological implications related to climate change and pollution.

1. Introduction

Molting is a fundamental biological step in birds, during which they renew their plumage to ensure optimal functionality, thermal insulation, and flight capability. The transformative phase is essential for maintaining the health and survival of avian species, particularly in extreme environments where feather integrity is critical for their well-being.. [1,2] For penguins, whose dense, waterproof feathers are essential for thriving in cold marine habitats, molting is an indispensable and crucial life event. Unlike other birds, penguins undergo a synchronous molt, replacing all their feathers over a short, intense period while fasting and remaining on land. This unique molting pattern underscores penguins' metabolic and physiological challenges, making them an ideal model for studying the interplay between molt, physiology, and environmental factors.[3] In recent years, disrupting in molting patterns have raised concerns about penguin populations, particularly in captivity. Emerging evidence suggests that environmental changes, dietary shifts, and rising pollution levels may impact penguins' health. Climate change, for example, has altered the availability of key nutrients in natural habitats, potentially delaying molt or impacting feather regeneration. [4,5] These issues are amplified in zoological habitats, where dietary formulations, water quality, and environmental management practices must closely mimic natural conditions. Understanding the factors' influencing molting is therefore critical for the conservation and welfare of captive penguins and for gaining insight into the influence of climate changes and pollution on the survival of wild populations. The molting period involves substantial physiological changes, including the mobilization and accumulation of mineral elements into feathers.[6] For example, Mg and Ca are vital for structural integrity and feather formation[7], while Fe plays a key role in pigmentation and oxygen transport [8]. Na and K are critical for maintaining metabolic and physiological processes, and trace elements, such as Ni and Pb, can further reflect environmental exposure or anthropogenic impact [9–13]. As chemical archives, feathers can offer a window into the interplay between an animal's biology and its environment during molt. Comparing pre- and post-molt feather element composition provides the opportunity to explore how external environmental factors and internal biological processes shape elemental profiles during a transformative phase. Despite the importance of molting, little is known about these interactions and their effects on penguins' health. This gap is particularly relevant in studies conducted in controlled environments, such as zoos, where variables like diet, habitat conditions, and management practice can significantly affect the element

composition of feathers.[14] Site-specific differences emphasize the need to adapt management practices to optimize mineral intake and support penguin health.

1.1 Literature Insights on Feathers Elements Content

Penguin feathers as bioindicators of environmental contamination and physiological states have acquired significant attention in recent years. Different studies have demonstrated the utility of feathers as bioindicators for assessing the accumulation of trace elements and metals in seabirds, reflecting both geogenic sources and anthropogenic impacts. [15,16] For instance, Mikoni et al. (2017) highlighted the role of heavy metals, such as lead (Pb) and cadmium (Cd), as markers of industrial pollution, while elevated levels of magnesium (Mg) and strontium (Sr) often correlate with natural geological features.[17] Moreover, feathers have been used to compare metal exposure in wild and captive birds, offering insights into how habitat, diet, and management practices influence metal accumulation[18,19]. The research by Jerez et al. (2011) emphasized the geographic variability in feather element profiles, linking local soil and water compositions to metal concentrations in bird tissues. [16] Furthermore, studies on penguin species, including those in Antarctica, have illustrated the importance of integrating biomonitoring approaches to inform conservation strategies [15].

This study addresses these gaps by investigating the element composition in penguins' feathers associated with the molting process, focusing on pre- and post-molt differences. Molting, a key biological process influencing elemental accumulation, is analyzed to reveal temporal changes in trace element exposure and the physiological demands associated with feather growth and replacement. By linking these findings to biological outcomes and providing detailed comparisons across managed environments and molting phases, this research advances multielement feather analysis and lays the groundwork for future studies on temporal trends and health implications. Eighteen elements (Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sr, and Zn) were quantified in pre- and post-molt feathers of penguins housed in three institutions: Zoom Torino in Turin, Italy, ZooParc Overloon, Holland, and Zoo Magdeburg, Germany. All samples were washed, mineralized with acid digestion, and analyzed with advanced analytical techniques, such as Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) and High-Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS). Advanced chemometric techniques (MANOVA and Factor Analysis) were applied to identify key elements influencing the molting process. This approach allowed us to explore site-specific differences in feather element composition

and assess the interaction between environmental exposure, physiological factors, and the molting process. The findings of this research have the potential to deepen our understanding of penguins' ecology and physiology, offering practical implications on zoo management, providing information to optimize care practices for captive penguins, and ensuring their health and welfare. Furthermore, these insights contribute to broader ecological studies highlighting the interaction between physiological and environmental exposure during molt, providing information about the influence of climate change and pollution on the survival of wild populations.

2. Materials and Methods

2.1 Zoological institutions

The study was promoted by Fondazione Zoom, an Italian organization dedicated to the conservation of biodiversity and the promotion of environmental education, research, and sustainable development initiatives. Samples were collected in three zoological institutions affiliated with the European Association of Zoos and Aquaria (EAZA); the three institutions are Zoom Torino in Turin, Italy, Overloon ZooParc, The Netherlands, and Magdeburg Zoo, Germany. At Zoom Torino, the penguin habitat is meticulously designed to simulate the coastal regions of Africa, providing both educational and conservation opportunities for visitors. The facility actively engaged in breeding programs to enhance the species' genetic diversity. Overloon ZooParc features extensive enclosures that facilitate natural behaviors among the penguins, with staff dedicated to monitoring animal health and welfare and implementing innovative feeding strategies to ensure optimal nutrition throughout the molting process. Magdeburg Zoo has a prominent role in the rehabilitation and conservation of aquatic birds. Participating in international breeding programs and contributing to research efforts enhanced their understanding of penguins' health and ecological needs. Together, these institutions provide a valuable framework for studying the effects of environmental conditions and management practices on penguin welfare, particularly the molting process and exposure to environmental pollutants.

2.2 Feather collection

Feather samples were methodically collected in two distinct phases for each penguin: pre-molt and post-molt. Initially, samples were gathered approximately two weeks before the onset of the molt in spring 2023, aimed at assessing the condition of feathers in their fully grown state. Subsequent collection occurred post-molt, approximately 4-6 weeks later, to observe a new feathers state. The collection protocol was standardized to ensure as much as possible; comparable samples were collected in different zoos. Primarily, feathers were taken, retrieving naturally shed feathers found in the penguin enclosure. In cases where shedding was insufficient, non-invasive sampling was conducted by collecting feathers from specific regions of the penguin's body, such as the back and wings, using sterile scissors. The veterinary teams of each zoo carried out all the procedures with extreme care to avoid causing stress or discomfort to the animals. Samples were stored in labeled plastic containers, with careful classification according to the individual penguin, the date of collection, and the zoological institution of origin. Due to logistical constraints, the Magdeburg Zoo was not accessible for feather collection pre- and post-molt; therefore, a mixture of feathers from the two periods, collected in the penguins' nest, was provided for each individual. While this limits a direct quantitative comparison with the other institutions, the mixed sample still offers valuable insights. The composite samples were analyzed to assess environmental differences in pollutant exposure between the three areas of study (Table 1). This approach allows us to identify potential disparities in environmental contamination between the institutions, providing an important context for understanding how local environmental conditions may influence feather composition. Table 1 summarizes the number and kind of samples collected in each facility. A total of 26 penguins were included in the study, 9 of them providing a mixed sample from Magdeburg Zoo, 11 individuals from Zoom Torino, and 6 penguins from Overloon ZooParc samples were collected pre- and post-molt for the individuals from Zoom Torino and Overloon ZooParc (Supplementary Table 1). In addition, to ensure the welfare of the penguins and to avoid excessive stress, some samples could not be obtained in sufficient amounts to support multiple analytical replicates. Consequently, only standard deviations and an average relative standard deviation percentage (RSD%) are reported.

Table 3 Samples campaign in the three areas of study

Origin	N° of samples	N° of penguins tested	Sample type
Zoom Torino	21	11	Pre- and Post-molt
Overloon ZooParc	12	6	Pre- and Post-molt
Magdeburg Zoo	9	9	Composite

2.3 Instruments and Reagents

The solvents used to wash the feathers were a 0.01% Trixon-X-100 solution, methanol (CH₃OH), and High Purity Water (HPW) produced with Millipore Milli-Q Plus system (18.2 MΩ cm). Sample acid digestion was performed in tetrafluoromethoxyl (TFM) vessels, using a Milestone MLS-1200 Mega (Milestone, Sorisole, Italy) microwave oven. All solutions were prepared in HPW and with reagents of analytical grade. Standard solutions were prepared from 1000 mg L⁻¹ Sigma-Aldrich solutions. Nitric acid (HNO₃, 65%, Sigma Aldrich), and hydrogen peroxide (H₂O₂, 30%, Sigma Aldrich) were also used. Based on the expected concentration levels of target elements, analytes of interest were determined with a Perkin Elmer Optima 7000 (Perkin Elmer, Norwalk, CT, USA) Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) or with an Element 2 (Thermo Fisher Scientific, Waltham, Massachusetts, United States), High-Resolution Inductively Coupled Plasma Mass Spectrometer (HR-ICP-MS).

2.4 Analytical method

The sample wash pre-treatment procedure was based on Squadrone et al. (2016) work [18]. It involves different steps aimed at removing external contaminants. Firstly, the feathers were immersed in 20 mL of a 0.01% Trixon-X-100 solution for four hours. After this step, the feathers undergo two rinses with deionized or HPW, each with a final volume of 20 mL, to eliminate the previous residual solvent. The third step involved two washes using 20 mL CH₃OH. Finally, feathers were rinsed with 20mL of ultrapure water to ensure the removal of residual solvents and were dried under a laminar flow hood until they reached a constant mass.

Washed samples were then mineralized by wet digestion with acid and oxidant. In this case, between 0.05 and 0.1 g of feathers, depending on availability, were subjected to microwave digestion with 7 mL of HNO₃ (70 % v/v) and 1.5 mL of H₂O₂ (30 % v/v). All samples solution was then filtered with Whatman 0.45 µm filters and diluted to 30 mL with HPW and analyze. Mineralization efficiency and

accuracy of the analytical method were verified by processing the Certified Reference Materials (CRM) ERM®-DB001, namely human hair, containing 5 (Cu, Zn, As, Cd, Pb) of the 18 analytes quantified in this study. Recovery percentages ($R \% = (\text{mg kg}^{-1} \text{ detected} / \text{mg kg}^{-1} \text{ certified}) \times 100$) ranged from 85.63% to 90.23% and are reported in Table 2. The selection of CRM ERM®-DB001 was necessitated by the absence of reference materials precisely matching the feather sample matrix. Consequently, a keratin-based material was chosen to ensure structural similarity in matrix composition with the samples under analysis.

Table 4 Results obtained from the analysis of CRM ERM®-DB001

Analyte	Certified value $\pm$ U [mgKg ⁻¹]	Obtained value $\pm$ U [mgKg ⁻¹]	%Error	%Recovery
As	0.044 $\pm$ 0.006	0.05000 $\pm$ 1*10 ⁻⁵	9.83	90.1
Cd	0.125 $\pm$ 0.007	0.110300 $\pm$ 9*10 ⁻⁶	11.6	88.4
Cu	33 $\pm$ 4	26.9 $\pm$ 0.7	9.77	90.2
Pb	2.1 $\pm$ 0.2	1.9500 $\pm$ 2*10 ⁻⁴	14.4	85.6
Zn	210 $\pm$ 10	182 $\pm$ 3	13.0	87.0

All the reagents were of analytical grade. Standard solutions were prepared by diluting 1000 mg L⁻¹ Sigma-Aldrich solutions with high-purity, 0.45 μ m filtered water (HPW, Milli-Q, Millipore, 18.2M Ω cm). Nitric acid (65%, Sigma Aldrich, St. Louis, Unites States) and hydrogen peroxide (30%, Sigma Aldrich) were used for the mineralization procedure.

2.5 Statistical treatment

A chemometric treatment was performed to explore the correlation between all the variables and the similarity or difference between samples. Data were pretreated and processed using XLSTAT software (Addinsoft, Paris, France). For elements with concentration below the limit of detection (LOD) in more than 40% of the measurements, the variables were not considered for the statistical treatment. In the case of a few missing data (concentration lower than the LOD), a random value was set between zero and the detection limit. MANOVA and Factor Analysis were employed to assess differences in metal concentrations in penguin feathers during pre- and post-molt periods and between samples from different origins, allowing for the simultaneous analysis of elements considered as dependent variables. This multivariate approach captures overall patterns and

interactions between metals and samples that might be overlooked in univariate analyses, providing a comprehensive view of how molt and geographic origin influence metal profiles.[20] To interpret MANOVA results, it is necessary to note that lambda represents the magnitude of effect that the independent variable (i.e., observations) exerts on the dependent variables (i.e., element concentrations), with a lower value indicating a greater difference between groups. The observed F value serves as the test statistic to determine whether significant differences exist between the groups. The degrees of freedom denoted as GDL1 and GDL2, correspond to the numerator and denominator, respectively, in the calculation of the F-test. The critical F value, or the threshold, must be exceeded by the observed F value for the result to be deemed significant. Finally, the p-value indicates the probability that the observed outcomes could have occurred under the null hypothesis, thus serving as a measure of statistical significance. These parameters were evaluated in different tests comprehended in the MANOVA evaluation (Wilks, Hotelling-Lawley, Pillai, Roy tests). Instead, a Factorial Analysis was performed to identify the dataset's underlying structure, reduce the dataset's dimensionality, and group correlated metal concentrations into factors representing shared sources or biological processes influencing metal accumulation. Factor Analysis was carried out by Varimax rotation of principal components obtained by Principal Component Analysis (PCA), to minimize the variability between factors and simplify the comprehension of the system. Varimax rotation was chosen to obtain a clear factor separation, minimizing variable overlap and better understanding their relationships.

3. Results and Discussion

3.1 Element concentrations in feathers (univariate discussion)

The concentrations of elements in the feathers of sampled penguins are presented in Supplementary Table 2 and 4, and generally decrease in the order: Ca > Mg > K > Na > Zn > Ni > Fe > Al > Cu > Sr > Mn > Ba > Pb > As > Cd > Mo. Co and Cr were below the LOD for all the samples analyzed. This dataset includes several Potentially Toxic Elements (PTEs), namely Al, As, Cd, Cr, Cu, Fe, Mn, Mo, Ni, Pb, and Zn. Among these, many elements are essential to animals, whereas others (As, Cd, Pb) have high toxicity even at low concentrations and warrant attention due to their potential health impacts on penguins' organisms and low toxicity thresholds. The As concentrations in the dataset range from 0.04 to 0.350 mg kg⁻¹, significantly below the cited dangerous threshold of 10 mg kg⁻¹ associated in the literature with different toxic effects, including reduced growth, reproductive impairments, and

increased mortality. [21] Furthermore, the levels of As quantified are lower than 3 mg kg^{-1} , which in avian tissue is often considered indicative of elevated exposure, suggesting that the penguins examined in this study may not be exposed to elevated concentrations of As.[22] The Cd concentration in the samples is relatively low, ranging from 0.012 to 0.274 mg kg^{-1} , which is comparable to data reported in studies examining various penguin species across different geographical areas.[15,16,18,19,23] These values are highly below 50 mg kg^{-1} , which is often cited as a threshold for kidney dysfunction in birds.[24] Similarly, the concentrations of Pb vary between 0.009 and 1.05 mg kg^{-1} across different groups, remaining well below the established limits of concern for wildlife (Supplementary Table 2 and 3 and Figure 1 of supplementary material). Toxicity levels for lead in birds are typically reported as 200 mg kg^{-1} , indicating that the observed values do not pose an immediate risk.[10] Even at these levels, lead chronic exposure can impair neurological function, growth, and reproduction in birds, making this an important element to monitor over time. In the present study, the observed concentration range aligns with values reported in the literature, suggesting that the areas under investigation do not exhibit significant contamination by this element.

Beyond these three highly toxic elements, the dataset also highlights significant variations in the concentrations of essential minerals, such as Fe and Ca, which play critical roles in physiological functions. Calcium, a key element for bone and feather development[7], was present in consistently high concentrations across all samples, indicating adequate availability for the Supplementary Table 2 and 3 and Figure 1 of supplementary material). The concentration range varies from 365 to 12200 mg kg^{-1} , consistent with the values reported in the literature for penguin feathers.[15,17–19] Iron concentrations vary widely between samples, with values ranging from 0.030 to 110 mg kg^{-1} . While these levels are not considered toxic, the variability could reflect differences in dietary intake, molt stages, or zoo management practices. Elevated iron concentrations are crucial for oxygen transport and feather pigmentation, particularly during phases of rapid feather growth.[8] Additionally, Mg and Na show noteworthy differences across samples. Magnesium, vital for biochemical processes,[25] fluctuates between 30.7 and 1380 mg kg^{-1} , while sodium, crucial for maintaining electrolyte balance,[26] ranges from 4.80 to 359 mg kg^{-1} . These variations might indicate different dietary compositions, environmental conditions in each zoo, and the water sources provided to the penguins.

Particularly in Magdeburg Zoo, data analysis revealed distinct patterns that differ notably from other zoos. Magdeburg samples showed higher sodium levels, which could indicate unique local dietary inputs or differences in water mineral content. These insights point to the necessity of adapting zoo management practices to local conditions to optimize mineral intake and ensure the health of captive animals. These minerals are vital for bone health and overall physiological functions, and their deficiency could lead to health issues such as bone demineralization or other metabolic disorders in penguins. [27] Further investigations at Magdeburg might focus on isolating specific factors contributing to this variation, thereby refining our understanding of environmental and dietary impacts on the mineral accumulation in penguin feathers.

3.2 Pre-molt and post-molt comparison

The dataset on element concentrations in penguin feathers was summarized in Supplementary Table 4 as maximum and minimum for pre-molt and post-molt groups. Supplementary Table 5 reports the average standard deviations and RSD for each element. This section evaluates the variation between these groups. The concentrations of elements in penguin feathers pre- and post-molt, sampled from the zoos of Turin and Overloon, reveal relevant variations between the two phases and across the different locations. In particular, the concentrations of analytes in pre-molt samples are relevantly higher for many analytes in the two sample sets. Some examples are Ca and Mn. The range of Ca in Turin pre-molt varies from $2.17 \cdot 10^3$ to $1.22 \cdot 10^4$ mg kg⁻¹, while the post-molt range is from $3.50 \cdot 10^2$ to $2.94 \cdot 10^3$ mg kg⁻¹, whereas in Overloon, it is slightly lower: from $2.63 \cdot 10^3$ to $9.74 \cdot 10^3$ mg kg⁻¹ in the pre-molt set and $7.4 \cdot 10^2$ to $3.65 \cdot 10^3$ mg kg⁻¹ in post-molt one. Similarly, Mn concentrations are higher in the pre-molt sample set in both zoos (26.49-3.44 for Turin and 36.4-7.47 mg kg⁻¹ for Overloon samples) than in post-molt ones (1.65-5.88 for Turin and 1.44-3.68 mg kg⁻¹ for Overloon samples). The ranges of each analyte are reported in Supplementary Table 4 and can be visualized in Figure 1 of supplementary material. These differences may be linked to the physiological preparation for molting, where the metabolic demand for keratin synthesis and Ca deposition in growing feathers is heightened. Mn's involvement in antioxidant defense systems further underlines the significance of these elements during the molting cycle. Understanding these patterns provides insights into the nutritional and physiological status of penguins, with potential applications for zoo management practices and conservation strategies.

Furthermore, pre molt feathers have been exposed to the environment for a longer time than post-molt ones, and they may have assimilated a larger portion of elements from soil, water or particulate matter. It must be recalled that feathers are washed before analysis, to remove the contribution of dust possibly deposited on them. Indeed, in both zoos, the variance in element concentrations is consistently higher before molt compared to post-molt, indicating a broader range of element accumulation before feather shedding. For instance, in Turin, the data variability of the group for Ca is significantly larger pre-molt than post-molt (890 mg/kg²), reflecting greater variability in calcium content before molt. A similar pattern is observed for other elements, such as Mg and Fe, where pre-molt variability is notably higher, as the boxplots in Figure 1 of the supplementary material shown in Turin's sample.

Overall, the significantly higher concentration and data variability pre-molt across most elements' points to more variable environmental exposure or metabolic accumulation before the molt, while post-molt feathers exhibit more uniform concentrations, reflecting the elimination of excess metals during the shedding of feathers. These findings underline the importance of molt as a critical period for detoxification in penguins. The significant differences between pre- and post-molt groups were assessed through the MANOVA application, as explained in the next paragraphs.

3.3 Pre- and post-molt comparison (chemometric analysis)

In the chemometric processing of the dataset for comparing pre- and post-molt samples, the samples from Magdeburg Zoo were not included as they represent a composite of pre- and post-molt conditions, which could not be distinctly categorized into either group. The MANOVA results indicated significant differences in element concentrations in penguin feathers when comparing pre-molt and post-molt samples from Torino and Overloon Table 3. This confirms that feather samples collected in different molting phases exhibit varying concentrations of elements, demonstrating the capacity of feathers to accumulate mineral elements and remove them from the organism. Specifically, the rejection of the null hypothesis across multiple tests (Wilks, Hotelling-Lawley, Pillai, and Roy) demonstrates that the molting process significantly influences metal retention or elimination. The consistent p-value across tests (0.032) highlights the robustness of these findings and suggests that the molt period could be an important factor in element regulation. Furthermore, the results imply that the concentration of elements might impact the molt conduction itself, potentially affecting the efficiency or timing of this critical biological process. Further analysis is

needed to explore the specific elements involved and their biological implications during molt and underscore the utility of feather analysis for monitoring both short-term and long-term environmental conditions, offering a valuable tool for assessing the impacts of pollution and climate change on penguin populations.

Table 3 Results obtained by MANOVA to assess the molting phase role in the concentration of the analytes considered.

	Wilks	Hotelling-Lawley	Pillai	Roy
Lambda	0.295	2.39	0.705	2.39
F observed value	2.54	2.54	2.54	2.54
GDL1	16	16	16	16
GDL2	17	17	17	17
F critic value	2.29	2.29	2.29	2.29
p-value	0.0320	0.0320	0.0320	0.0320

In order to further assess this issue, a factorial analysis was performed on the same portion of the dataset, which confirmed pivotal insights into the environmental and dietary factors influencing bioaccumulation patterns. **Factor 1**, accounting for 41.19% of the variance, has strong correlations with **Mg (0.799)**, **Mn (0.667)**, **Sr (0.853)**, **Ca (0.877)** and **Ba (0.791)** (**Table 4**). These elements point to an environmental influence, particularly reflecting geogenic origins. For instance, **Mg** and **Sr** are naturally abundant in soils, particularly in geologically active regions. **Ca** and **Mn** are crucial for biological processes,[28] but their presence in high concentrations in the feathers also hints at environmental accumulation from the soil. Post-molt samples might exhibit higher concentrations of these elements due to the accumulation during feather regrowth. Furthermore, this highlights the interplay between environmental accumulation and internal biological processes during molt. As noted by Squadrone et al. (2016), the variability of these elements can also signal localized environmental conditions.[18] Feathers absorb minerals from the environment, and during the molting period, birds could experience changes in diet or environmental exposure that affect these levels. Factor 1 also shows a high correlation with Cd (0.765). Thus, natural and anthropogenic factors blend in this analysis, with the factorial loadings revealing these dual influences in elemental accumulation patterns, a discussed below.

Factor 2, which accounts for 13.54% of the variance, has notable inverse correlations with As (-0.807), Mo(-0.668), and a high correlation with Ni (0.598) as shown in Table 4. As is typically associated with

anthropogenic sources, such as industrial pollution [21], it highlights the importance of monitoring human activities in influencing elemental accumulation. In this study, As concentrations were similar to those reported by Squadrone et al. (2016,2018, 2019), and lower than concentrations found in the work of Metcheva et al.(2006), suggesting effective industrial regulation in these regions.[15,18,19,23] [15,18,19] Mo also has a high anticorrelation with Factor 2, similar to As, suggesting that these two elements could have a common origin or be influenced by the same environmental processes. Despite being an essential trace element involved in numerous enzymatic processes, Mo can be generated from both natural and anthropogenic sources, such as industrial or agricultural activity. Its presence in this factor may indicate a contribution from human-induced contamination, although further investigation is needed to distinguish between different sources. Conversely, Ni shows a notable correlation with Factor 2, suggesting that its elevated concentration (mainly in Turin samples) is due to geologic factors more than anthropogenic ones. The elevated Ni levels in Torino reflect its natural geogenic origin from serpentinite-rich soils, as corroborated by D'Amico et al. (2014). Such findings provide critical insights into how geological features and local industrial practices interact to influence bioaccumulation patterns in penguin feathers.[11,12] Different works that analyze Ni in penguin feathers collected in the metropolitan area of Turin found similar concentrations. The opposite correlation of the three elements is indicative that, while Ni is mostly influenced by geogenic processes, As and Mo presence could be regulated by different factors, probably connected to anthropogenic activity and their consequential presence in biological systems. Factor 3 accounts for 12.55% of the variance and shows significant correlations with Na (0.606) and K (0.545) (Table 4). These elements are critical in metabolic and physiological processes, suggesting that Factor 3 captures variations related to physiological differences in the birds, potentially linked to diet or the birds' electrolyte balance. [13] The high correlation with Na and K might indicate that the differences observed in this factor are more closely related to dietary intake or water source changes post-molt, rather than direct environmental contamination. The increase in Na and K levels post-molt may reflect physiological changes associated with feather regeneration, as birds might assimilate more minerals during this recovery phase. In the context of factorial analysis, Factor 3 reflects these internal physiological variations rather than external environmental accumulation. This complements Factors 1 and 2, which are more reflective of environmental accumulation and geogenic or anthropogenic influences. In conclusion, the Factorial analysis suggested that variations in the concentrations of elements are not only due to contaminations or

environmental factors but can be explained also with the natural biological processes of feather growth, metabolism and diet, especially during molt phases.

Table 4 Correlations between variables and the factors derived from factorial analysis conducted on pre- and post-molt samples

Elements	F1	F2	F3	F4
Al	0.517	0.111	-0.509	0.256
As	0.347	-0.807	-0.329	-0.141
Ba	0.791	0.317	-0.146	-0.228
Ca	0.877	0.098	0.123	0.392
Cd	0.765	0.284	-0.246	-0.195
Cu	0.542	-0.243	-0.404	-0.505
Fe	0.251	-0.365	-0.554	0.484
K	0.539	-0.245	0.545	-0.160
Mg	0.799	0.434	0.377	-0.065
Mn	0.667	-0.194	-0.049	0.634
Mo	0.228	-0.668	0.127	-0.206
Na	0.590	-0.276	0.606	-0.118
Ni	0.481	0.598	-0.332	-0.305
Pb	0.693	-0.197	-0.328	-0.367
Sr	0.853	0.121	0.143	0.367
Zn	0.659	-0.200	0.375	-0.040

3.4 Comparative Analysis of Samples from Different Zoos (chemometric analysis)

The same chemometric methods were applied comprehensively across the entire dataset to assess the differences and correlations among the various zoological locations. This analysis included the mixed samples from the Magdeburg Zoo to evaluate potential environmental or management influences on each sample set since no distinction has been made according to the molting period. The MANOVA results are shown in Table 5. The rejection of the null hypothesis across multiple statistical tests strongly suggests that the geographic location of the zoos significantly influences element accumulation in their feathers. The Wilks' Lambda value of 0.004, alongside highly significant p-values (<0.0001) for all tests, demonstrates that the habitat of the penguins is a key factor in determining the variation in element concentrations. The observed F-values for Wilks (21.8), Hotelling-Lawley (21.8), Pillai (21.8), and Roy (29.8) tests exceed their corresponding critical values, further confirming the robustness of the findings. The consistently significant outcomes across all

tests highlight that geographic location is a major determinant of element bioaccumulation in feathers. This could be due to differences in habitat, water quality, or food availability in the regions in which the penguins live. In particular, the next step will consist of the analysis of the feed eaten by the penguins. Further research is required to investigate which elements are most impacted by these environmental factors and to explore the potential biological implications of these findings for penguin health and conservation efforts.

Table 5 Results obtained by MANOVA to assess the sampling locations' role in the concentration of the analytes considered.

	Wilks	Hotelling-Lawley	Pillai	Roy
Lambda	0.004	28.9	1.86	18.3
F observed value	21.8	21.8	21.8	29.8
GDL1	32	32	32	16
GDL2	50	40	52	26
F critic value	1.67	1.73	1.66	2.05
p-value	<0,0001	<0,0001	<0,0001	<0,0001

As to the factorial analysis of the whole dataset, Factor 1 showed a high correlation with several elements (Table 6), including Ba(0.808), Ca (0.807), and Cd (0.840). These findings could indicate that Factor 1 potentially reflects physiological or environmental accumulation patterns common across the dataset. The implication of these correlations suggests that Factor 1 captures the general patterns of how these elements accumulate in the penguins, likely driven by consistent environmental exposure (e.g., dietary intake and/or habitat conditions).

Instead, Factor 2 majorly displayed strong inverse correlation with As (-0.718) and Pb (-0.405). As and Pb are known environmental pollutants often linked to industrial activities, waste, or other anthropogenic sources. [29,30]Therefore, Factor 2 could capture geographical or industrial differences between the penguin habitats or diets, especially in areas where contamination levels are higher.

On the other hand, Factor 3 showed high negative or positive correlation values with elements such as Ni (-0,715) and Mg (0,454), suggesting a reflection of either localized environmental contamination or exposure to these elements through their natural penguin diets or habitats. Ni variation could originate both from anthropogenic and natural sources. As reported in the previous

paragraph, the natural geological characteristics of the Turin Area include a high presence of serpentinite rocks, which enrich the soil with Ni and Cr, contributing to higher baseline levels of these elements in the local environment, and affecting their concentrations in local flora and fauna. [11,12] The results provide insights for improving zoo management practices and conservation strategies. By identifying elemental differences linked to local environmental conditions, this study highlights the role of captive penguins as sentinels for monitoring pollution and ecological changes. Additionally, the pre-and post-molt comparisons provide a framework for assessing how environmental and dietary factors impact feather development, with potential applications for wild populations. These findings could inform conservation strategies aimed at mitigating the impacts of pollution and climate change on seabirds globally.

One limitation of this study is the inclusion of mixed feather samples from Magdeburg Zoos, which did not allow for a clear distinction between pre- and post-molt phases. While these samples still provide valuable insights into elemental concentrations and environmental conditions, they limit the ability to make direct temporal comparisons between molting stages. Future studies should prioritize the collection of distinct pre- and post-molt feather samples from all study sites to facilitate more robust temporal analyses. Additionally, logistical constraints in sample collection and the small sample size for some zoos may have influenced the statistical power of our findings. Expanding the sample size and increasing the number of replicates across all institutions will be critical for future investigations to validate and extend these results.

Table 6 Correlations between variables and the factors derived from the factorial analysis conducted on the complete dataset

	F1	F2	F3	F4
Al	0.623	-0.209	-0.148	0.486
As	0.479	-0.718	0.387	-0.145
Ba	0.808	0.107	-0.354	-0.060
Ca	0.807	0.437	0.260	0.263
Cd	0.840	-0.075	-0.393	-0.005
Cu	0.608	-0.462	-0.174	-0.249
Fe	0.307	-0.404	0.338	0.638
K	0.488	0.223	0.331	-0.484
Mg	0.819	0.419	-0.209	-0.196
Mn	0.709	0.091	0.454	0.460
Mo	0.355	-0.418	0.359	-0.400
Na	0.261	0.392	0.420	-0.376
Ni	0.585	-0.010	-0.715	0.050
Pb	0.784	-0.405	-0.150	-0.187
Sr	0.847	0.400	0.210	0.226
Zn	0.709	0.024	0.177	-0.283

4 Conclusions

This study addressed the element compositions of *African penguin* feathers during pre- and post-molt phases in three different captive habitats. The results show higher concentrations and a higher variability in pre-molt samples for most elements; in particular, significant differences in Ca, Mg, Mn, and Na levels suggest an active process of mobilization and accumulation of mineral elements during the feather growth, resulting in a high variability of element content at the end of the molting cycle. Elevated Ca and Mn levels in pre-molt feathers indicate the mineral demands of feather keratinization, while changes in Mg and Na levels may reflect metabolic shifts and electrolyte balance regulation during feather regrowth. While MANOVA tests confirm the significant differences between pre-and post-molt samples, Factorial analysis underlines distinct geogenic and anthropogenic influences on elements like Sr and Ni, with higher concentrations linked to local geological conditions, such as serpentinite-rich soils in Torino. Zoo-specific differences were also confirmed as significant by the MANOVA tests. These variances may reflect the dietary and

environmental inputs, empathizing the role of the local environment and management practice influencing feathers element content. These findings underscore the need of tailored management strategies to assess the environmental and nutritional needs of captive penguins. In addition, the zoo-specific differences confirmed the potentiality of feathers as bioindicators of mineral elements content of the habitat examined, offering at the same time valuable insights into how zoo management practices influence elemental accumulation during molting. These findings have implications for improving captive penguin welfare and understanding broader ecological and environmental dynamics. While this study provides a robust foundation, it is not without limitations. The use of mixed feather samples from Zoo Magdeburg precludes a direct comparison of pre- and post-molt stages at that site. Future research should aim to address this limitation by standardizing sample collection protocols across all institutions. Analyzing animals' feed, expanding the sample size and incorporating additional zoos or, if possible, wild colonies will also help validate these findings and provide a more comprehensive understanding of the interplay between environmental, dietary, and physiological factors during the molting process.

By identifying elemental differences linked to local environmental conditions, this study highlights the role of captive penguins as sentinels for monitoring pollution and ecological changes. Additionally, the pre-and post-molt comparisons provide a framework for assessing how environmental and dietary factors impact feather development, with potential applications for wild populations. These findings provide insights for improving zoo management practices, thus enhancing the welfare of captive penguins and contributing to the ecological understanding and to the implementation of conservation strategies aimed at mitigating the impacts of pollution and climate change on this endangered species.

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Supplementary material

Supplementary Table 1 Details of samples included in the study

Penguin	Date of Sampling	PRE/POST molt or TOT	Origin
Brie 1	02/05/23	PRE	Turin
Charlotte 1	02/05/23	PRE	Turin
Chocolate 1	02/05/23	PRE	Turin
Dred 1	02/05/23	PRE	Turin
Funny 1	02/05/23	PRE	Turin
Homer 1	02/05/23	PRE	Turin
Jerry 1	02/05/23	PRE	Turin
Joker 1	02/05/23	PRE	Turin
Kyr 1	02/05/23	PRE	Turin
Pepe 1	02/05/23	PRE	Turin
Violet 1	02/05/23	PRE	Turin
Brie 2	30/08/23	POST	Turin
Charlotte 2	30/08/23	POST	Turin
Chocolate 2	30/08/23	POST	Turin
Dred 2	30/08/23	POST	Turin
Funny 2	30/08/23	POST	Turin
Jerry 2	30/08/23	POST	Turin
Joker 2	30/08/23	POST	Turin
Kyr 2	30/08/23	POST	Turin
Pepe 2	30/08/23	POST	Turin
Violet 2	30/08/23	POST	Turin
Purple 1	12/04/23	PRE	Overloon
Grey 1	12/04/23	PRE	Overloon
Pink 1	12/04/23	PRE	Overloon
Black/Blue 1	12/04/23	PRE	Overloon
Green/Pink 1	12/04/23	PRE	Overloon
Purple/Blue 1	12/04/23	PRE	Overloon
Purple 2	29/06/23	POST	Overloon
Grey 2	29/06/23	POST	Overloon
Pink 2	29/06/23	POST	Overloon
Black/Blue 2	29/06/23	POST	Overloon
Green/Pink 2	29/06/23	POST	Overloon
Purple/Blue 2	29/06/23	POST	Overloon
Erna	-	COMPOSITE	Magdeburg
Fritz	-	COMPOSITE	Magdeburg
Helmut	-	COMPOSITE	Magdeburg
Ida	-	COMPOSITE	Magdeburg
Julius	-	COMPOSITE	Magdeburg
Klaus	-	COMPOSITE	Magdeburg
Lady	-	COMPOSITE	Magdeburg
Mr. Pebody	-	COMPOSITE	Magdeburg
Petra	-	COMPOSITE	Magdeburg

Supplementary Table 2 Concentration of analytes (mgkg⁻¹) in feather samples part 1

Sample ID	Origin	Molt period	Al	As	Ba	Ca	Cd	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	Pb	Sr	Zn
1_M_C	Magdeburg	COMPOSITE	16.9	0.050	1.51	2930	0.020	9,99	28.6	397	2656	4.37	0.02	243	2.01	0.150	8.71	92.7
2_M_C	Magdeburg	COMPOSITE	8.59	0.050	2.46	3760	0.020	10.5	24.3	184	197	2.30	0.03	221	3.08	0.150	9.36	114
3_M_C	Magdeburg	COMPOSITE	5.10	0.050	3.79	3650	0.020	11.5	20.2	131	175	2.06	0.03	196	2.55	0.100	9.30	103
4_M_C	Magdeburg	COMPOSITE	7.00	0.050	3.72	3230	0.010	10.8	20.3	108	119	5.13	0.02	294	2.58	0.130	7.45	103
5_M_C	Magdeburg	COMPOSITE	10.3	0.050	2.36	3670	0.020	11.5	22.1	165	151	2.12	0.02	211	3.74	0.170	8.81	104
6_M_C	Magdeburg	COMPOSITE	5.79	0.050	2.34	2830	0.020	10.3	17.2	126	118	2.60	0.04	199	3.43	0.120	6.59	107
7_M_C	Magdeburg	COMPOSITE	4.33	0.050	2.53	2820	0.020	10.3	16.0	175	150	2.30	0.03	165	2.26	0.090	5.29	101
8_M_C	Magdeburg	COMPOSITE	9.77	0.050	3.25	4040	0.020	8.66	26.5	136	138	3.62	0.02	190	2.48	0.130	9.09	106
9_M_C	Magdeburg	COMPOSITE	5.83	0.060	2.87	4170	0.020	10.8	16.8	145	175	2.54	0.02	203	2.18	0.120	8.84	112
11_O_PRE	Overloon	PRE	16.7	0.350	3.93	2630	0.080	47.1	72.2	394	145	7.47	0.11	211	8.41	0.960	9.43	159
12_O_PRE	Overloon	PRE	32.7	0.150	3.08	4360	0.050	10.84	66.7	190	272	15.4	0.07	192	7.22	0.400	15.0	160
13_O_PRE	Overloon	PRE	17.2	0.100	7.68	8960	0.090	30.7	64.3	197	523	32.0	0.07	112	10.8	0.660	36.3	161
14_O_PRE	Overloon	PRE	64.6	0.190	3.81	9740	0.100	11.3	110	362	568	36.3	0.10	201	10.7	0.630	39.9	167
15_O_PRE	Overloon	PRE	34.2	0.120	1.56	4360	0.040	9.26	39.7	105	229	14.7	0.04	120	3.34	0.250	14.7	145
10_O_MID	Overloon	MID	21.5	0.080	1.23	176	0.030	9.51	40.1	158	129	4.68	0.03	247	3.57	0.300	3.57	93.1
10_O_POST	Overloon	POST	12.4	0.080	0.710	740	0.020	7.91	29.9	124	50.6	3.37	0.04	7213	3.81	0.220	1.77	97.9
11_O_POST	Overloon	POST	6.99	0.080	0.410	650	0.010	6.50	23.6	<LOD	44,3	3.68	0.02	<LOD	1.86	0.210	1.44	98.5
12_O_POST	Overloon	POST	9.26	0.100	0.930	723	0.020	6.93	29.0	120	60.7	3.42	0.03	72.1	4.84	0.220	1.70	114
13_O_POST	Overloon	POST	7.61	0.100	0.980	389	0.030	6.24	35.3	1,7	< LOD	2.44	0.05	4.78	4.42	0.240	<LOD	90.9
14_O_POST	Overloon	POST	7.66	0.060	0.520	365	0.020	7.82	21.9	<LOD	30.7	1.14	0.03	<LOD	2.53	0.150	0.66	94.8
15_O_POST	Overloon	POST	28.8	0.080	0.860	655	0.020	7.11	35.4	<LOD	50.5	2.59	0.03	<LOD	2.77	0.160	1.78	103

Supplementary Table 3 Concentration of analytes (mgkg⁻¹) in feather samples part 2

Sample ID	Origin	Molt period	Al	As	Ba	Ca	Cd	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	Pb	Sr	Zn
16_Z_PRE	Turin	PRE	14.6	0.070	8.84	3200	0.080	18.8	0.030	134	562	7.35	30	103	35.6	0.940	12.6	156
16_Z_POST	Turin	POST	16.4	0.060	2.03	2500	0.040	8.06	0.060	297	477	5.88	0,06	191	8.79	0.250	7.72	166
17_Z_PRE	Turin	PRE	9.92	0.060	6.22	3550	0.090	10.9	0.050	565	660	5.56	0.050	186	32.5	0.540	11.8	180
17_Z_POST	Turin	POST	17.1	0.070	2.80	1930	0.100	13.2	0.050	284	405	3.20	0.050	124	47.9	0.340	6.66	116
18_Z_PRE	Turin	PRE	7.93	800	3.33	2180	0.060	22.8	16.4	261	393	3.58	0.030	126	54.8	0.430	8.81	132
18_Z_POST	Turin	POST	9.65	0.060	4.96	1620	0.040	13.3	19.4	264	423	1.65	0.050	195	65.2	0.430	5.70	112
19_Z_PRE	Turin	PRE	21.4	0.080	7.59	12200	0.090	10.6	0.040	420	1380	24.5	0.0400	359	33.9	0.460	53.4	178
19_Z_POST	Turin	POST	12.4	0.080	4.61	2800	0.060	11.5	0.130	215	664	3.14	0.130	151	14.1	0.340	9.77	153
20_Z_PRE	Turin	PRE	32.3	0.0800	7.14	4160	0.090	18.9	46.8	166	716	9.39	0.040	84.4	87.5	0.290	16.2	149
20_Z_POST	Turin	POST	4.84	0.08	3.12	2950	0.050	18.9	28.5	146	649	2.59	0.030	100	48.6	0.210	10.3	182
21_Z_PRE	Turin	PRE	12.6	0.040	1.88	2170	0.060	14.2	0.030	117	394	3.44	0.030	87.1	27.2	0.210	7.06	108
22_Z_PRE	Turin	PRE	58.4	0.070	8.25	3660	0.160	24.42	0.030	105	640	8.14	0.030	56.1	98.1	0.530	13.7	119
22_Z_POST	Turin	POST	8.30	0.1000	5,09	2230	0.100	20.0	0.060	145	536	1.72	0.060	227	39.0	0.490	8.29	142
23_Z_PRE	Turin	PRE	30.3	0.110	5.19	3150	0.110	14.3	0.070	306	706	5.08	0.070	223	39.4	0.500	12.5	143
23_Z_POST	Turin	POST	15.6	0.080	2.11	1530	0.070	10.7	0.100	263	407	<LOD	0.100	184	580	0.310	4.17	152
24_Z_PRE	Turin	PRE	62.5	0.110	18.3	1040	0.27	19.9	64.9	182	1150	14.8	0.030	98.7	86.5	0.630	42.3	17
24_Z_POST	Turin	POST	19.0	0.2000	9.79	2070	0.13	37.9	0.150	200	377	2.60	0,150	156	20.3	0.850	5.15	153
25_Z_PRE	Turin	PRE	14.1	0.100	16.4	9320	0.13	32.6	0.040	346	969	12.8	0,04	224	82.4	0.860	43.0	140
25_Z_POST	Turin	POST	3.12	0.060	2.19	2890	0.03	100	16.6	367	526	5.00	0.060	132	14,7	0.230	8.98	153
26_Z_PRE	Turin	PRE	65.1	0.100	7.18	3680	0.12	35.2	78.2	144	599	10.1	0.030	83.9	137	1.05	11.0	132
26_Z_POST	Turin	POST	6.82	0.080	4.22	2410	0.05	14.0	19.3	255	6324	1,92	0.040	146	27.2	0.240	8.16	149

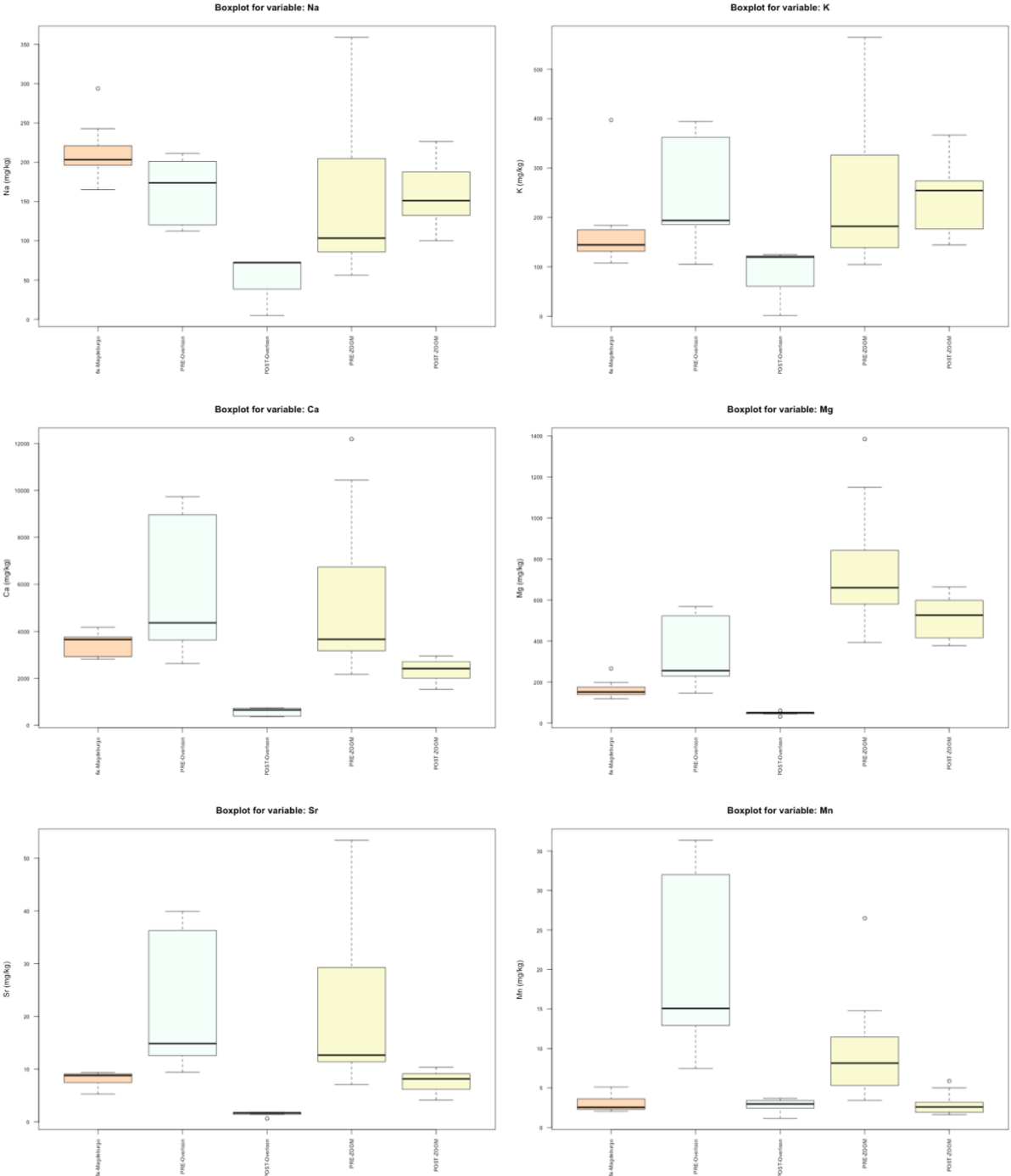
Supplementary Table 4 Summary of data on elements in penguin feathers: maximum, minimum, variance. The following groups are address: Turin and Overloon pre and post molt; all data

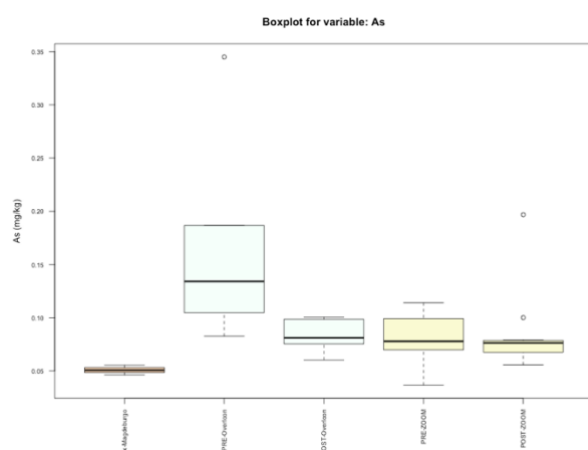
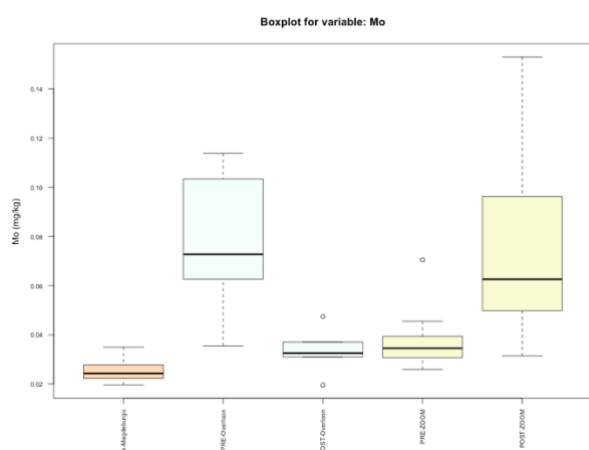
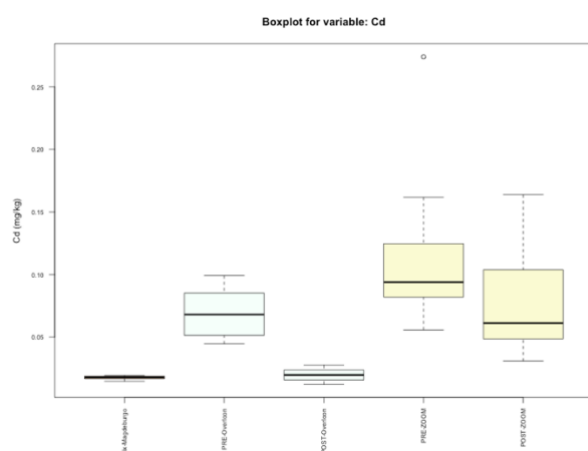
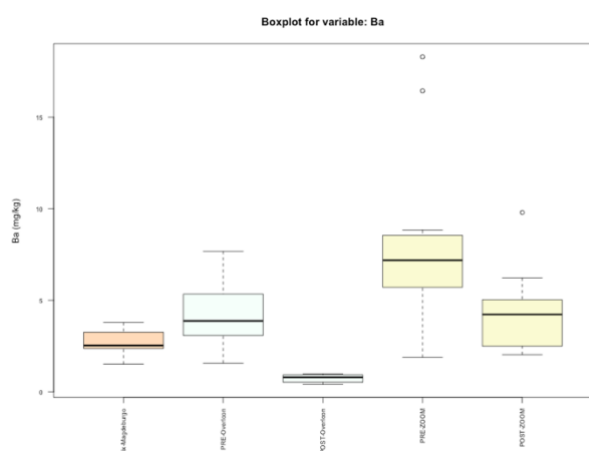
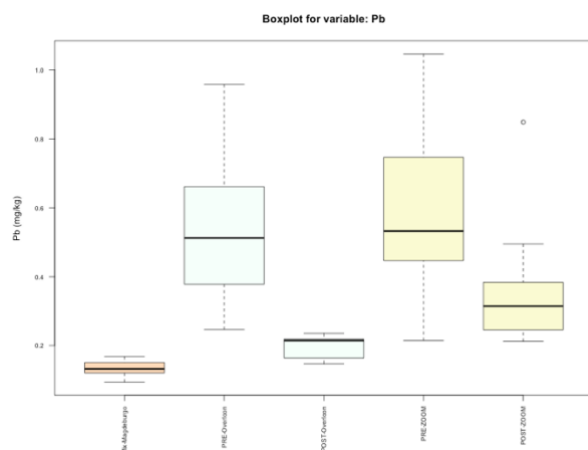
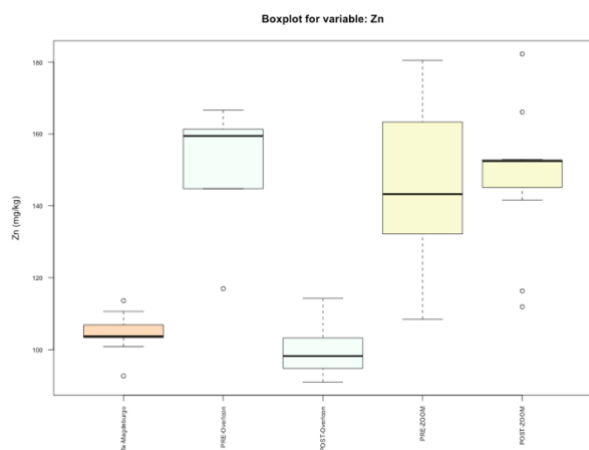
	Al	As	Ba	Ca	Cd	Cu	Fe	K	Mg	Mn	Mo	Na	Ni	Pb	Sr	Zn
Max total	65.1	0.345	18.30	12200	0.274	47.1	110	565	1385	36.4	0.153	359	137	1.05	53.4	182
Min total	3.12	0.037	0.408	365	0.012	6.24	0.026	1.70	30.7	1.14	0.020	4.78	1.86	0.094	0.655	90,9
Turin_max pre molt	65.1	0.114	18.30	12200	0,274	35.1	78.2	565	1380	26.5	0.071	359	137	1.05	53.4	180
Turin_max post molt	19.0	0.197	9.79	2950	0.164	37.9	35.4	367	664	5.88	0.153	227	66.7	0.848	10.3	182
Turin_min premolt	7.93	0.037	1.87	2170	0.056	10.6	0.026	105	393	3.44	0.026	56.1	27.1	0.214	7.06	108
Turin_min post molt	3.12	0.056	2.02	1530	0.031	8.06	0.047	145	377	1.65	0.031	100	5,8	0.212	4.17	112
Overloon_max pre molt	64.6	0.345	7.67	9740	0.099	47.1	110	395	568	36.4	0.114	211	10.8	0.958	39.9	167
Overloon_max post molt	28.8	0.100	0.979	740	0.028	7.91	35.4	125	60.7	3.68	0.047	72.2	4.84	0.235	1.78	114
Overloon_min premolt	16.7	0.083	1.56	2630	0.045	9.26	39.7	105	145	7.47	0.036	112	3.34	0.246	9.43	117
Overloon_min post molt	6,,99	0.060	0.408	365	0.012	6.24	21.9	1.70	30.7	„14	0.020	4.78	1.86	0.147	0.655	90.9

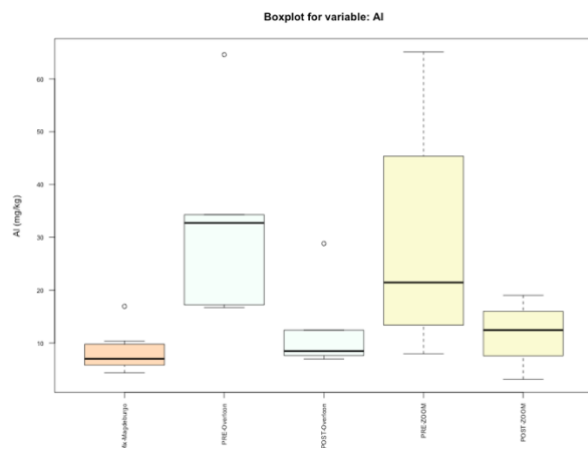
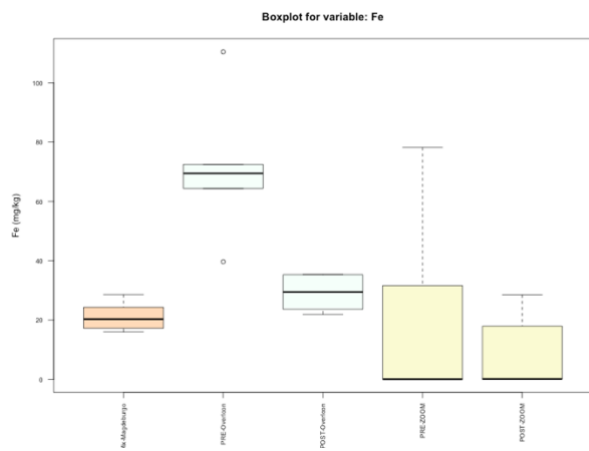
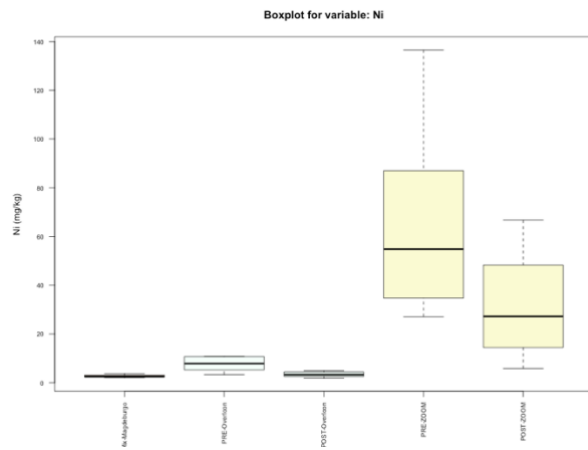
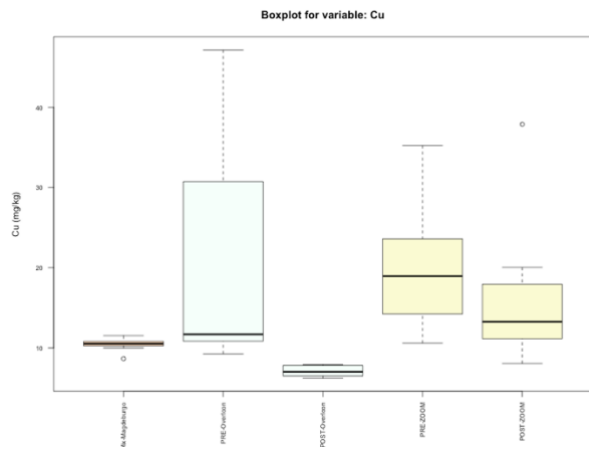
Supplementary Table 5 Average Standard deviation and RSD%

Element	Standard Deviation	RSD%
Al	2.130	16.9
As	0.010	12.41
Ba	0.900	24.18
Ca	377	10.71
Cd	0.010	17.98
Cu	1,6	13.34
Fe	5.16	22.07
K	57.2	22.34
Mg	23.5	8.94
Mn	0.830	15.46
Mo	0.010	18.62
Na	36.0	17.57
Ni	3.62	21.92
Pb	0.060	19.59
Sr	1.45	14.92
Zn	4.79	14.92

Figure 1 Boxplot of all the variables taken into account







General Conclusions

This work explored the potential of plants and animals as environmental bioindicators, applying a multidisciplinary perspective that unifies environmental chemistry, phytotherapy, and conservational strategy. Aligning with the program of the Doctoral School in Sustainable Development Cooperation, the entire project aimed to evaluate the potentiality of biological matrices as tools to estimate ecosystem health, environment monitoring, and the safety of traditional phytotherapy remedies.

The first study led to the development of a simple, on-site, and economic voltammetric method to quantify Cd and Pb in endemic officinal plants used in traditional Malagasy medicine, as well as in officinal plants collected in Italy. Through experimental design, it was possible to obtain a significant improvement in sensitivity, accuracy, and repeatability compared to the starting procedure, in which operative conditions had been chosen with the OVAT approach. The method, which uses a glassy carbon electrode modified with mercury film *in situ*, has shown excellent analytical performance, providing the possibility of carrying out the quantification of the two considered PTEs with a simple and economical method, representing a valid alternative to traditional laboratory techniques. In particular, this protocol permits to assess the risk associated with the consumption of medicinal plants in a context with limited resources, such as in Madagascar.

The second chapter regards the analysis of essential and non-essential elemental content in endemic officinal plants of Madagascar to evaluate the safety of use and their potential as bioindicators of the ecosystem state. The study quantified elements in three different plant species collected in three parks of Madagascar with different anthropic impacts, highlighting elevated concentrations of different PTEs in soil (Co, V, and Cr), potentially due to the proximity of the Ambatovy mine. However, the majority of leaf samples show concentration below the European legal low limit, suggesting the existence of physiological mechanisms of regulation in the uptake of elements by plants. These results underline the need for systematic environmental monitoring and greater knowledge of local biogeochemical cycles in tropical areas, which are fundamental to ensuring the conservation of biodiversity and the protection of human health in vulnerable contexts.

The third chapter extended the application of the non-invasive approach to the biomonitoring of lemurs, endemic endangered species of Madagascar. The characterization of element content in the

samples taken in the two bioparks (Turin, Italy, and Antananarivo, Madagascar) highlights how the biological matrices reflect distinct exposition dynamics; fur dataset reflects the soil pattern, indicative of chronic bioaccumulation in a long-term period, while feces dataset reveals a higher sensitivity to short-term changing as the seasonal variation. The multivariate data processing made it possible to distinguish the samples both based on geographical origin and the sampling season, suggesting that climate factors influence the availability of elements in environment compartments, with direct implications for individual metabolism. The obtained results confirm the value of a cross-section approach, offering a solid base for future study, bio-park management, and conservational strategy.

In the last work, the analysis of African penguin feathers showed the presence of a relationship between molt and element bioaccumulation. The molt represents a key physiological phase in which birds renew their plumage, allowing recent exposure to environmental contaminants to be traced through the analysis of new feathers. Data show concentration patterns and biological factors, offering new scientific perspectives for the use of feathers as a non-invasive matrix to evaluate the health of individuals and their habitat. In addition, the comparison between individuals housed in different biopark in Europe (Turin, Magdeburg, and Overloon) underline different patterns of element concentrations, suggesting the influence of environmental, dietary, and management factors specific to each facility.

Considered together, the four studies of this thesis offer a valid and coherent contribution to the development of economic, simple and effective methodologies to evaluate the health of ecosystems. This work addresses portable solutions and ethical approaches, which were adopted as a base to adapt advanced techniques and methodology to fragile contexts, such as developing countries and endangered species. The added value of this work lies in its ability to link sophisticated chemical analyses with concrete goals of sustainability, safety, and conservation, promoting an effective integration between scientific research and local needs. The realization of this work was made possible thanks to close international cooperation, which represented not only an opportunity for scientific and cultural exchange but also a key element to ensure the applicability and relevance of the proposed solutions in heterogeneous environmental and socio-economic contexts.

This integrated approach lends itself to being extended to other application areas, including food safety, phytotherapy, pharmacovigilance, and participatory environmental monitoring. In conclusion, the work carried out lays a solid foundation for further research aimed at enhancing biodiversity and promoting sustainable practices based on reliable scientific data, in line with the Sustainable Development Goals and the One Health approach.