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Accumulation and spatial distribution of arsenic and phosphorus in the fern *Pityrogramma calomelanos* evaluated by micro X-Ray fluorescence spectrometry

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Abstract

The accumulation and spatial distribution of arsenic and phosphorus in the As-hyperaccumulator fern *Pityrogramma calomelanos* were investigated with micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF). Ferns were grown in half-strength Hoagland nutrient solution without and with 1.0, 10 or 30 x 10^{-3} mol L^{-1} As during three weeks. Microchemical As and P maps in different areas of the pinna were obtained by μ-EDXRF, and the same elements were also determined in pelletized powdered samples from the pinna, stipe and root. The reference method for the determination of As and P in the fern samples was a validated method based on microwave-assisted acid digestion followed by ICP OES analysis. Correlations between X-ray characteristic emission intensities and the corresponding As and P mass fractions obtained from the analysis of pelletized test samples exhibited linear correlation coefficients higher than 0.98 (n = 42). Better root mean square errors of prediction (RMSEP) were obtained when different calibration models were built with pinna, stipe and root test samples separately. Arsenic was accumulated mainly in the pinna midrib, secondary veins, and apical and marginal regions of the pinnule of *P. calomelanos*, causing alterations in the P distribution. The proposed μ-EDXRF method is an appropriate analytical tool for simultaneously mapping As and P in *P. calomelanos*. In addition, useful information for either environmental monitoring or phytoremediation studies regarding As contamination is properly obtained.

Keywords

EDXRF, Elemental mapping, As-hyperaccumulator, plant nutrition, phytoremediation.

Introduction

Arsenic, recognized as a potential contaminant of great concern, is worldwide distributed in nature, mainly as arsenopyrite (FeAsS), the most common arsenic mineral.[1] Parent materials containing arsenopyrite and other As-bearing sulfides are oxidized when exposed to atmospheric oxygen and water, thus releasing this potentially toxic element.[2] This natural phenomenon is called acid mine drainage[3,4] and it is the main cause of high As levels in freshwater reservoirs.[2] Anthropogenic activities, such as the intensive use of arsenical pesticides, mining, fossil-fuel burning and disposal of As-enriched wastes, can also substantially enhance the As contamination.[5] High arsenic levels in soil and groundwater may lead to deleterious effects to the ecosystem, and efforts towards environmental monitoring and remediation programs must be implemented in order to minimize the organisms' exposure to this element.[6-8]

Plants naturally growing in metal(loid)-contaminated soils are adapted to survive in this stressful environment and are classified into three main categories: metal excluders, indicators and accumulators/hyperaccumulators.[9] The majority of plant species are excluders, which contain low levels of potentially toxic elements in their aerial tissues, even when exposed to higher concentrations of contaminants. Indicators present metal(loid)s into their aboveground biomass, thus reflecting the elemental concentration in the soil. Accumulators/hyperaccumulators are able to increase metal(loid) internal sequestration, translocation and accumulation into their fronds to levels that far exceed those usually found in the soil.[9,10]

Arsenic-hyperaccumulator species, *e.g. Pteris vittata*[11] and *Pityrogramma calomelanos*[12] differ from As-accumulators by having metalloid mass fractions higher than 1 % m m^{-1} dry matter. These hyperaccumulator plants are promising organisms in environmental monitoring and in remediation programs.[13,14] In this sense, arsenic

concentration in leaves of hyperaccumulators can be taken into account for evaluating the input of As in the soil/water system by natural/anthropogenic sources. These plants can be harvested to reduce potential As contamination, thereby limiting the metalloid entry into the food chain, a strategy known as phytoremediation.[15]

In environmental studies, the quantitative determination of trace elements in plant materials has been generally accomplished by atomic absorption spectrometry (flame, graphite furnace, hydride generation), inductively coupled plasma (ICP) optical emission spectrometry or ICP-mass spectrometry.[16,17] These methods often require an *a priori* chemical treatment for the decomposition of the organic material, usually involving microwave-assisted digestion with nitric acid and hydrogen peroxide.[18-20] Micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) is a fast and non-destructive method that has been successfully applied to the determination of macro- and micronutrients in plant materials.[21] Its suitability to simultaneous multielemental determinations combined with its high spatial resolution make μ-EDXRF a versatile screening tool for elemental mapping in plants.

Recently, a benchtop μ-EDXRF instrument was used for investigating the Al[22] spatial distribution in leaves of plants from High Altitude Rocky Complexes, Southeast Brazil. Synchrotron radiation micro X-ray fluorescence spectrometry (SR-μ-XRF) was also successfully used for mapping As,[23] Tl,[24] Pb,[25] Cd,[26] Se[27] and Zn[28,29] in hyperaccumulator plants.[30]

Some studies have investigated both the As spatial distribution and its speciation in *Pteris vittata*.[31-36] Regarding *P. calomelanos*, there are important contributions dealing with arsenic mapping,[37,38] however, to the best of the authors' knowledge, there are no studies on simultaneous arsenic and phosphorus mapping in this fern species. This issue deserves special attention because it has been demonstrated that arsenate exhibits a

strong physiological competition by the phosphate uptake systems in higher plants[39], but interference of As in P metabolism of hyperaccumulator ferns remains as a controversial issue.[40]

In the present contribution, μ-EDXRF was evaluated for the simultaneous As and P microchemical mapping in sporophytes of *Pityrogramma calomelanos* grown in absence and in As-enriched solutions. Moreover, the validation of a μ-EDXRF method for the quantitative determination of As and P in pelletized powdered fern samples is presented.

Experimental

Sporophytes of *Pityrogramma calomelanos* L. (Link) (Pteridaceae) were obtained through *in vitro* culture of spores, and subsequently gametophytes, in Murashige and Skoog (MS) medium.[41] The young sporophytes were cultivated in commercial substrate Plantmax® in a greenhouse of the Plant Growth Unit at Universidade Federal de Viçosa, Minas Gerais state, Brazil, at 25 ± 5 ºC. Ferns at 4-5 frond stage (n = 10) were transferred to a hydroponic system with half-strength Hoagland nutrient solution,[42] pH 5.5, under continuous aeration. After an acclimatization period of 4 weeks, the Hoagland nutrient solution containing 1.0 x 10^{-3} mol L^{-1} As was used with half of the ferns. Arsenic was supplied as sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). The remaining ferns were grown in Hoagland nutrient solution[42] without As (control). There were five replicates per treatment and each replicate involved a 2.2 L pot containing one plant. Plants were exposed during 21 days and the solution was renewed weekly. Similarly, a second experiment with ferns at 5-7 frond stage (n = 20, five replicates per treatment) were carried out in the absence and in the presence of 1.0, 10 and 30 x 10^{-3} mol L^{-1} As, under the same exposure time.

At the end of the experiments, pinnae, stipes, and roots were sampled, washed with deionized water and oven-dried at 60 ºC until constant weight. In order to obtain flat surfaces required for elemental mapping, previously dried pinna samples were manually pressed between two decontaminated glass plates.

ICP OES analysis

Dried samples were powdered in a ball mill and sieved through a 200-mesh (74 μm) stainless steel sieve. The sieved material (test sample for ICP OES) was accurately weighed (*ca.* 50 mg) in triplicate in closed TFM® digestion vessels (ETHOS 1600, Milestone, Italy). To each vessel, 5.0 mL of 2.8 mol L^{-1} HNO_3 and 2.0 mL of H_2O_2 30 % m m^{-1} were added. The microwave heating program is shown in Table 1. After cooling to room temperature, the final solutions were transferred to volumetric flasks and the volume was made up to 10 mL with deionized water. The resulting digests were analyzed by ICP OES, with a dual view iCAP 6500 Duo optical emission spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a cyclonic spray chamber and a PEEK Mira Mist® nebulizer from the same manufacturer. The following ICP OES measurement conditions were used: 27 MHz generator frequency; 1.2 kW RF applied power; 1.5 mL min^{-1} sample flow rate; 12, 0.5 and 0.6 L min^{-1} argon flow rates for plasma, auxiliary and nebulizer, respectively; 15 s measurement time. Arsenic and phosphorus emission lines (As I 197.262 nm and P I 213.618 nm) were monitored in the axial and radial viewing mode, respectively.

μ-EDXRF analysis

μ-EDXRF analysis were performed using a benchtop spectrometer (μEDX-1300, Shimadzu, Kyoto, Japan) furnished with a Rh X-ray tube with polycapillary lenses as

X-ray convergence method, and a Si(Li) semiconductor detector (with 30 mm² detection area) sealed with a 8 μm thick Be window. The whole spectra comprised 4096 channels with a resolution of approximately 150 eV (FWHM) at 5.9 keV. Spectrum energy calibration was daily performed before each analysis batch by using an aluminum alloy (A 750 calibration standard). This was done for adjusting offset and system conversion gain (10 eV/channel) parameters.

The limits of detection (LODs) were calculated as $3.3 \times \frac{s}{b}$, where s is the estimated standard deviation from the background (BG) intensity of each Kα peak (P Kα 2.01 keV and As Kα 10.54 keV) from the spectra of 10 pelletized pinna test samples. In each pellet 30 different test portions were irradiated (50 μm X-ray spot size). The BG intensities were calculated by the equipment software. The term "b" in the expression for LOD calculation is the slope of the calibration curve.

Pellet preparation and method calibration

Pellets of pinna, stipe, and root were prepared in a pneumatic press (Perkin Elmer, Waltham, MA, EUA) from previously ground and sieved samples by transferring 0.2 g of each sieved portion to a 13 mm internal diameter stainless steel die set and applying 8 t cm^{-2} for 5 min. Pellets with approximately 1 mm thickness were used as test samples for μ-EDXRF quantitative analysis. Fourteen ground and sieved samples were selected from each fern part (pinna, stipe and root) totalizing 42 pellets (test samples).

Three lines, comprising 30 measurement points each, were randomly selected on the pellet surface. Each measurement point (50 μm X-ray spot size) was analyzed during 10 s by keeping 100-μm distance between two adjacent sampling sites. The operational conditions are shown in Table 2.

Linear regression models were calculated to correlate As and P mass fractions determined by ICP OES with the corresponding Kα lines intensities (cps μA^{-1}), as recommended elsewhere,[21] using either the data from all test samples (n = 42) and also separately for each fern part (n = 14).

Microchemical As and P mapping

Regarding microchemical mapping, the selected fern parts were horizontally fixed with adhesive tape onto a 4 μm thick Mylar film previously assembled in an XRF sample cup, which was placed in a sample holder specially designed for transmitted image measurements. Sampling areas of 4 mm x 3 mm (80 x 60 measurement points; 50 μm step per site) were selected on the basal and apical portions of a pinnule, including the pinna midrib for simultaneous As and P mapping. Pinna samples with similar thickness and position in the stipe were chosen. A sampling area of 10 mm x 7.5 mm (200 x 150 measurement points; 50 μm step per site) embracing the whole pinnules, attached to the pinna midrib, was also mapped. The following peaks: P Kα 2.01 keV, As Kα 10.54 keV and Rh Kα Compton 18.96 keV were monitored for the construction of each microchemical map.

The following measurement conditions were used for mapping: 200 μA and 50 kV for X-ray tube current and voltage, respectively and *ca.* 10 % detector dead time. The elemental mapping was performed in three different pinnule samples (either for basal or apical portions) of both As-treated pinna and the control pinna. In order to circumvent the intermediate-thickness sample effect, which may affect the As detection in the analyzed matrix, the scattered radiation method was used.[43,44] In this case, the Rh Kα Compton peak was selected as internal standard to compensate for the inherent differences in thickness and density along the mapped pinna regions. The software

SigmaPlot® version 11 (San Jose, CA, USA) was used for the construction of the microchemical maps.

The mapped pinnule samples by μ-EDXRF were photographed with a stereo microscope (SZX7 Olympus) equipped with an EVOLT E-300 Olympus digital camera (Olympus Optical). Figure 1 illustrates the morphological characteristics of *P. calomelanos* highlighting the assayed pinna regions.

Estimation of mass *per* unit area of pinna regions

Mass *per* unit area (g cm^{-2}) of selected pinna regions (midrib and pinnule blade) were estimated by weighing portions of midrib and pinnule blades and determining the area of the selected portions by using the software Image-Pro Plus® version 4.5 (Rockville, MD, USA). This procedure was performed in triplicate.

Statistical analysis

The software Origin version 8 (Northampton, MA, USA) was used to perform the linear regression analysis. For each linear regression model, the corresponding linear correlation coefficient (r) and the root mean square error of prediction (RMSEP) were calculated as described elsewhere.[45]

Results and discussion

Determination of As and P by ICP OES

The quality of the measurements of As and P made by ICP OES after microwave-assisted acid digestion (reference method) was checked by determining the mass fraction of arsenic in the certified reference material BCR-060 (*Lagarosiphon*

major), and also the mass fractions of phosphorus in NIST SRM 1515 (Apple leaves) and NIST SRM 1547 (Peach leaves). No significant differences were observed between the certified and found values after applying the Student's *t*-test at 95 % confidence level.

Pityrogramma calomelanos ferns from the control treatment presented arsenic mass fractions of 1.6 to 2.7 mg kg^{-1} for pinna and stipe, and of 9 to 52 mg kg^{-1} for roots. Ferns exposed to different As doses presented arsenic mass fractions ranging from 580 to 8800 mg kg^{-1} in pinnae, 70 to 6600 mg kg^{-1} in stipes, and 200 to 7800 mg kg^{-1} in roots. Higher As mass fractions were noted for the pinna from ferns exposed to 10 and 30 x 10^{-3} mol L^{-1} As that also presented apical and marginal necrosis in the old fronds.

The phosphorus mass fractions ranged from 3.8 to 11 g kg^{-1} in pinnae, 2.4 to 4.2 g kg^{-1} in stipes, and 3.8 to 6.6 g kg^{-1} in roots. In the first experiment, As promoted diminution of P contents in ferns, but no appreciable changes were observed in the samples from the second experiment. Ferns at 5-7 frond stage have probably a high capacity to maintaining P status in roots and pinnae than younger ferns.

μ-EDXRF analysis of pelletized samples

A fragment of a typical μ-EDXRF spectrum obtained from the analysis of a pelletized pinna of *P. calomelanos* is shown in Figure 2. Fragments of the spectra containing the characteristic Kα peaks of As and P from the analysis of pelletized test samples of pinnae were used to estimate the limits of detection (LODs) of 103 mg kg^{-1} As and 1.0 g kg^{-1} P by μ-EDXRF. Limits of quantification (LOQs) of 309 mg kg^{-1} As and 3.0 g kg^{-1} P were estimated as three times the obtained LODs.[21]

Figure 3 shows the linear regression models calculated from the emission line intensities of As Kα 10.54 keV and P Kα 2.01 keV, and their corresponding elemental

mass fractions (mg kg^{-1} As and g kg^{-1} P) found in the pressed pellets of all fern materials, with linear correlation coefficients (r) higher than 0.98 (n = 42) for both As and P calibration models. The higher RMSEP values obtained for phosphorus (as high as 1230 mg kg^{-1} P) in relation to arsenic (from 227 to 671 mg kg^{-1} As) can be derived from the lower XRF sensitivity and, consequently, higher LOD values for the detection of low atomic number elements.[46]

The regression models were even better when pelletized powdered samples from the same fern parts were used for calibration. This behavior was confirmed by better RMSEP values and linear correlation coefficients when this calibration strategy was used. As the RMSEP values represent an estimation of the predictive capacity of the calibration models, their lowest values are a good indication that possible matrix effects were minimized.

In general, the coefficients of variation of μ-EDXRF measurements carried out in all pelletized fern test samples varied from 1.0 to 17 % (n = 3 sampling lines, 30 sites per line, 50 μm X-ray spot size).

It should be pointed out that, in addition to the abovementioned figures of merit, one shall consider that the intensities of the characteristic X-ray emission lines of Ca, K, S, Fe and Mn (Figure 2) can be similarly correlated with their corresponding mass fractions in the pelletized fern materials, as already demonstrated for macro- and micronutrients with pellets of powdered sugar cane leaves.[21]

Microchemical mapping

Microchemical qualitative maps were obtained in order to investigate As and P distribution patterns in the pinna. To evaluate the necessity for applying the correction strategy based on the scattered radiation method,[43,44] the data generated from the mass

per unit area of the assayed pinna regions were compared with theoretical data, which were calculated with the equations described elsewhere[47] by using cellulose as the sample matrix.

The final values obtained with the help of the aforementioned equations[47] were corrected taking into consideration the angle between the energy dispersive detector axis and the plane of the test sample. Based on this, a cellulose matrix can be considered thick at mass *per* unit area higher than about 0.01 g cm^{-2} for phosphorus (P Kα 2.01 keV) and approximately 1 g cm^{-2} for arsenic (As Kα 10.54 keV). In the present study, the estimated mass *per* unit area was 0.0093 ± 0.0002 g cm^{-2} for the pinnule blade and 0.025 ± 0.005 g cm^{-2} for the pinna midrib. Therefore, the scattered radiation method based on the use of the Rh Kα Compton peak as internal standard was applied only for arsenic.

Arsenic maps for control samples did not show any significant distribution trend in both basal and apical analyzed areas (Figure 4). In the basal portion of pinnule from ferns treated with 30 x 10^{-3} mol L^{-1} As, it was observed a preferential As localization in the pinna midrib and in the secondary ribs (Figure 4 a, As Kα/Rh Kα Compton map). The apical portion of pinnules from the 30 x 10^{-3} mol L^{-1} As treated fern also showed a preferential accumulation of As in the vascular regions (Figure 4 b, As Kα/Rh Kα Compton map). Additionally, the As Kα/Rh Kα Compton maps obtained for whole pinnules indicated an increased gradient of the metalloid towards the margin, being the pinna tip the main As hotspot (Figure 5).

Phosphorus distributions between vascular and non-vascular areas were similar for ferns of both treatments. Control and As-treated samples showed a high P concentration in internerval regions, which represent the photosynthetic areas, and a low P concentration in the pinna midrib and pinnule veins, which can be considered as As

hotspots (Figure 4). The same tendency was observed when comparing As and P maps of whole As-treated pinnules (Figure 5). As-treated ferns showed a higher P concentration in the basal portion of the pinnule whereas control samples presented a higher P concentration in the apical region (Figure 4). A possible explanation for these results is addressed in the next comments.

Comments on As and P localization in ferns compartments

The importance of the proposed method for simultaneously investigating the distribution of arsenic and phosphorus in the As-hyperaccumulator fern *Pityrogramma calomelanos* by μ-EDXRF is further demonstrated by briefly commenting and suggesting possible mechanisms for explaining the results reported here.

Arsenic was not detected in the control samples, indicating that the mass fractions were below the estimated LOD (103 mg kg^{-1} As). In As-treated ferns, arsenic was preferentially located along the veins and in the apical and marginal regions of the pinna, in accordance with previous report for *Pteris vittata.*[33] This distribution pattern corroborates with the mechanism of As transport from root to shoot, which is driven by transpiration, similarly to several other elements.[48,49] Arsenic is transported though xylem to the lamina of the frond, where it seems to be sequestered in the vacuole.[50]

Arsenate is the dominant As species in aerobic soils.[51] In *Pteris vittata,* arsenate is the main As species transported though the xylem sap when arsenic is supplied as an inorganic form.[52] Francesconi *et al.*[12] analyzed *Pityrogramma calomelanos* ferns grown in an As-contaminated soil (presenting 6-12 % of water-extractable As with 97 % of this as arsenate). The ferns presented 60 % of water-extractable arsenic from their rhizoids, and the largest fraction (95 %) as arsenate. Once arsenate is uptaken by fern roots, most of it can be reduced to arsenite, mainly in the frond.[33,34] Vacuolar

sequestration of arsenite represents a strategy for As detoxification in the hyperaccumulator ferns.[51] In the present study, the observed higher As accumulation pattern in pinna tissues may have caused toxic effects, as observed by the occurrence of brownish-orange necrotic areas in the pinna tip. This arsenic-mediated phytotoxicity may have induced the observed decay in the phosphorus content in the apical portion of the As-treated pinna (Figures 4 and 5, P Kα maps). Similarly, Tu *et al*.[53] evaluated the effects of arsenate and phosphate on As and P accumulation patterns in roots and fronds of *P. vittata*. In this study[53] it was found a decrease in the phosphate accumulation in the roots and fronds at high arsenate doses. Another possible explanation for this behavior can be derived from the competition between arsenate and phosphate. In this sense, the P transport along the pinnule is impaired, thus dropping its concentration in the apical region.

Conclusions and outlook

This is the first study to propose a validated analytical method using μ-EDXRF for quantitative determination and mapping As and P simultaneously in *Pityrogramma calomelanos,* a well-recognized As-hyperaccumulator fern.

The analysis of pelletized powdered fern samples was also addressed as a powerful tool for plant nutrition diagnosis, as this information proved to be useful for explaining possible induced phosphorus deficiency in As-accumulating plants. The results obtained here demonstrate the usefulness of this method to detect bioaccumulation of As in plants, especially when cultivated in As-enriched soils or irrigated with contaminated water. In addition, in view of the presented results, further systematic studies on physiological mechanisms involved in As species and P

distribution in pinna tissues of *Pityrogramma calomelanos* L. (Link) under As exposure are encouraged.

In short, the proposed strategy provides important information regarding environmental monitoring and phytoremediation studies to be obtained in a fast and non-destructive way.

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Tables

Table 1. Microwave heating program for plant acid digestions

Step	Time (min)	Target temperature (°C)	Condition
1	3	160	Ramping
2	2	-	No heating
3	5	200	Ramping
4	15	200	Holding

Table 2. μ-EDXRF operational conditions

Instrumental parameter	Operating condition
Irradiated diameter	50 μm
Measurement time for pellet interrogation	300 s
Analyzed spectra region	1– 40 keV
Measuring atmosphere	Atmospheric air
Monitored peaks	P Kα 2.01 keV, As Kα 10.54 keV and Rh Kα Compton 18.96 keV
X-ray tube voltage	50 kV
X-ray tube current for pellet interrogation	100 μA
Maximum sample size	200 (W) x 300 (D) x 40 (H) mm
Maximum stroke	X-Y - 100 mm x 100 mm Z - 40 mm

Captions for figures

Figure 1. Morphological characteristics of *P. calomelanos* emphasizing the pinna regions used for arsenic and phosphorus microchemical mapping by μ-EDXRF. (a) Sporophyte. (b) Pinna. (c) Basal portion of the pinnule and (d) Apical portion of the pinnule.

Figure 2. Fragment of typical μ-EDXRF spectrum related to a pressed pellet of pinna of *P. calomelanos* with 1600 mg kg^{-1} As and 11 g kg^{-1} P. Experimental conditions: 50 μm spot size and 300 s measurement time.

Figure 3. Linear regression models adjusted to correlate As (a-d) and P (e-h) mass fractions determined by ICP OES and μ-EDXRF intensity (cps μA^{-1}). Data from all samples and separately for each fern part (pinna, stipe and root).

Figure 4. μ-EDXRF microchemical maps for As and P of the basal (a) and apical (b) portions of the pinnule of *Pityrogramma calomelanos* from control ferns and ferns exposed to 30 x 10^{-3} mol L^{-1} As. For As maps the Rh Kα Compton peak was used as internal standard. Legend: pm = pinna midrib; sv = secondary vein. Arrow = tertiary ribs. Bars length = 1.0 mm. Experimental conditions: 50 μm spot size and 120 min. irradiation time.

Figure 5. μ-EDXRF microchemical maps for As and P of whole pinnules of *Pityrogramma calomelanos* ferns exposed to 30 x 10^{-3} mol L^{-1} As. For As maps the Rh

Kα Compton peak was used as internal standard. Bars length = 2.0 mm. Experimental conditions: 50 μm spot size and 750 min. irradiation time.

Figure 1

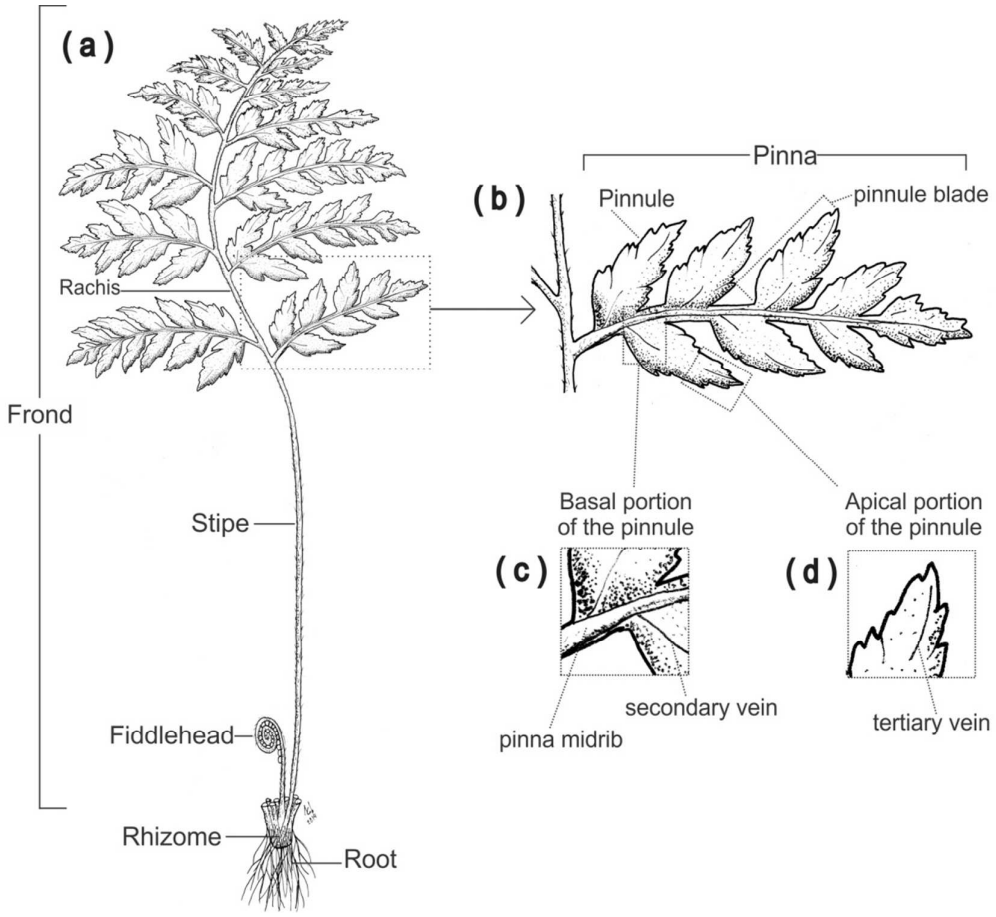


Figure 2

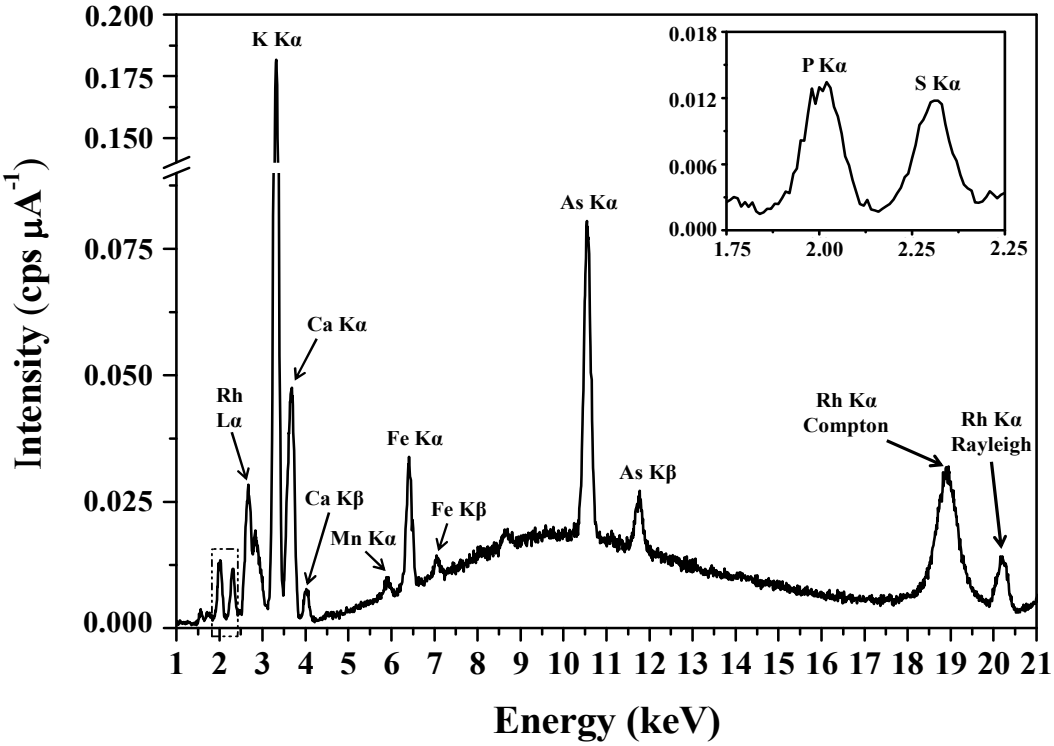
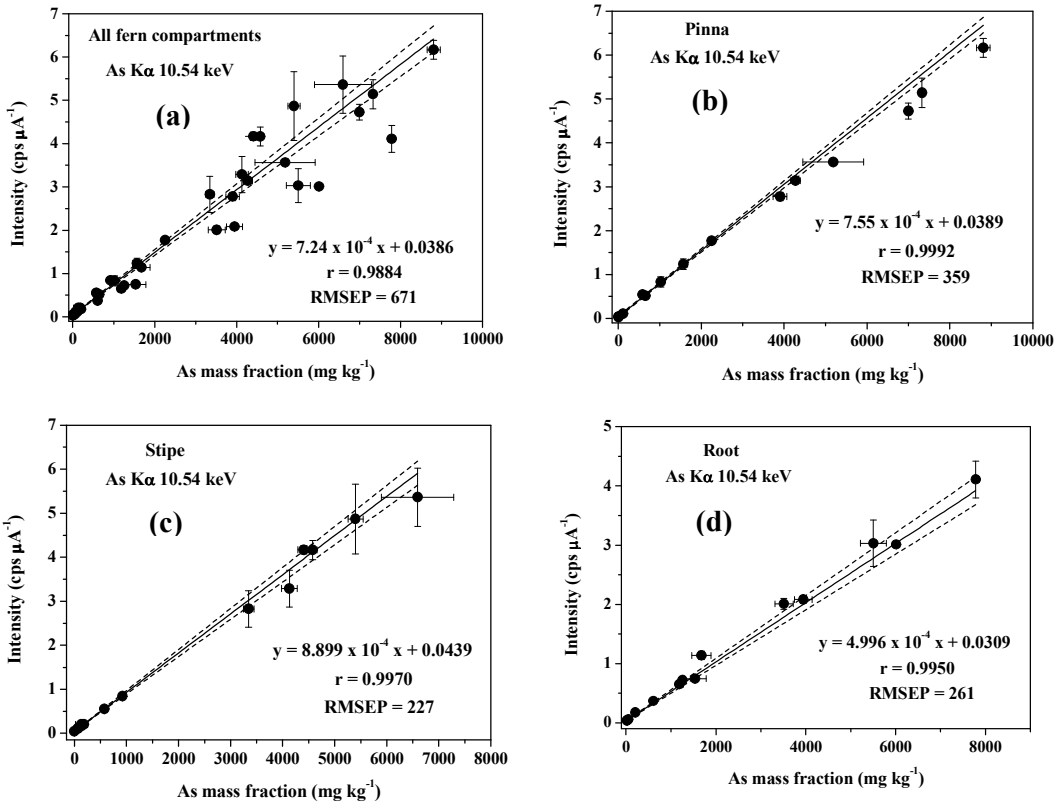


Figure 3



Continuation... Figure 3

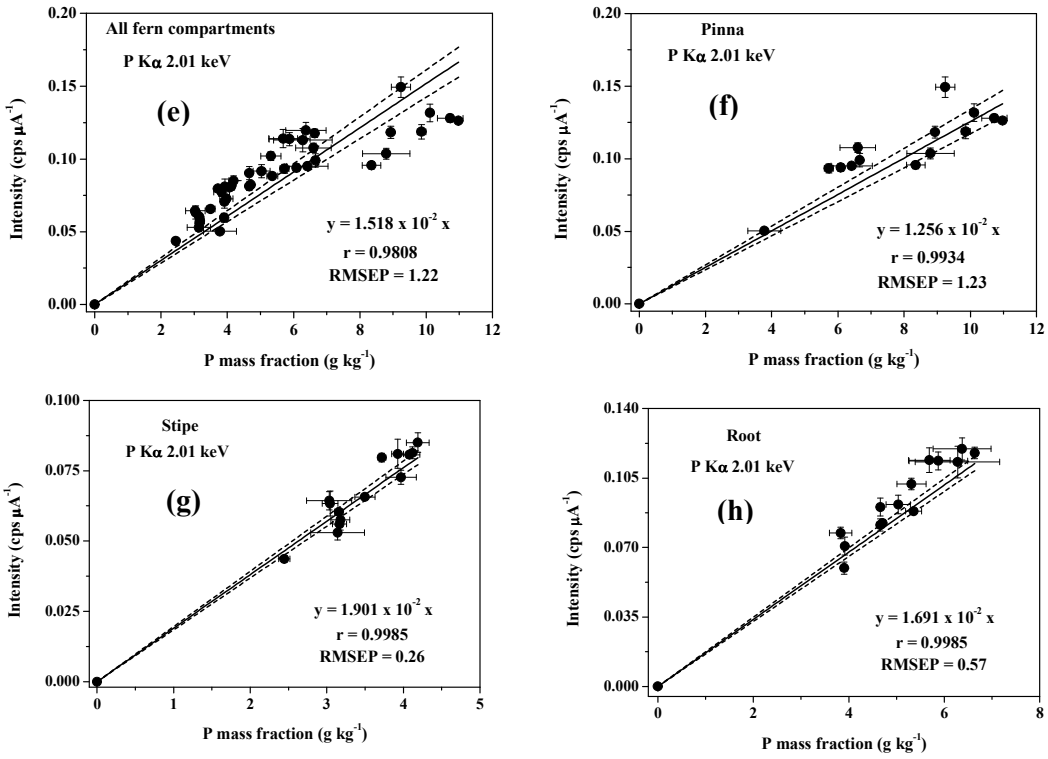


Figure 4

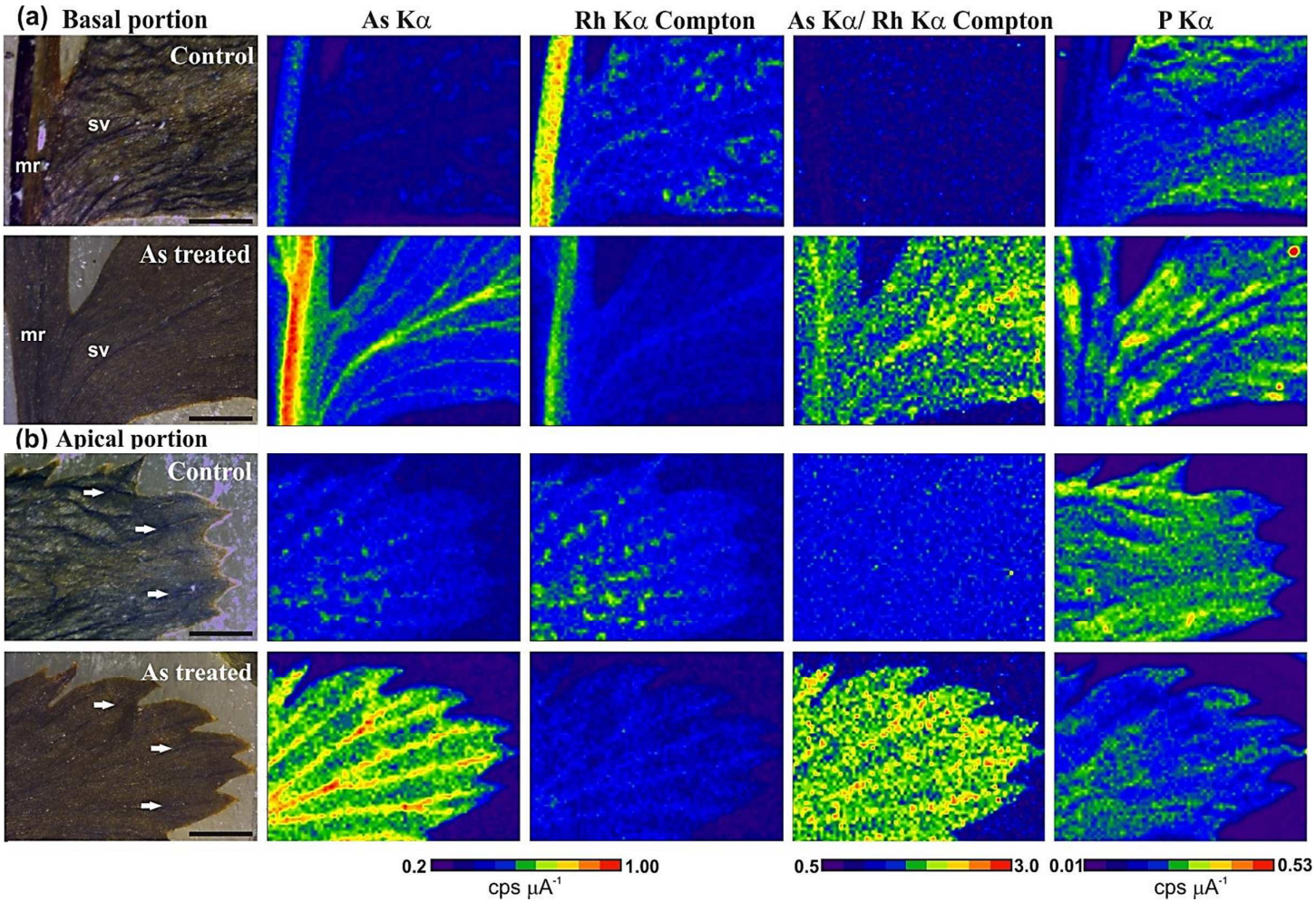
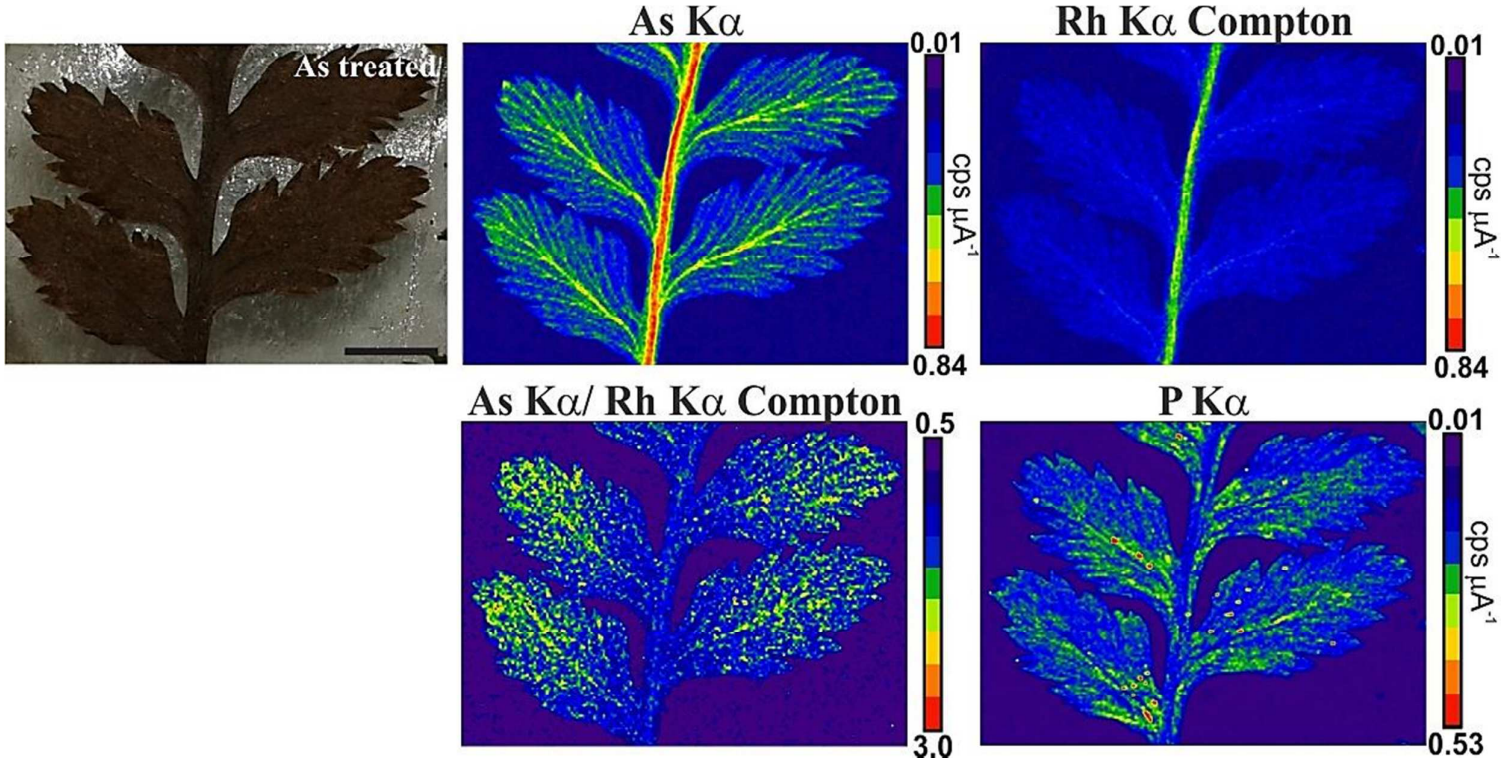
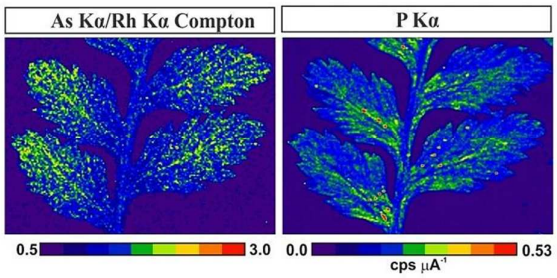


Figure 5





μ-EDXRF is a strong analytical tool enabling the simultaneous mapping of As and P in the As-hyperaccumulator fern *Pityrogramma calomelanos.*