Arsenic in *Pteris vittata* is localized to the cell wall in a water-soluble state

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Abstract

Pteris vittata L., a hyperaccumulator of As, has great potential in environmental remediation. Localization analysis of As and Ca, K, S, and P in arsenic-exposed and unexposed *P. vittata* pinnae was performed using an electron probe micro analyzer. A comparison of section preparation methods revealed that the As in *P. vittata* is water-soluble and not tightly bound to the tissue. Further elemental mapping revealed that the localization of S was not consistent with that of As. Therefore, the formation of a complex of low-molecular-weight thiols and As, which is a common detoxification mechanism in plants, may be a limited in *P. vittata*. It was confirmed that As was localized around the cell wall when the cell was in a healthy state, and that the distribution of As expanded to the whole cell when the cell was damaged. These results suggest that *P. vittata* may retain As extracellularly to inhibit damage to healthy cells. However, in the periphery of pinnae, which is more susceptible to damage, As was distributed throughout the cell, eventually leading to browning.

Introduction

There have been reports of soil and groundwater contamination by naturally occurring arsenic (As) in many countries worldwide. In Southeast Asian countries, such as Vietnam, Myanmar, and Nepal, tens of millions of people are feared to have health problems due to As contamination in groundwater[1,2]. Existing environmental remediation methods can reliably remove pollution, but they are expensive and can only be applied to a limited number of areas. In addition, it is imperative that the methods are sustainable, as environmental pollution of natural origin requires continuous measures. Therefore, there is a need for low-cost and sustainable measures to reduce the environmental pollution caused by As over a wide area. In recent years, phytoremediation, a method of environmental purification using plants that accumulate high concentrations of certain heavy metals, has attracted much attention. Phytoremediation is characterized by its low cost and environmental impact compared with existing physical environmental remediation methods and its applicability to a wide range of contaminants at low concentrations[3]. To date, over 700 species of plants have been found to accumulate high concentrations of certain elements, including those that are harmful to living organisms, such as As[4]. A hyperaccumulator of As, *Pteris vittata* L., was discovered by Ma et al. in 2001 and was reported to accumulate more than 20,000 ppm of As in the aboveground part of the plant[5]. In general, plants either do not absorb harmful elements at all, or absorb only a small amount depending on the concentration in the environment. Therefore, studies on the mechanism of super-accumulation of specific elements, especially harmful elements such as As, are underway. It is known that *P. vittata* primarily absorbs arsenate from the environment and reduces it to arsenite, which accumulates in the plant body[6,7]. As arsenite is known to be more toxic than arsenate, various mechanisms for the detoxification of As have been proposed. Additionally, attempts have been made to elucidate the point where it accumulates in the body at high concentrations after being reduced to toxic As. Detoxification by sequestration of toxic substances into vacuoles or formation of complexes with specific substances is considered a general cellular detoxification mechanism[8,9]. In particular, detoxification by the formation of low-molecular-weight thiols that specifically bind to As has been

explained as a detoxification mechanism common to other toxic elements as a reason for the accumulation of As in *P. vittata* after its reduction to arsenite[10]. In particular, the mechanism by which phytochelatin, a compound containing multiple thiols, is coordinated with a single As molecule to form a complex is well known[11]. However, since the amount of low-molecular-weight thiols is limited relative to the amount of As that can be accumulated by *P. vittata*, the above-mentioned detoxification mechanism alone cannot explain the As hyper-accumulation capacity of *P. vittata*.

In recent years, studies have been conducted on the localized sites of As in ferns, and micro X-ray fluorescence (μ-XRF) analysis has been attempted as a method of direct elemental mapping with high resolution and magnification. Hokura et al. studied the localization of As, K, and Ca elements on the surface of small plumage fragments of *P. vittata* by μ-XRF and reported different distribution conditions depending on the growth stage[12]. Furthermore, they also reported a lack of As accumulation in sporocytes, revealing that As does not accumulate evenly throughout the plant tissue. In addition, Datta et al. reported that As accumulated in *P. vittata* may be localized to the extracellular cell wall, rather than intracellularly[13]. However, there is a concern that the condition of the sample may change during the analysis because the sample is irradiated with a powerful beam in μ-XRF. There is a need to develop a method that enables tissue- and cell-level analyses while maintaining the state of biological samples. In addition to μ-XRF, an electron probe micro analyzer (EPMA) can be used to map the elements in a sample. An EPMA is a device that collects characteristic X-rays generated by irradiating a sample with electrons and obtains information on the distribution of elements in the sample. The irradiation transitions the orbital electrons of the material to produce characteristic X-rays, but the electronic state of the material instantly returns to the ground state, making it difficult to change the properties of the sample. Examples of applications to biological samples include mapping of metal elements in biological tissues of dust lung and liver cirrhosis. Since the resolution is about 0.5 μm, it is also possible to examine the localization at the cellular level as well as at the tissue level. In this study, we used an EPMA to perform mapping at the tissue and cell levels to (1) compare changes in the localization of As and other abundant elements in As-exposed and unexposed *P. vittata* pinnae and (2) compare the localization of each element near the leaf vein and at the edge in As-exposed *P. vittata* pinnae. We aimed to elucidate why the As hyperaccumulator, *P. vittata*, can accumulate As at high concentrations, especially by comparing its localization with that of sulfur in low-molecular-weight thiols.

Results And Discussion

Analysis of As and essential elements by ICP-AES

The concentrations of As, Ca, K, Mg, P, and S are shown in Fig. 1, and the concentrations of trace elements B, Cu, Fe, Mn, Mo, and Zn are shown in Fig. 2. The As-unexposed *P. vittata* accumulated 1.02 ± 0.56 mg As/kg. The concentrations of essential elements in the As-unexposed *P. vittate* were as follows: Ca, 7062 ± 1159 mg/kg; K, 8080 ± 721 mg/kg; Mg, 1964 ± 387 mg/kg; P, 7837 ± 2103 mg/kg; S, 7142 ± 2552 mg/kg; B, 19.0 ± 5.2 mg/kg; Cu, 5.17 ± 0.63 mg/kg; Fe, 68.2 ± 14.7 mg/kg; Mn, 28.0 ± 6.4 mg/kg; Mo,

1.30 ± 0.16 mg/kg; and Zn, 30.2 ± 7.04 mg/kg, which is consistent with the values of previous studies[15]. Conversely, the concentration of As in the As-exposed *P. vittata* was much higher, at 4563 ± 257 mg/kg. There was more browning of the outer periphery of pinnae under high As concentrations compared with the normal condition. The essential element concentrations in the As-exposed *P. vittata* were as follows: Ca, 6283 ± 208 mg/kg; K, 8779 ± 601 mg/kg; Mg, 2111 ± 286 mg/kg; P, 18591 ± 4582 mg/kg; S, 9962 ± 1964 mg/kg; B, 13.6 ± 4.8 mg/kg; Cu, 5.89 ± 1.09 mg/kg; Fe, 81.2 ± 20.5 mg/kg; Mn, 28.2 ± 6.7 mg/kg; Mo, 1.34 ± 0.18 mg/kg; and Zn, 37.8 ± 4.22 mg/kg. These concentrations were almost identical to those in the As-unexposed *P. vittata*. However, K, P, S, Fe, and Zn concentrations were higher in As-exposed *P. vittata* than unexposed *P. vittata*, whereas concentrations of Ca and B were lower. Similar increases and decreases in essential element concentrations with the accumulation of As have been reported in previous studies[15]. Yamazaki et al. reported a negative correlation between As and Ca concentrations in the pinnae of *P. vittata*, and proton-induced X-ray emission (PIXE) analysis showed that Ca concentrations decreased as As concentrations on the surface of pinnae increased[16]. In addition, Sugawara et al. reported a negative correlation of As with B, K, and P concentrations in pinnae when As exposure was increased in a stepwise manner[15]. In general, K is an indicator of plant activity, and it is well known that growth enhancement occurs in the presence of trace amounts of As in *P. vittata*. Therefore, the concentration of P in plants tended to increase due to the growth promotion associated with exposure to As. Moreover, S is a constituent element of low-molecular-weight thiols related to the detoxification of toxic substances in plants, which could explain the increase in S with increasing As. Based on these results, we decided to map four essential elements, P, S, Ca, and K, which were correlated with As and can be measured simultaneously with As in EPMA analysis.

Localized mapping of As, P, S, Ca, and K in the pinnae of *P. vittata* by EPMA

Frozen thin-layer sections of As-exposed and unexposed *P. vittata* pinnae analyzed by ICP-AES, as described above, were prepared using paraffin embedding and cryomicrotome, and elemental mapping was performed by EPMA. Figure 3 shows the results of the elemental mapping of pinnae of *P. vittata* prepared using the paraffin-embedding method, where the signals of elements such as Ca in the cell tissue were confirmed, but the signals of As and K were very weak or almost absent. It is generally believed that K is present in plant cells in a water-soluble ionic state at a concentration of 100 mM or more[17]; therefore, it is likely that K was eluted from the tissue during the sectioning process. These results suggest that As is not only present as arsenite but also accumulates in a water-soluble state without strong tissue binding. Figures 4 and 5 show the results of elemental mapping of As-exposed and unexposed pinnae of *P. vittata*, respectively. Analysis of the entire cross-section of the pinnae confirmed that even water-soluble elements were retained in the sample, unlike the sections prepared using the paraffin-embedding method, although frost was observed around the sample. The As signal was confirmed only in the As-exposed pinnae and not at all in the unexposed pinnae.

The behavior of As was compared with that of the four other essential elements measured. The S and P signals were strong in places where the As signal was also strong, indicating similar localization.

However, no clear change in the behavior of Ca and K with the accumulation of As was observed. The results of Nano-SIMS and μ-XRF analyses of As-accumulated rice by Moore *et al*. showed that the localization sites for As and S were consistent and the signal intensity was positively correlated, whereas the localization sites for As and P were not consistent[18]. Thus, although the localization of S was consistent with the general mechanism of plants involved in the detoxification of As, the behavior of P may be unique to the fern. The cross-sectional analysis showed a tissue-level elemental mapping result consistent with the ICP-AES results as a trend of the entire pinnae. Therefore, in order to further analyze a narrower area, we decided to analyze two parts of the cross-section of the pinnae, the central part near the leaf vein and the periphery of the pinnae (Fig. 6). The accumulation mechanism of As is expected to be different in these two sites because the area around the leaf vein tends to be healthy while the area around the petiole tends to brown and die when exposed to As. Elemental mapping of As, Ca, K, S, and P in a narrow area near the pinnae vein of the As-exposed fern is shown in Fig. 7. It was confirmed that As was localized to the cell wall and not inside the cell. In addition, S was localized in the area where the As signal was relatively weak. However, the localization of P was completely different from that of As. The localization of K was relatively consistent with that of As, and Ca was localized near the epidermis of the pinnae. S and P were also present in the cells.

Elemental mapping of As, Ca, K, S, and P in a narrow area around the periphery of the pinnae of the As-exposed fern is shown in Fig. 8. The signal of As tended to be stronger in the vicinity of the leaf veins, consistent with the presence of As at the base and at the periphery of the leaf, as reported in previous studies. Consistent with the results of the analysis near the foliar veins, As was not localized near the epidermal tissue in the periphery, but in the fence-like or spongiform tissues. More interestingly, while As was localized near the cell wall in the vicinity of the veins, As was not clearly extracellularly localized in the periphery of the pinnae, but was distributed throughout the cell. The localizations of As, K, and S are partially consistent with each other, and the K signal was particularly strong. In addition, Ca was distributed throughout the apical region, which was very different from the distribution of As in the vicinity of the veins. The localization of P and As was quite different from the results of the analysis near the foliar veins, especially P, which did not localize near the epidermal tissue of the pinnae.

Comparison of the elemental mapping results in the narrow area near the leaf vein and near the periphery of the pinnae of *P. vittata* confirmed some major differences. First, we found that As was localized near the cell wall in the foliar vein, while it was present in the entire cell tissue in the periphery. As reported in previous studies, pinnae tend to die off from the periphery when As concentrations are high in *P. vittata*[15]. Therefore, it was expected that As localization around the veins, which are relatively healthy, would be different from that around the periphery. Although the periphery of the pinna of *P. vittata* was not dead, the presence of As in the cells even in this state suggested that death from the periphery occurs when As is not isolated from the cells for a long period of time. As and K tended to localize similarly in both the vicinity of the vein and the periphery, but the behavior of S, which tended to coincide with As, was different between the vicinity of the vein and the periphery. In the analysis near the fovea, where the cell condition is considered to be relatively healthy, S tends to be negatively correlated with the strength of the

signal itself, although S is consistent with the localization of As. As mentioned above, small molecule thiols containing S as a constituent element are known to reduce toxicity by forming specific complexes with arsenite, which is the main chemical form of As present in *P. vittata*[19]. Therefore, although the As and S signals were expected to be positively correlated, they were in fact different. This is because, as mentioned earlier, As and S can be positively correlated in terms of both localization and abundance if only small thiols are used to detoxify As. However, these results suggest that *P. vittata* also has other mechanisms of detoxification. Alternatively, although the localization of As and S was consistent in the periphery, the overall low signal of S may be due to the high level of As present in the periphery. Since this trend has been observed in the vicinity of the leaf veins, the behavior of S in relation to As is, to some extent, consistent in pinnae. For Ca, the localized sites of As were completely different from those of As in the vicinity of the foliar veins and were observed only in the vicinity of epidermal cells, whereas the localized sites of As tended to coincide to some extent with those of As in the periphery. Ca plays a role in the pectin end of the cell wall in plants and is known as a constituent element of the cell wall[17]. Therefore, it is likely that Ca was localized near epidermal cells, which seemed to have a relatively strong structure. Conversely, at the periphery, Ca was relatively co-localized with As, K, and P. Considering that K is mainly present in the intracellular protoplasm and P is mainly present in nucleic acids and other intracellular substances, the structure of the cell itself is not maintained at the periphery of the cell, suggesting that the localization sites of elements with different properties are consistent. Therefore, it is thought that this situation is maintained for a long period of time, and eventually death from the periphery occurs.

Methods

P. vittata plants purchased from FUJITA Co. Tokyo, Japan. An experiment was carried out with *P. vittata* plants, which were approximately 20 cm long in plumage and had been grown for 3 months under hydroponic conditions. *Pteris vittata* plants was transplanted into 1/10 Hoagland culture medium containing 10 mg/L of arsenate ($Na_2HAsO_4 \cdot 7H_2O$, Wako Chemical) and further grown under hydroponic conditions for 2 weeks in a growth chamber (25°C, 3500 lux, 16 h light period). The culture medium was renewed once every 3 d, and the As concentration was set to 10 mg/L each time. Some of the pinnae of *P. vittata* after the end of cultivation were dewatered in 10% formalin, and thin layered sections were prepared using the paraffin embedding method. In addition, cryo-microtomes were used to generate frozen thin-layer sections. At the same time, paraffin-embedded and frozen pinnae sections of *P. vittata* plants grown under the same conditions in 1/10 Hoagland culture medium only without the addition of arsenate were also prepared. To further confirm the concentration of As-containing elements in pinnae, five pinnae from As-exposed and unexposed *P. vittata* plants were collected from the same frond. Pinnae were dried at 60°C for 2 d and digested using the wet-ashing method with HNO_3 at 120°C for 90 min. The concentrations of As and essential elements were determined using inductively coupled plasma atomic emission spectroscopy (ICP-AES) with the test solution, which was filled up to 10 mL with Milli-Q water and filtered through a 0.45 μm membrane filter[14].

Pinnae were taken from the fronds analyzed by ICP-AES, and thin sections were prepared using the paraffin-embedding and freezing methods. In the paraffin-embedding method, plant samples were treated with 10% formaldehyde solution for 24 h, dehydrated, and embedded in paraffin to make thin sections using a microtome. For the freezing method, fresh plant samples were sliced using a cryomicrotome and thin sections were prepared. Paraffin-embedded and frozen thin-layer sections of *P. vittata* were analyzed using an EPMA (EPMA-8050G, Shimadzu Co., Ltd.). The thin sections were pretreated with carbon deposition (approximately 5 nm thick) before analysis, and the localization of As and other elements was confirmed by mapping. The acceleration voltage of the EPMA was set to 15 kV and the irradiation current was 50 nA. For elemental mapping of the entire cross-section of the pinnae the measurement pitch was set to 20 μm and the integration time was set to 120 ms/point. For elemental mapping in a narrow area, the measurement pitch was set to 1 μm and the integration time was set at 60 ms/point. This ensured the detection sensitivity of elements and prevented a temperature rise caused by electron irradiation. All experiments were performed in accordance with relevant guidelines and regulations.

Data availability statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

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Additional Information

Author contributions

K.S: Conceptualization, Methodology, Validation, Investigation, Writing – Original Draft, Visualization, Project administration, Funding acquisition. **H.H:** Investigation, Data Curation, Writing – Review & Editing. **C.I:** Resources, Supervision, Funding acquisition.

Competing Interests

The authors declare no competing interests.

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Figures

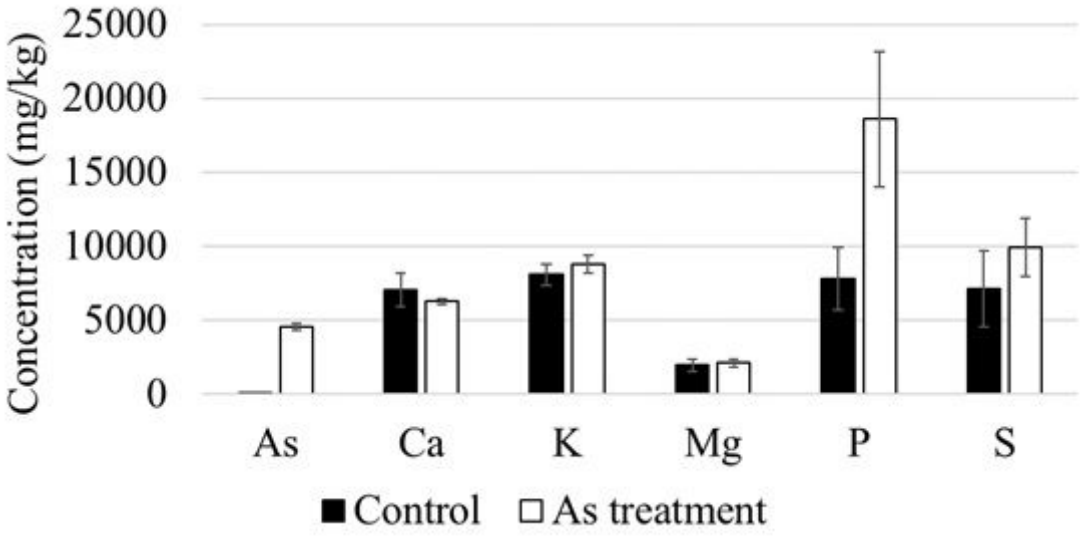

Figure 1

Major nutrients in pinnae of As-exposed (black bar) and control (white bar) Pteris vittata. Error bars represent standard deviations

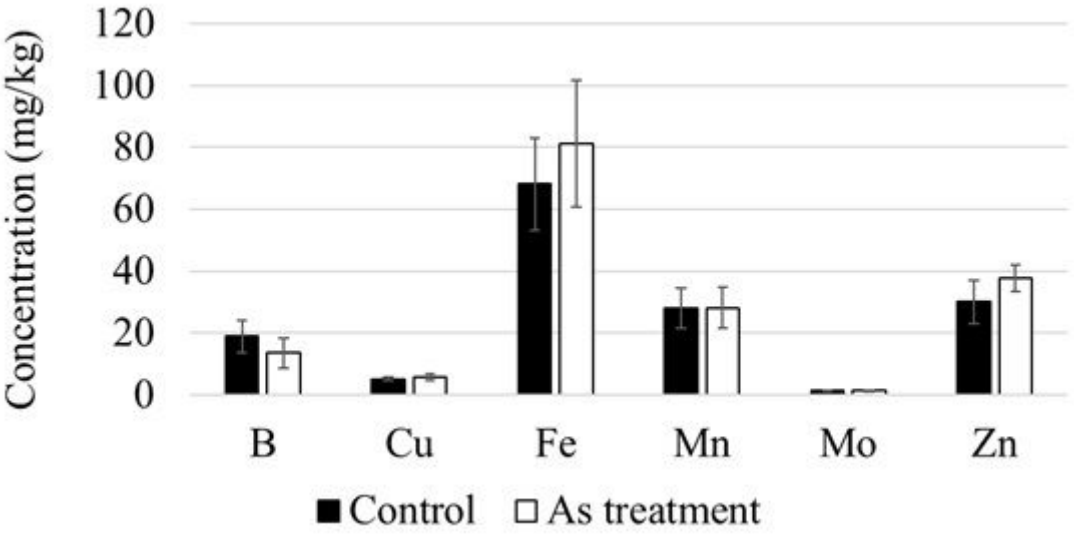

Figure 2

Minor nutrients in pinnae of As-exposed (black bar) and control (white bar) Pteris vittata. Error bars represent standard deviations.

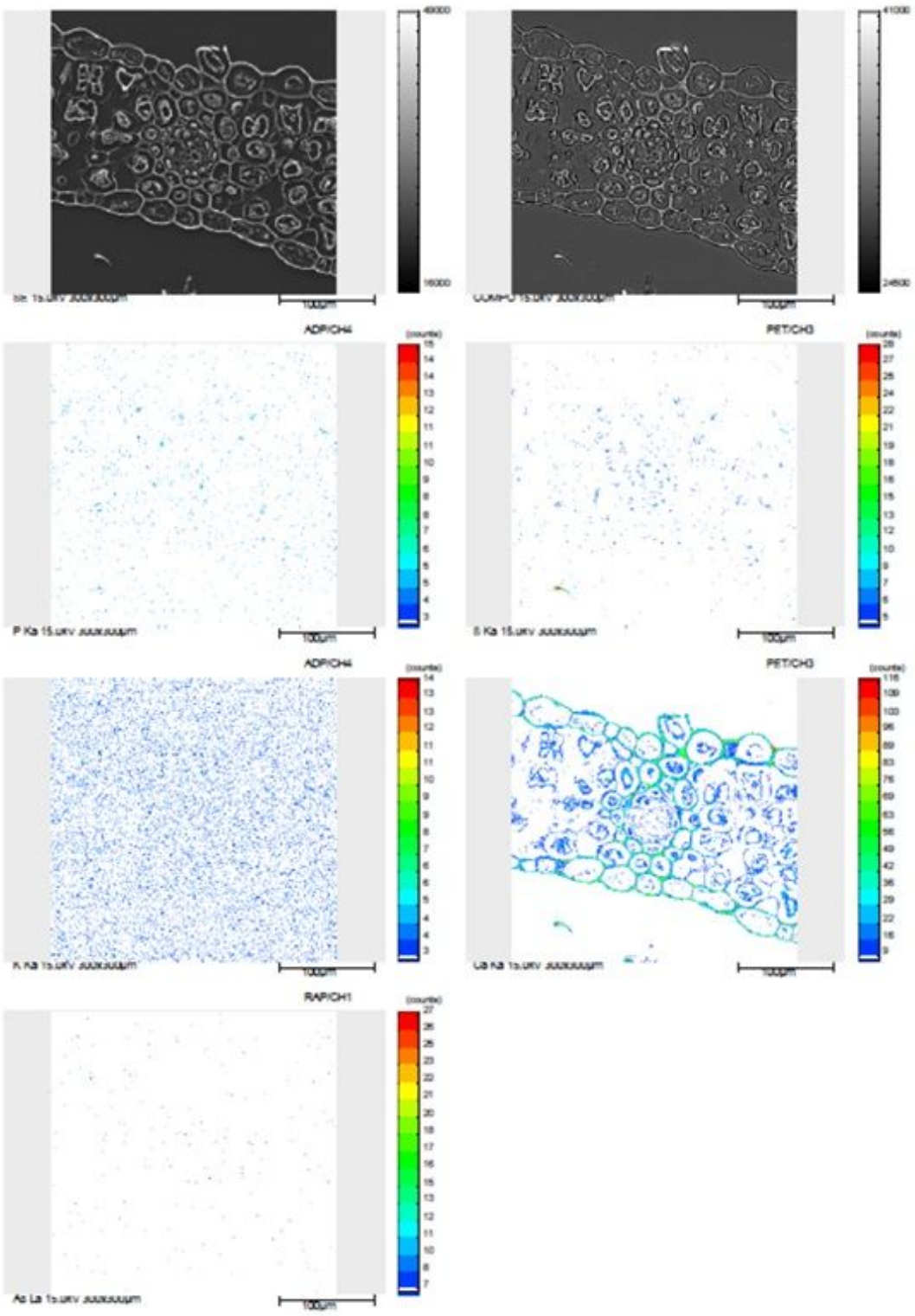

Figure 3

Element mapping of paraffin-embedded pinnae sections of As-exposed Pteris vittata using an electron probe micro analyzer

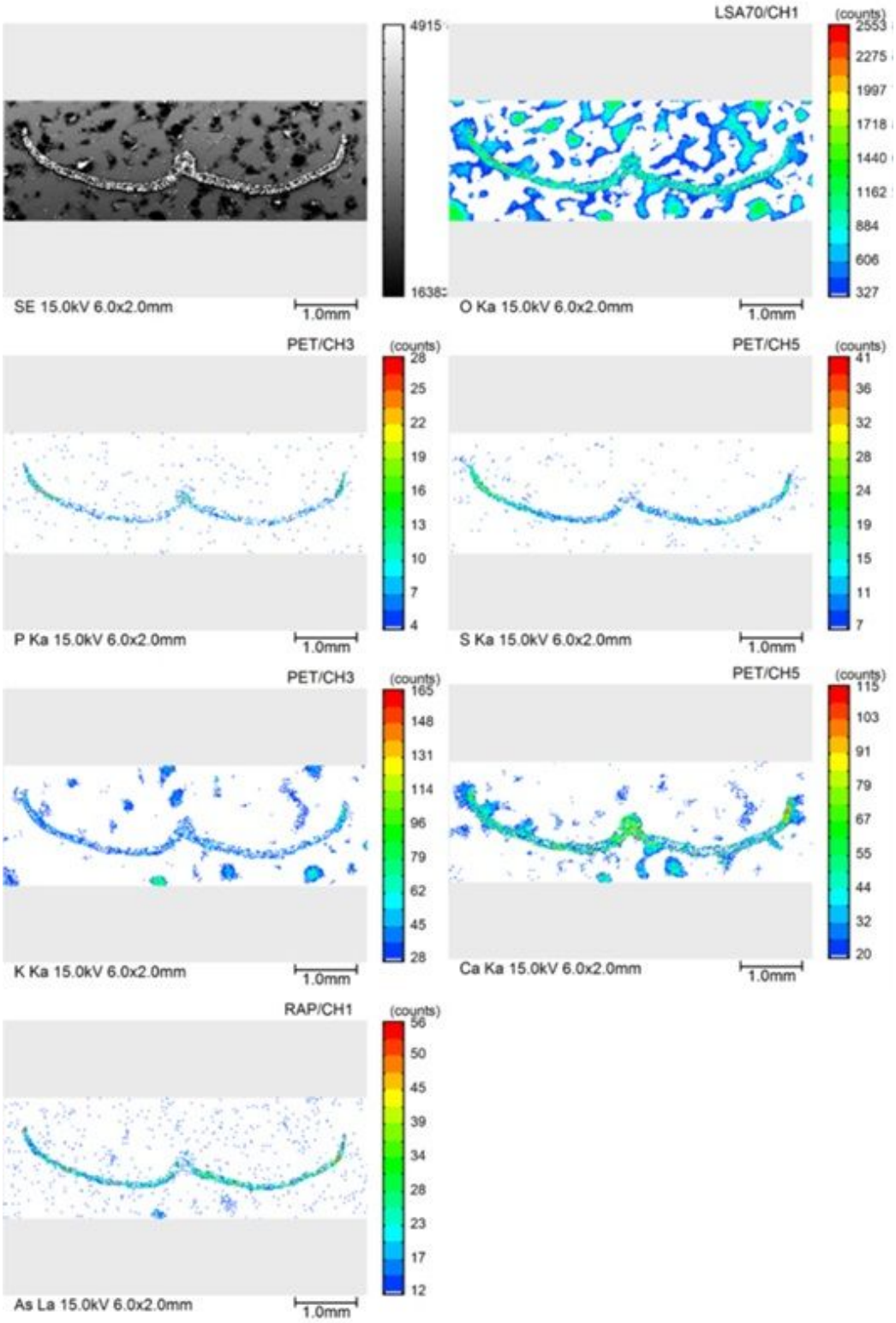

Figure 4

Element mapping of frozen thin-layer pinnae sections of As-exposed Pteris vittata using an electron probe micro analyzer.

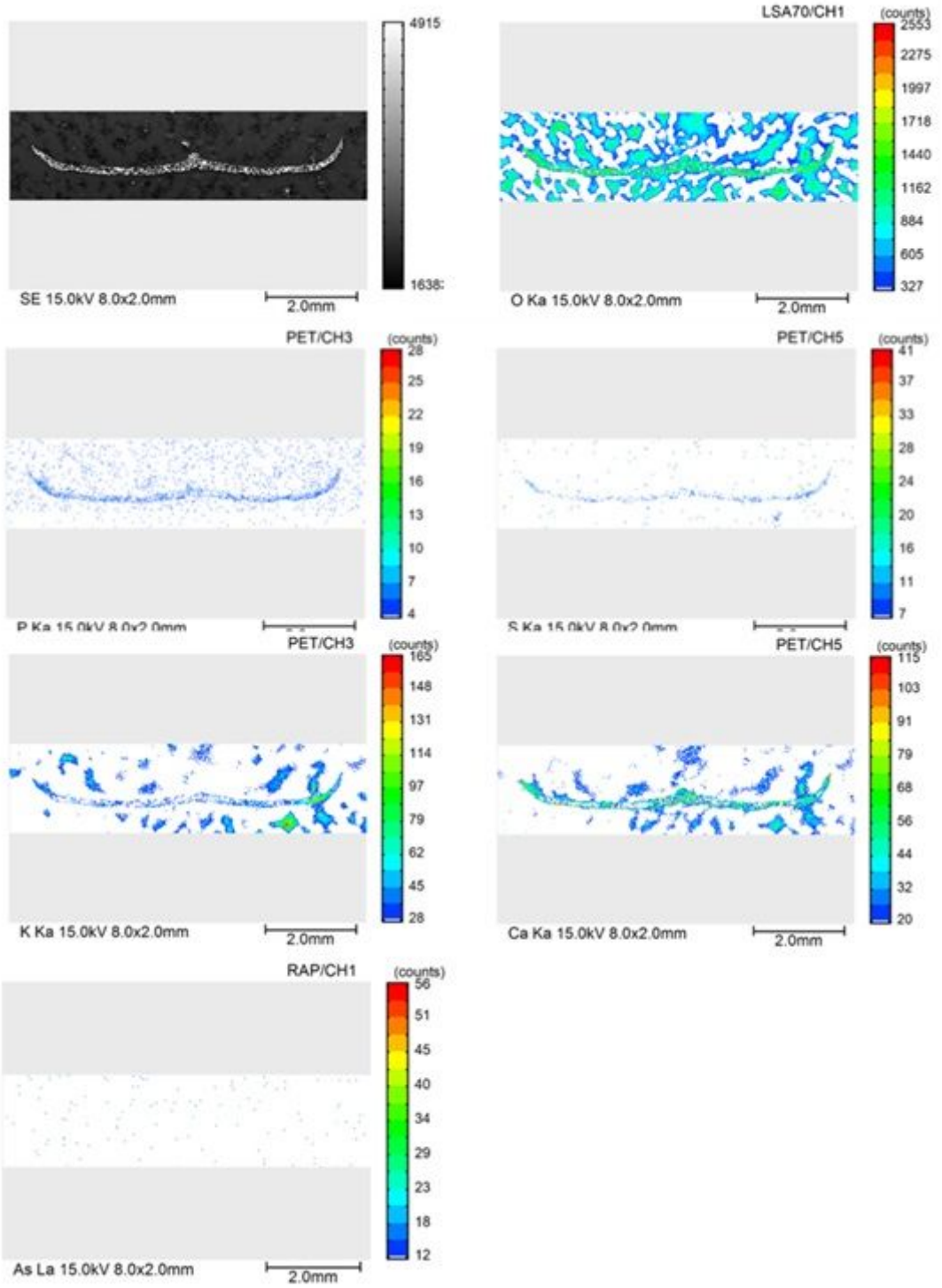

Figure 5

Element mapping of frozen thin-layer pinnae sections of Pteris vittata using an electron probe micro analyzer.

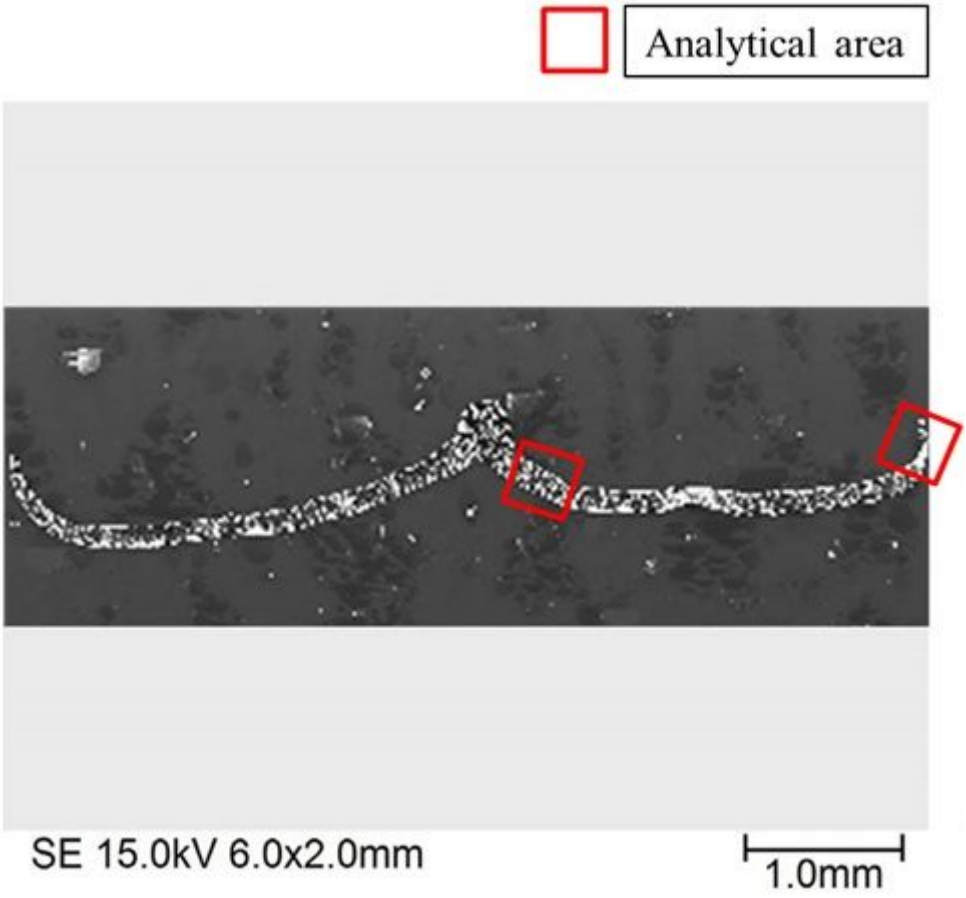

Figure 6

Magnified analytical area of pinnae of As-exposed Pteris vittata.

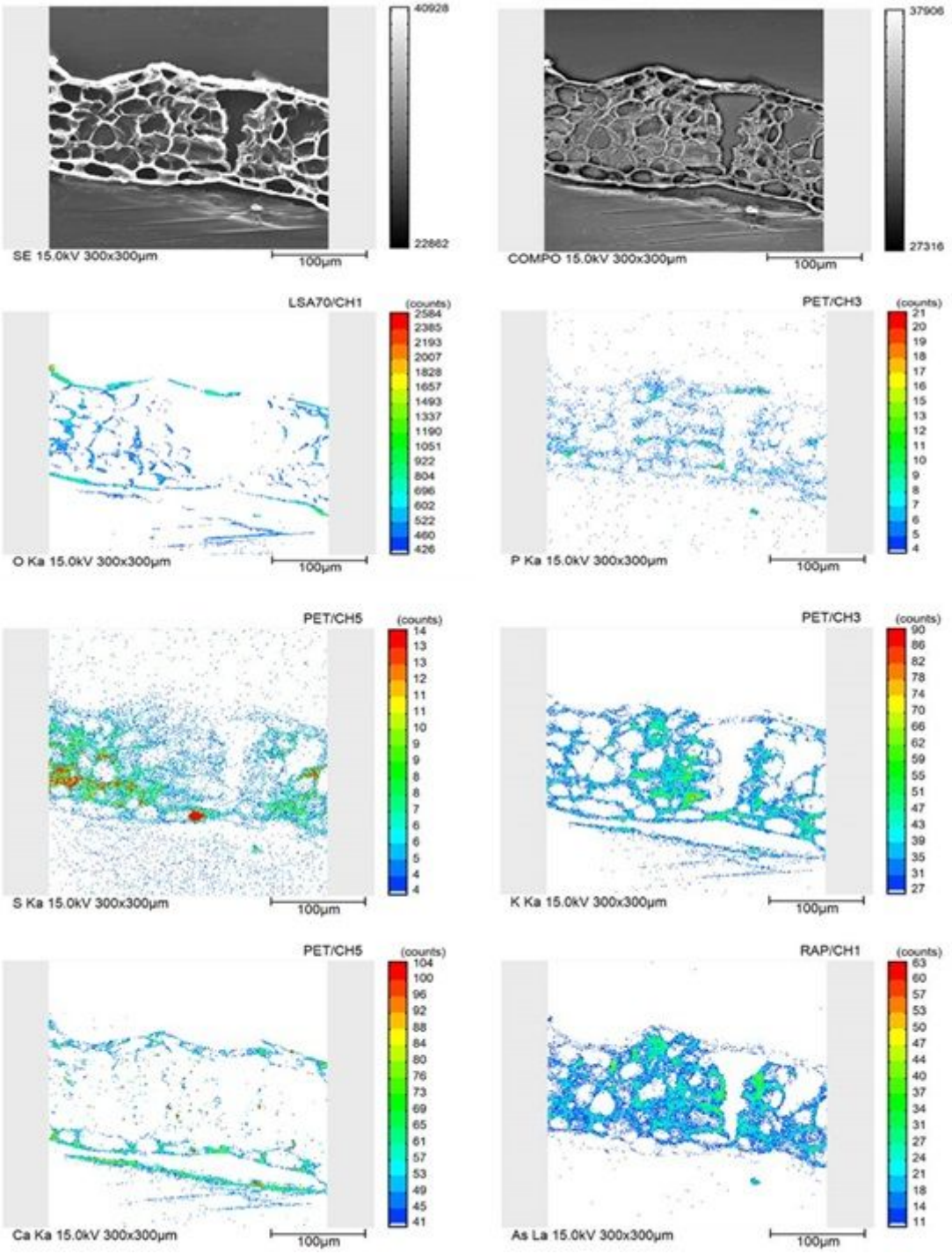

Figure 7

Element mapping of nearby midrib area of frozen thin-layer pinnae sections of As-exposed Pteris vittata using an electron probe micro analyzer.

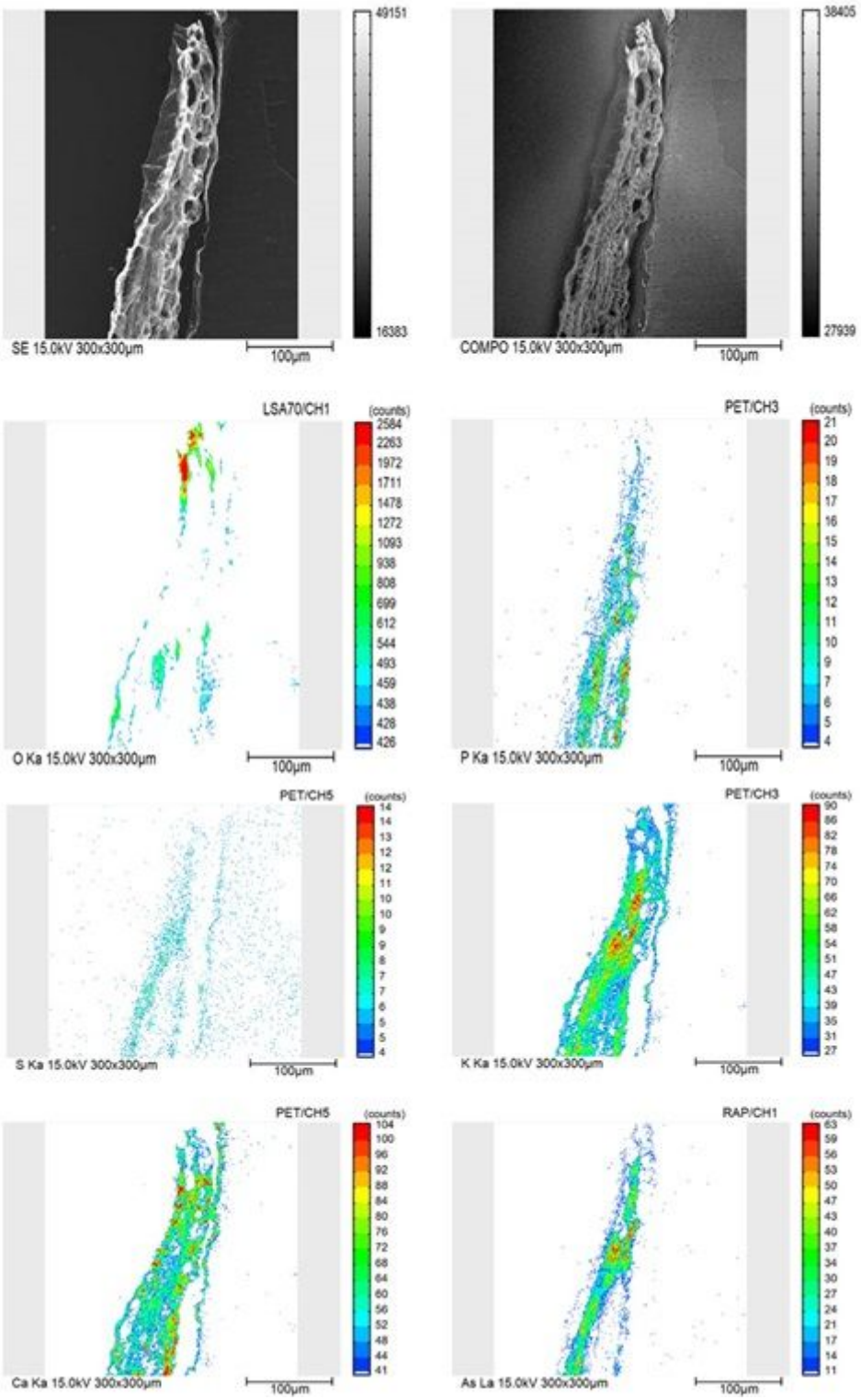

Figure 8

Element mapping of nearby outer edge area of frozen thin-layer pinnae sections of As-exposed Pteris vittata using an electron probe micro analyzer.