



Article Application of X-ray Spectroscopic Techniques to Determine the Inorganic Composition and Sulfur Chemical Speciation of the Amazonian Plant *Bixa orellana*

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Abstract: *Bixa orellana* is a plant that has a variety of uses, such as applications in the food and cosmetic industries, as well as culinary uses, and body painting for Indigenous people. Despite its versatility, few studies have explored its inorganic composition, and its sulfur chemical speciation has only been assessed from the point of view of sulfurous amino acids. Here, we report on the inorganic composition of *Bixa orellana* fruits, pericarps, and seeds obtained using Wavelength Dispersive X-ray Fluorescence (WD-XRF) and sulfur chemical speciation using X-ray Absorption Near-Edge Structure (XANES). Our results show that the seed is a source of potassium, calcium, magnesium, and phosphorous. But also, the pericarp, which is considered waste, contains a high amount of nutrients. From the XANES measurements, the distribution of the oxidation state of the sulfur atom was obtained, and it was shown that although several oxidation states of sulfur are present, oxidized sulfur (sulfate) is the dominant form of sulfur in all samples.

Keywords: Bixa orellana; WD-XRF; XANES; sulfur chemical speciation

1. Introduction

Bixa orellana (*Bixa orellana* L.) is a plant from the Bixaceae family, whose trees are commonly known as *urucum* or *colorau* [1]. Its fruits are red- or yellow-colored, and the seeds are covered by an arillus rich in carotenoids, such as bixin, used for the production of natural colorings [2] and as body paints by Indigenous people [3]. It has also been used in sacred rituals since ancient times by the First Nations of America [4]. Natural colorings such as bixin are gaining increasing importance [5] due to concerns surrounding the use of some synthetic colorings in food, which are associated with human health risks [6]. The seeds of *Bixa orellana* are also used for culinary purposes by the Amazonian people, in the form of a powder spice known as *colorifico* or *colorau* [3], which is made by mixing powdered seeds (or its extract) with corn flour, vegetable oil, and salt [7]. The seed without the arillus also has nutritional value, with high amounts of phosphorous, proteins, and amino acids, such as tryptophan and lysine [7,8]. This plant also has many applications in natural medicine. Nonetheless, there is a shortage of studies related to its medicinal properties, as pointed out in [7]. There is a wide variability in this plant, which propagates through its seeds. Although it is presumed to have a Native American tropical origin [3],



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Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). due to its many uses, it is currently cultivated in Mexico and South America as well as in tropical and subtropical regions spanning Africa and Asia [9].

Despite its widespread use, few studies have reported the inorganic composition of the macro- and micronutrients in *Bixa orellana* fruits and seeds [8,10,11]. Notably, the chemical speciation of sulfur (S) remains unexplored. Sulfur plays a critical role in amino acid biosynthesis, coenzyme formation, seed quality maintenance, and germination regulation [12]. Due to its widespread use in the food industry and cosmetics, there is a serious need for fast and reliable techniques to determine its inorganic composition. This is an important requirement for the food industry, in order to determine its nutritional value, and for the cosmetic industry. In the present study, the inorganic composition of *Bixa orellana* was determined using Wavelength Dispersive X-ray Fluorescence spectrometry. Sulfur chemical speciation was performed using the X-ray Absorption Near-Edge Structure (XANES) technique using synchrotron light. Sulfur is usually taken up from the soil via plant roots in the form of sulfate, undergoing a complex series of reactions. Sulfur is typically present in plants in several oxidation states [13]. From the perspective of a number of important spectroscopic techniques, sulfur is considered a "silent element" [14]; however, modern spectroscopic techniques in the X-ray region not only provide information about quantification but can also reveal the oxidation state of sulfur in different plant parts, providing insights into the homeostasis and metabolism of sulfur in different plant tissues. Techniques, such as those used in this study, also require a minimum sample preparation, usually without the need for chemical digestion, extraction, or preparation, which is advantageous for chemical speciation studies since it avoids the risk of any alterations in the constituents. This study is part of a wider study of the macro- and micronutrient compositions of plants from the Amazon rainforest using advanced spectroscopic techniques [15,16]. The samples considered in this study came from the states of Pará and Amazonas in the northern region of Brazil.

2. Materials and Methods

2.1. Plant Material

Plant material was collected from the Amazonian rainforest. Fruits belonging to two different regions were collected. The first one (named "whole fruit" in the tables) was collected in Altamira, State of Pará (3°23′003″ S 51°23′005″ W). The second sample, collected at the Campus of the Instituto Nacional de Pesquisas da Amazônia (INPA), located in Manaus, State of Amazonas (3°05′49.2″ S 59°59′06.3″ W), was separated into pericarps and seeds. The materials were dried in a drying chamber at 50 °C until a constant mass was achieved and then milled, preserving their biological properties. A voucher of the first sample was deposited in the Herbarium of the National Institute of Amazonian Research (INPA) under the number, 259,202. The second sample was registered in the Genetic Heritage Management Council (SISGEN/MMA) of Brazil under the number, ACB5AAA.

2.2. Wavelength Dispersive X-ray Fluorescence (WD-XRF)

The measurements performed using WD-XRF and the validation of this method are described in detail elsewhere [15,16], and thus we only offer a brief description here. The inorganic composition of the plant material was determined using a 1 kW WD-XRF Spectrometer (S8 Tiger, Bruker, Karlsruhe, Germany) at the Federal University of Rio de Janeiro. It was equipped with three analyzing crystals covering elements ranging from fluorine to uranium: XS-55 (F to Mg), PET (Al to Cl), and LiF200 (K to U) [17]. Two detectors mounted on a goniometer could detect fluorescent lines ranging from 0.5 eV to 50 eV: a gas proportional counter (F to V) and a scintillation counter (Cr to U). The spectrometer also featured a rhodium (Rh) tube with a beryllium window, well-suited for plant sample analysis since rhodium itself is rarely found in these samples. The spectrometer's software included a standardless application based on fundamental parameters, which was used to obtain quantitative results from the measured spectra. Quantification based on fundamental

parameters makes use of incident beam intensity and tube spectra, detector efficiency, fluorescence yield, and photoabsorption cross sections, among other parameters [18,19]. This kind of application allows for quantitative analysis without the need to develop a specific calibration curve. It is nevertheless necessary to keep in mind that this is possible but with some detriment in accuracy, which for this application has been determined to be approximately 5% in major components and as much as 20% for elements with low atomic numbers or with concentrations close to the limit of detection [20]. In order to validate this method, a standard reference material from NIST (CRM 1570a from spinach leaves) was measured. Our measurements [15] support the results previously reported in [20]. All samples were milled to a fine powder, supported by a low Z material, and measurements were performed in helium atmosphere at reduced pressure in a 17 min routine. The sample holder was assembled with a 4 μ m thin film (3525 Ultralene-SPEX[®]SamplePrep, Metuchen, NJ, USA), and a 34 mm mask was used to delimitate the irradiated area.

Standardless applications require information about matrix compositions. For plant material, cellulose provides a good accuracy level for this application [20]. Measurements were made in triplicate, and the standard deviation of the mean is reported.

2.3. X-ray Absorption Near-Edge Structure (XANES)

XANES measurements have also previously been described in detail [15,16]. Briefly, measurements were performed in the Soft X-ray Spectroscopy beamline (SXS) of the Brazilian Synchrotron Laboratory (LNLS Brazil) [21,22]. The beam energy was tuned within a selected photon energy range, encompassing the sulfur K-edge. A Si (111) double crystal with an energy bandwidth of 0.5 eV was used in the monochromatization of the incident beam. In this beamline, XANES measurements were performed in total electron yield (TEY) mode using an electrometer (Keithley, Cleveland, OH, USA, Model 6514) and in fluorescence yield (FL) mode using a silicon drift detector (SuperFast SDD, AMPTEK, Bedford, MA, USA, Model XR-100 SDD). Energy calibration was carried out by measuring the XANES spectrum of an ultrapure molybdenum foil in the TEY mode and setting the zero of the second derivative of the spectra to 2520 eV [21].

Spectra around the sulfur K-edge were obtained from 2400 to 2600 eV in the photon energy range, with the following: increments of 2 eV in the ranges between 2400–2440 eV and 2560–2600 eV; steps of 1 eV in the ranges between 2440–2465 eV and 2500–2560 eV; and steps of 0.2 eV in the 2465–2500 eV energy range. The latter interval covers the main absorption near edge for the sulfur K-edge (\approx 2470 eV). The first normalization of the spectra was carried out with respect to the beam intensity by measuring it using a gold mesh placed before the sample. The second normalization, a step height normalization, was performed using the procedure available in the ATHENA software package (Demeter_0.9.24) [23]; the wide photon energy range of the pre-edge and post-edge regions allows for a precise step height normalization. Spectra of the plant samples were measured in the fluorescence mode, since due to the sulfur dilution in these samples, self-absorption issues were not expected.

A collection of standards was previously measured in order to obtain a library of spectra of sulfur functional groups with the aim of carrying out sulfur chemical speciation in different plant tissues [15,16]. The set of standards is composed of thiols (cysteine, reduced glutathione), thioether (methionine), disulfide (cystine, oxidized glutathione), sulfoxide (dimethyl sulfoxide (DMSO)), sulfone (L-methionine sulfone), sulfonic acid (cysteic acid), organic sulfate (chondroitin sulfate), and inorganic sulfate (zinc sulfate). Solid standards were purchased from Sigma Aldrich (Burlington, MA, USA), and they were milled and measured in the TEY mode in order to avoid the distortion of the spectra caused by self-absorption. For DMSO, a solution with a 0.4% mass of sulfur was prepared, and the spectrum was obtained in fluorescence mode. A special Teflon sample holder, designed for liquid samples and covered with a 4 μ m X-ray film (3525 Ultralene-SPEX[®]SamplePrep), was used.

To check for reproducibility and stability, a cysteine spectrum was measured several times during the course of spectra acquisition for the plant samples and standards. Cys-

teine was chosen because it has been proven that its spectra remain unchanged following repeated irradiation [24]. The white line in the cysteine NEXAFS spectrum was observed at 2471.3 eV, whereas its second derivative determination was observed at 2470 eV.

3. Results

3.1. WD-XRF

The absolute concentration (measured in $\mu g g^{-1}$) of the inorganic components determined through WD-XRF and the corresponding standard deviation of triplicate measurements are presented in Table 1 [16]. For Sample 1 (from State of Pará), the sample was constituted of the whole fruit, dried, and milled, whereas for Sample 2 (from State of Amazonas), the seeds and pericarp were separated from each other and dried and milled.

Table 1. Concentration (\pm SD) of inorganic components in the *Bixa orellana* samples as determined via WD-XRF.

Measured Concentrations ($\mu g g^{-1}$)						
	Sample 1	Sample 2				
	Whole Fruit	Pericarp	Seed			
Mg	3200 ± 140	1150 ± 50	1990 ± 50			
AÌ	ND *	105 ± 5	ND			
Si	540 ± 30	280 ± 20	120 ± 10			
Р	3720 ± 70	2570 ± 50	3750 ± 40			
S	2240 ± 50	2450 ± 70	1850 ± 30			
Cl	3220 ± 40	5070 ± 60	1010 ± 20			
Κ	$21,070 \pm 340$	$20,720 \pm 360$	$14,\!510\pm220$			
Ca	6340 ± 110	7570 ± 130	2020 ± 60			
Mn	33 ± 3	ND	ND			
Fe	70 ± 2	103 ± 3	43 ± 3			
Cu	50 ± 1	60 ± 4	40 ± 1			
Zn	50 ± 6	ND	63 ± 5			
Rb	210 ± 12	ND	ND			

* ND: Not detected or below the quantification limit.

Potassium was the element found in the highest concentration in fruit, pericarp, and seeds. Calcium had the next highest concentration, and in Sample 2, it was more concentrated in the pericarp than in the seeds. Our results for seeds are consistent with previously reported measurements [10,11], but for Sample 1 (whole fruit) and Sample 2 (pericarp), we observed relatively higher values. Magnesium is another macronutrient present at a high concentration, and our results are in agreement with previously reported measurements taken using different methods [10,11]. The two samples showed a high content of phosphorous, mostly in the seeds, while sulfur was present in both samples at similar concentrations. From Sample 2, it can be seen that chlorine was present at a higher concentration in the pericarp. The results for essential micronutrients, such as manganese, iron, copper, and zinc are in the ppm order. Our results for manganese content were relatively lower than previously reported values in [10] but in agreement with [11]. Interestingly, rubidium was present in Sample 1 (from Altamira, State of Pará region). It is an alkaline element whose presence might be related to the high concentration of potassium. In a previous study carried out on Andira surinamensis [15], a plant used in traditional medicine and from the same region of Amazonian rainforest (Pará), a similar pattern was observed: samples showed higher concentrations of calcium than potassium as well as high concentrations of strontium and barium, which are elements of the same group of calcium. It is well known that the presence of some non-essential elements is intrinsically related to the soil where the plants grow. These results may suggest that non-essential elements can be acquired through the same paths of some essential elements from the same chemical families [25].

From Table 1, it is also possible to infer that the pericarp accumulates higher concentrations of macronutrients, such as potassium, calcium, and sulfur. The same behavior is observed with respect to micronutrients, iron, and copper. This result reinforces that the pericarp, currently considered a waste product in applications of *Bixa orellana*, may be considered as a source of important nutrients.

3.2. XANES

XANES spectra of the fruit, pericarp, and seeds were measured around the sulfur K-edge, and the results are shown in Figures 1-3 [16], respectively.



Figure 1. Sulfur K-edge XANES spectra of the *Bixa orellana* fruit (Sample 1). Linear Combination Fitting and components are plotted together with experimental data.



Figure 2. Sulfur K-edge XANES spectra of the *Bixa orellana* pericarp (Sample 2). Linear Combination Fitting and components are plotted together with experimental data.



Figure 3. Sulfur K-edge XANES spectra of *Bixa orellana* seeds (Sample 2). Linear Combination Fitting and components are plotted together with experimental data.

The three spectra have a strong resemblance in shape, with three characteristic features around 2471 eV, 2474 eV, and 2480 eV. Intensity differences are observed in the main features from one spectrum to the other. With the objective of determining the sulfur functional groups that account for each feature, the sulfur K-edge spectrum of reference compounds was measured in identical experimental conditions for a set of standards covering the main functional groups of sulfur in different oxidation states. These spectra were then used to perform a best fit analysis of the sample spectra. Spectral compositions were determined using a Linear Combination Fitting technique in ATHENA [23] software. The best fit was determined by fitting several combinations of standards and choosing the combination that provided better fitting parameters; these combinations were achieved using the same software algorithm. When more than one standard with the same sulfur functional group was available, the fitting was tested by changing the standards to determine which standard better fitted the spectra. The set of standards comprised reference compounds including thiols (as cysteine and reduced glutathione), thioether (as methionine), disulfide (as cysteine and oxidized glutathione), sulfoxide (as dimethyl sulfoxide), sulfone (as L-methionine sulfone), sulfonic acid (as cysteic acid), organic sulfate (as chondroitin sulfate), and inorganic sulfate (as zinc sulfate), measured under the same conditions. The contribution of these functional groups to each spectrum and the standard used in the fitting are presented in Table 2 [16].

Table 2. Results of LCF of Bixa orellana	fruit, pericarp,	and seeds.
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Compound	Functional Group	Oxidation State [26–28]	Sample 1	Sample 2	
			Whole Fruit %	Pericarp %	Seed %
Oxidized glutathione	disulfide	0.2	12.9 ± 2.0	6.6 ± 2.4	18.2 ± 1.7
Methionine	thioether	0.5	18.2 ± 1.8	14.9 ± 2.2	24.8 ± 1.6
DMSO	sulfoxide	2	10.2 ± 0.7	8.6 ± 0.9	11.0 ± 0.5
Cysteic acid	sulfonic acid	5	9.6 ± 0.5	9.3 ± 0.6	3.1 ± 0.5
Inorganic sulfate	inorganic sulfate	6	48.2 ± 0.5	61.0 ± 0.6	43.0 ± 0.5

For all three spectra, the feature at 2480 eV is the most intense and is explained by the presence of at least two highly oxidized groups: inorganic sulfate and sulfonic acid. The feature at 2474 eV indicates the predominance of one intermediate oxidized species,

such as a sulfoxide group. Finally, in the feature at 2471 eV, contributions from thioether (methionine) and disulfide (oxidized glutathione) were observed in all spectra.

4. Discussion

The concentrations of macro and micronutrients from *Bixa orellana* fruits and seeds are presented in Table 1. In Table 2, we present the sulfur chemical speciation based on synchrotron radiation spectroscopic measurements. The results presented in Table 1 confirm that, using WD-XRF, it is possible to efficiently obtain fast and reliable results regarding the inorganic composition of *Bixa orellana* fruits and seeds, with minimal sample preparation. Furthermore, a great advantage of working with this technique is that it provides a full screening of all inorganic constituents of the seeds, allowing for a complete determination of the presence of some elements otherwise not identified in specifically developed calibration curves, such as chlorine or rubidium, which are reported in this study. This is possible because the full analysis mode available with the standardless application allows all fluorescence emission lines to be scanned.

The results obtained using WD-RXF show that the seeds of Bixa orellana are a source of nutrients such as potassium, magnesium, calcium, and phosphorus. Potassium is present in samples in concentrations of a few percent, highlighting that these seeds may be considered an interesting source of potassium compared to other foods [29,30]. The calcium content is not as high as the potassium content, but it is higher than those commonly found in other edible seeds [30]. Magnesium, which is related to energy absorption, enzymatic regulation, and structural functions in plant tissues, is also more concentrated in the seeds than in the pericarp. Our results show that Bixa orellana seeds have similar magnesium levels compared to other seeds [30]. The phosphorus concentration is higher in the seeds compared to the pericarp, likely due to the high metabolic activity associated with this tissue. The same observations of macronutrient content apply to the pericarp, which is usually taken just as a sub product in most applications of this plant. In a previous study carried out by two Brazilian Research Institutions, EMBRAPA and CPATU, it was observed that the pericarp might accumulate more nutrients than the seed [11]. In the present study, we proved that the pericarp has similar nutrient concentrations to those found in the seeds. In the case of calcium and potassium, it may even surpass the concentrations found in seeds. This brings our attention to the fact that important nutrients might be wasted in the production of urucum, for instance, which is widely used in the cosmetic and food industries.

Although a few studies focused on the inorganic composition of Bixa orellana fruits and seeds, none of them were performed using the WD-XRF technique. Our results are, in any case, in good agreement with previously reported measurements performed with specimens collected in different regions and analyzed using different methods. For instance, in a study carried out with specimens collected in Guatemala [8] and analyzed using chemical methods, only results for calcium, phosphorus, and iron were reported. From those results, it can be observed that the content of phosphorous and iron is fairly similar in all samples and in agreement with our results. The results presented for calcium, on the other hand, show slightly lower concentrations compared to the present results. Moreover, we observed a similar trend in the distribution, with pericarp accumulating a higher amount of calcium, phosphorus, and iron than the seeds. This variation in mineral distribution may be related to soil nutritional characteristics and genetic factors. However, due to its role as a protective tissue, the pericarp is expected to exhibit a high concentration of calcium, providing greater rigidity to this tissue [31]. Studies conducted by Vijayan et al. [32] indicate that higher concentrations of iron and copper in the seed coat are associated with water impermeability. Moreover, differences in the distribution of mineral nutrients between the pericarp and the seeds may occur not only due to the mechanical strength of this structure but also to its ability to confer chemical resistance against predators [31]. In a recent study carried out with samples collected in the state of São Paulo, Brazil, and analyzed by ICP-MS [10], a comprehensive analysis was performed, and results for 16 macro and micronutrients as well as contaminants were reported for the seeds. The results for macronutrients, such

as calcium, magnesium, and potassium, are on the same order of magnitude (percentage range) but slightly higher than those obtained here, while results for phosphorus were not reported. The results for micronutrients, such as copper, iron and zinc (in order of ppm), are also presented here, with iron showing the greatest variability in measured values, and zinc and copper exhibiting little variability. Finally, our results are in better agreement with those from samples collected in a nearby region in the state of Pará, which were analyzed using atomic absorption spectrometry [11]. This suggests that the differences found in nutritional content are likely due to the climatic and cultivation conditions of the plant.

In summary, the WD-XRF standardless application used in this study can provide reliable results for the inorganic composition of plant material. It is fast and requires minimal sample preparation, and it is adequate for the purpose of evaluating the content of macro and micronutrients. However, it has limitations regarding the determination of the presence of heavy metals, such as mercury or lead. The limits of detection (LOD) for these metals are reported to be in the order of 10 μ g g⁻¹ using a WD-XRF standardless application in the organic matrix [20]; however, international standards regarding the presence of toxic heavy metals in food are around 0.1–0.2 μ g g⁻¹ [33]. In the present study, we did not detect those heavy metals in our samples.

Using WD-XRF, we observed that sulfur is slightly more concentrated in the pericarp than in the seeds. From XANES spectra, we gathered information regarding the distribution of oxidation states of sulfur atoms in different samples. XANES spectra are very sensitive to the chemical environment around the selected atom. This method is shown to be suitable for performing sulfur chemical speciation in several materials including the following: coal, petroleum, and asphaltenes [26,34–38]; soils and related samples [28,39–45]; biological systems [46–49]; plants [15,16,50]; and other materials [14,24,27,51–56].

In XANES, the chemical environment of an atom is probed by tuning the excitation energy and scanning the selected core electron binding energy. This method probes the excitation of core electrons for empty or partially filled molecular orbitals, providing information about the chemical binding of the selected atom in the molecule. In complex samples such as plants, the spectrum is a superposition of all the different oxidation states of the selected atom in the molecules. By applying data processing tools (Principal Component Analysis and Target Transformation [16]) and Linear Combination Fitting, it is possible not only to identify the oxidation states of sulfur in those molecules but also to determine the fraction of each oxidation state that computes to the sample spectrum. To achieve this, the normalized sample spectrum is modeled as a linear combination of the normalized spectra of model compounds, and the coefficients of each model represent the fraction of sulfur atoms in the sample that belong to the same sulfur chemical groups [50]. Therefore, in the XANES approach, it is important to have some knowledge of the samples in order to select the model compounds that provide a chemical meaning to the fitting, avoiding the problems related to the fact that similar sulfur functional groups may show differences in XANES spectra [53].

We measured a set of model compounds used to perform sample fitting with the purpose of obtaining a collection of model spectra of sulfur-containing chemicals with meaning in plants sulfur metabolism. From these fitting results, we gathered information on the chemical speciation and contribution of each species to the sample spectra.

In the XANES approach, speciation means the determination of the oxidation states and functional groups in which the selected atom is present as well as the fraction of each of those groups [50]. From Table 2, which shows the results of the LCF of XANES measurements, it can be seen that sulfate is the predominant form of sulfur in *Bixa orellana* seeds. This sulfate may serve as a reserve for protein synthesis and related metabolic processes during the early stages of seedling development [57]. Sulfur is delivered to the seed either in the form of sulfate or reduced sulfur compounds such as reduced glutathione or methyl methionine [58]. Sulfate that arrives in mature leaves through the xylem can be redistributed via the phloem to young leaves and developing fruits by specific membrane transporters [59]. Our results allow us to outline a profile of the sulfur forms present in *Bixa orellana* fruits and seeds. From our results, it is shown that sulfur accumulates in the seed in the form of sulfate and is also present in reduced sulfur forms (thioether and disulfide) that could be used in the development of the seedling. It is observed that sulfate is the predominant form of sulfur in the pericarp, from where it is transported to the developing seed. In the seed, its distribution changes a little, and more sulfur is accumulated in a reduced form than in the pericarp. However, inorganic sulfate is still the major form of sulfur in the seeds. The low number of sulfur-containing proteins in the seeds is also verified in this study, where the amount of sulfur belonging to thioether/disulfide groups is in the order of 0.5 to 0.7 g/100 g of the total mass, calculated from the total sulfur concentrations (Table 1) and the corresponding percentage of each sulfur type (Table 2). This is in agreement with the content of sulfur-containing amino acids in seeds of *Bixa orellana*, reported to be 0.4 g/100 g [60].

Our results highlight the fact that the seeds of *Bixa orellana* accumulate sulfur in an inorganic form that has an important energetic cost as a source of sulfur in the early stages of plant development. Future studies are needed to assess the accumulation and metabolism of sulfur in these stages.

5. Conclusions

Based on our results, we found that a standardless application based on the fundamental parameters of spectroscopic techniques in the X-ray region is a fast and reliable method for determining the inorganic composition of plant material for all elements, starting from Z = 9, and with concentrations ranging from the order of ppm to a few percent. This technique requires minimal sample preparation and maintains a reasonable level of accuracy. Additionally, XANES analysis provides valuable insights into sulfur speciation in plant tissue, delivering a complete profile of sulfur oxidation states without the need for chemical preparation, thereby avoiding any risk of altering the distribution of species.

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References

- Antar, G.M.; do Rio de Janeiro Jardim, B. Flora e Funga Do Brasil. Available online: https://floradobrasil.jbrj.gov.br/FB5745 (accessed on 17 May 2023).
- Stringheta, P.C.; Silva, P.I. Pigmentos de Urucum: Extração, Reações Químicas, Usos e Aplicações, 1st ed.; Gráfica e Editora Suprema: Viçosa, Brazil, 2008.
- 3. Moreira, P.A.; Lins, J.; Dequigiovanni, G.; Veasey, E.A.; Clement, C.R. The Domestication of Annatto (*Bixa orellana*) from Bixa Urucurana in Amazonia. *Econ. Bot.* 2015, *69*, 127–135. [CrossRef]
- Ravindran, P.N.; Divakaran, M.; Pillai, G.S. Other Herbs and Spices: Achiote to Szechuan Pepper. In *Handbook of Herbs and Spices*, 2nd ed.; Woodhead Publishing: Sawston, UK, 2012; Volume 2, pp. 534–556. [CrossRef]

- Dias, V.M.; Pilla, V.; Alves, L.P.; Oliveira, H.P.M.; Munin, E. Optical Characterization in Annatto and Commercial Colorific. J. Fluoresc. 2011, 21, 415–421. [CrossRef] [PubMed]
- Sasaki, Y.F.; Kawaguchi, S.; Kamaya, A.; Ohshita, M.; Kabasawa, K.; Iwama, K.; Taniguchi, K.; Tsuda, S. The Comet Assay with 8 Mouse Organs: Results with 39 Currently Used Food Additives. *Mutat. Res. Genet. Toxicol. Environ. Mutagen.* 2002, 519, 103–119. [CrossRef] [PubMed]
- Demczuk, B., Jr.; Hoffmann Ribani, R. Updates on Chemistry and Use of Annatto (*Bixa orellana* L.). *Rev. Bras. Pesqui. Em. Aliment.* 2015, 6, 37. [CrossRef]
- 8. Bressani, R.; Porta-España de Barneón, F.; Braham, J.E.; Elías, L.G.; Gómez-Brenes, R. Chemical composition, amino acid content and nutritive value of the protein of the annatto seed (*Bixa orellana* L.). *Arch. Latinoam. Nutr.* **1983**, *33*, 356–376. [PubMed]
- Raddatz-Mota, D.; Pérez-Flores, L.J.; Carrari, F.; Mendoza-Espinoza, J.A.; de León-Sánchez, F.D.; Pinzón-López, L.L.; Godoy-Hernández, G.; Rivera-Cabrera, F. Achiote (*Bixa orellana* L.): A Natural Source of Pigment and Vitamin E. *J. Food Sci. Technol.* 2017, 54, 1729–1741. [CrossRef] [PubMed]
- Baptistão, M.; Lorençatto, R.; Eberlin, M.N.; Simionato, A.V.C. Multielemental Characterization of *Bixa orellana* L. (Urucum) Seeds by Inductively Coupled Plasma Mass Spectrometry. *Food Bioproc. Tech.* 2023, *16*, 2521–2530. [CrossRef]
- Ferreira, W.d.A.; Falesi, I.C. Características Nutricionais Do Fruto E Teor De Bixina Em Urucu (Bixa orellana L.); Boletim. de Pesquisa Número 97-Embrapa; Embrapa: Brasília, Brazil, 1989; p. 31. Available online: https://www.embrapa.br/busca-de-publicacoes/ -/publicacao/381124/caracteristicas-nutricionais-do-fruto-e-teor-de-bixina-em-urucu-bixa-orellana-l (accessed on 16 May 2022).
- 12. Scherer, H.W. Sulphur in Crop Production-Invited Paper. Eur. J. Agron. 2001, 14, 81–111. [CrossRef]
- Chao, D.Y.; Baraniecka, P.; Danku, J.; Koprivova, A.; Lahner, B.; Luo, H.; Yakubova, E.; Dilkes, B.; Kopriva, S.; Salt, D.E. Variation in Sulfur and Selenium Accumulation Is Controlled by Naturally Occurring Isoforms of the Key Sulfur Assimilation Enzyme Adenosine 5'-Phosphosulfate REDUCTASE2 across the Arabidopsis Species Range. *Plant Physiol.* 2014, *166*, 1593–1608. [CrossRef]
 Jalilehvand, F. Sulfur: Not a "Silent" Element Any More. *Chem. Soc. Rev.* 2006, *35*, 1256–1268. [CrossRef]
- Gonzalez, J.C.; Simões, G.; Bernini, R.B.; Coutinho, L.H.; Stedile, F.C.; Nunez, C.V.; Vicentin, F.C.; De Souza, G.G.B. Elemental Concentration and Sulfur Chemical Speciation in the Amazonian Plant Andira Surinamensis Using Synchrotron Radiation Techniques (SR-XRF, XANES), RBS and WD-XRF. *Artic. J. Braz. Chem. Soc.* 2019, 30, 1887–1896. [CrossRef]
- 16. Gonzalez, J.C. Uso de Técnicas Espectroscópicas Avançadas Para Caracterização Elementar e Especiação de Enxofre Em Plantas Da Amazônia. Ph.D. Thesis, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, 2019.
- 17. Bruker. S8 Tiger Operator's Manual; Bruker: Billerica, MA, USA, 2009; p. 203.
- 18. Jenkins, R. X-ray Fluorescence Spectrometry; RSC Publishing: London, UK, 2008; Volume 2, ISBN 9783527618323.
- 19. Haschke, M. Micro-X-ray Fluorescence Spectroscopy; Springer: Berlin/Heidelberg, Germany, 2014; ISBN 9783319048635.
- Andersen, L.K.; Morgan, T.J.; Boulamanti, A.K.; Álvarez, P.; Vassilev, S.V.; Baxter, D. Quantitative X-ray Fluorescence Analysis of Biomass: Objective Evaluation of a Typical Commercial Multi-Element Method on a WD-XRF Spectrometer. *Energy Fuels* 2013, 27, 7439–7454. [CrossRef]
- Abbate, M.; Vicentin, F.C.; Compagnon-Cailhol, V.; Rocha, M.C.; Tolentino, H. The Soft X-ray Spectroscopy Beamline at the LNLS: Technical Description and Commissioning Results. J. Synchrotron. Radiat. 1999, 6, 964–972. [CrossRef]
- 22. Tolentino, H.; Cezar, J.C.; Cruz, D.Z.; Compagnon-Cailhol, V.; Tamura, E.; Martins Alves, M.C. Commissioning and First Results of the LNLS XAFS Beamline. J. Synchrotron. Radiat. 1998, 5, 521–523. [CrossRef] [PubMed]
- 23. Ravel, B.; Newville, M. ATHENA, ARTEMIS, HEPHAESTUS: Data Analysis for X-ray Absorption Spectroscopy Using IFEFFIT. *J. Synchrotron. Radiat.* **2005**, *12*, 537–541. [CrossRef] [PubMed]
- Simões, G.; Rodrigues, F.N.; Bernini, R.B.; Castro, C.S.C.; De Souza, G.G.B. A NEXAFS and Mass Spectrometry Study of Cysteine, Cystine and Insulin Irradiated with Intermediate Energy (0.8 KeV) Electrons. J. Electron. Spectros. Relat. Phenom. 2014, 193, 21–26. [CrossRef]
- Myrvang, M.B.; Hillersøy, M.H.; Heim, M.; Bleken, M.A.; Gjengedal, E. Uptake of Macro Nutrients, Barium, and Strontium by Vegetation from Mineral Soils on Carbonatite and Pyroxenite Bedrock at the Lillebukt Alkaline Complex on Stjernøy, Northern Norway. J. Plant Nutr. Soil Sci. 2016, 179, 705–716. [CrossRef]
- 26. Waldo, G.S.; Carlson, R.M.K.; Moldowan, J.M.; Peters, K.E.; Penner-hahn, J.E. Sulfur Speciation in Heavy Petroleums: Information from X-ray Absorption near-Edge Structure. *Geochim. Cosmochim. Acta* **1991**, *55*, 801–814. [CrossRef]
- 27. Vairavamurthy, A. Using X-ray Absorption to Probe Sulfur Oxidation States in Complex Molecules. *Spectrochim. Acta A Mol. Biomol. Spectrosc.* **1998**, *54*, 2009–2017. [CrossRef]
- Xia, K.; Weesner, F.; Bleam, W.F.; Helmke, P.A.; Bloom, P.R.; Skyllberg, U.L. XANES Studies of Oxidation States of Sulfur in Aquatic and Soil Humic Substances. Soil Sci. Soc. Am. J. 1998, 62, 1240–1246. [CrossRef]
- Rodushkin, I.; Engström, E.; Sörlin, D.; Baxter, D. Levels of Inorganic Constituents in Raw Nuts and Seeds on the Swedish Market. Sci. Total Environ. 2008, 392, 290–304. [CrossRef] [PubMed]
- Almeida, I.M.C.; Oliva-Teles, M.T.; Alves, R.C.; Santos, J.; Pinho, R.S.; Silva, S.I.; Delerue-Matos, C.; Oliveira, M.B.P.P. Oilseeds from a Brazilian Semi-Arid Region: Edible Potential Regarding the Mineral Composition. *Foods* 2020, *9*, 229. [CrossRef]
- 31. Huss, J.C.; Gierlinger, N. Functional Packaging of Seeds. New Phytol. 2021, 230, 2154–2163. [CrossRef]
- Vijayan, S.S.; Nagarajappa, N.; Ranjitha, H.P. Seed Coat Composition in Black and White Soybean Seeds with Differential Water Permeability. *Plant Biol.* 2023, 25, 935–943. [CrossRef]

- FAO; WHO. CF/14 INF/1. Joint FAO/WHO Food Standards Program CODEX Committee on Contaminants in Foods. 2021, pp. 8–47. Available online: https://www.fao.org/fao-who-codexalimentarius/sh-proxy/it/?lnk=1&url=https%253A%25 2F%252Fworkspace.fao.org%252Fsites%252Fcodex%252FMeetings%252FCX-735-14%252FINFO-DOC%252FCF14_INF01x.pdf (accessed on 8 June 2024).
- George, G.N.; Gorbaty, M.L. Sulfur K-Edge X-ray Absorption Spectroscopy of Petroleum Asphaltenes and Model Compounds. J. Am. Chem. Soc. 1989, 111, 3182–3186. [CrossRef]
- 35. Huffman, G.P.; Mitra, S.; Huggins, F.E.; Shah, N.; Vaidya, S.; Lu, F. Quantitative Analysis of All Major Forms of Sulfur in Coal by X-ray Absorption Fine Structure Spectroscopy. *Energy Fuels* **1991**, *5*, 574–581. [CrossRef]
- 36. Waldo, G.S.; Mullins, O.C.; Penner-Hahn, J.E.; Cramer, S.P. Determination of the Chemical Environment of Sulphur in Petroleum Asphaltenes by X-ray Absorption Spectroscopy. *Fuel* **1992**, *71*, 53–57. [CrossRef]
- Huffman, G.P.; Shah, N.; Huggins, F.E.; Stock, L.M.; Chatterjee, K.; Kilbane, J.J.; Chou, M.I.M.; Buchanan, D.H. Sulfur Speciation of Desulfurized Coals by XANES Spectroscopy. *Fuel* 1995, 74, 549–555. [CrossRef]
- Zhang, L.L.; Wang, C.L.; Zhao, Y.S.; Yang, G.H.; Su, M.; Yang, C.H. Speciation and Quantification of Sulfur Compounds in Petroleum Asphaltenes by Derivative XANES Spectra. *Ranliao Huaxue Xuebao/J. Fuel Chem. Technol.* 2013, 41, 1328–1335. [CrossRef]
- Vairavamurthy, M.A.; Maletic, D.; Wang, S.; Manowitz, B.; Eglinton, T.; Lyons, T. Characterization of Sulfur-Containing Functional Groups in Sedimentary Humic Substances by X-ray Absorption near-Edge Structure Spectroscopy. *Energy Fuels* 1997, 11, 546–553. [CrossRef]
- 40. Beauchemin, S.; Hesterberg, D.; Beauchemin, M. Principal Component Analysis Approach for Modeling Sulfur K-XANES Spectra of Humic Acids. *Soil Sci. Soc. Am. J.* 2002, *66*, 83–91. [CrossRef]
- 41. Scheinost, A.C.; Kretzschmar, R.; Pfister, S.; Roberts, D.R. Combining Selective Sequential Extractions, X-ray Absorption Spectroscopy, and Principal Component Analysis for Quantitative Zinc Speciation in Soil. *Environ. Sci. Technol.* **2002**, *36*, 5021–5028. [CrossRef]
- 42. Solomon, D.; Lehmann, J.; Martínez, C.E. Sulfur K-Edge XANES Spectroscopy as a Tool for Understanding Sulfur Dynamics in Soil Organic Matter. *Soil Sci. Soc. Am. J.* 2003, 67, 1721–1731. [CrossRef]
- 43. Bostick, B.C.; Theissen, K.M.; Dunbar, R.B.; Vairavamurthy, M.A. Record of Redox Status in Laminated Sediments from Lake Titicaca: A Sulfur K-Edge X-ray Absorption near Edge Structure (XANES) Study. *Chem. Geol.* **2005**, *219*, 163–174. [CrossRef]
- 44. Zhao, F.J.; Lehmann, J.; Solomon, D.; Fox, M.A.; McGrath, S.P. Sulphur Speciation and Turnover in Soils: Evidence from Sulphur K-Edge XANES Spectroscopy and Isotope Dilution Studies. *Soil Biol. Biochem.* **2006**, *38*, 1000–1007. [CrossRef]
- Prietzel, J.; Botzaki, A.; Tyufekchieva, N.; Brettholle, M.; Thieme, J.; Klysubun, W. Sulfur Speciation in Soil by S K-Edge XANES Spectroscopy: Comparison of Spectral Deconvolution and Linear Combination Fitting. *Environ. Sci. Technol.* 2011, 45, 2878–2886. [CrossRef] [PubMed]
- 46. Pickering, I.J.; Prince, R.C.; Divers, T.; George, G.N. Sulfur K-Edge X-ray Absorption Spectroscopy for Determining the Chemical Speciation of Sulfur in Biological Systems. *FEBS Lett.* **1998**, *441*, 11–14. [CrossRef] [PubMed]
- Rompel, A.; Cinco, R.M.; Latimer, M.J.; Mcdermott, A.E.; Guiles, R.D.; Quintanilha, A.; Krauss, R.M.; Sauer, K.; Yachandra, V.K.; Klein, M.P. Sulfur K-Edge x-Ray Absorption Spectroscopy: A Spectroscopic Tool to Examine the Redox State of S-Containing Metabolites in Vivo. *Proc. Natl. Acad. Sci. USA* 1998, 95, 6122–6127. [CrossRef] [PubMed]
- Prange, A.; Arzberger, I.; Engemann, C.; Modrow, H.; Schumann, O.; Tru, H.G.; Steudel, R.Y.; Dahl, C. In Situ Analysis of Sulfur in the Sulfur Globules of Phototrophic Sulfur Bacteria by X-ray Absorption near Edge Spectroscopy. *Biochim. Biophys. Acta* 1999, 1428, 446–454. [CrossRef]
- 49. Veronesi, G.; Koudouna, E.; Cotte, M.; Martin, F.L.; Quantock, A.J. X-ray Absorption near-Edge Structure (XANES) Spectroscopy Identifies Differential Sulfur Speciation in Corneal Tissue. *Anal. Bioanal. Chem.* **2013**, 405, 6613–6620. [CrossRef]
- De Souza, G.G.B.; Gonzalez, J.C. Near Edge X-ray Absorption Fine Structure (NEXAFS) Spectroscopy of Molecules of Biological Interest: From Chemically Pure to Complex Samples. In *Radiation in Bioanalysis*; Pereira, A., Tavares, P., Limão-Vieira, P., Eds.; Springer Nature: Berlin/Heidelberg, Germany, 2019; pp. 287–312, ISBN 978-3-030-28246-2.
- Wasserman, S.R.; Allen, P.G.; Shuh, K.; Bucher, J.J.; Edelstein, N.M. EXAFS and Principal Component Analysis: A New Shell Game. J. Synchrotron. Radiat. 1999, 6, 284–286. [CrossRef] [PubMed]
- Dey, A.; Okamura, T.A.; Ueyama, N.; Hedman, B.; Hodgson, K.O.; Solomon, E.I. Sulfur K-Edge XAS and DFT Calculations on P450 Model Complexes: Effects of Hydrogen Bonding on Electronic Structure and Redox Potentials. *J. Am. Chem. Soc.* 2005, 127, 12046–12053. [CrossRef] [PubMed]
- Sarangi, R.; Frank, P.; Hodgson, K.O.; Hedman, B. When Identical Functional Groups Are Not Identical: A DFT Study of the Effects of Molecular Environment on Sulfur K-Edge X-ray Absorption Spectra. *Inorganica Chim. Acta* 2008, 361, 956–964. [CrossRef]
- 54. Almkvist, G.; Boye, K.; Persson, I. K-Edge XANES Analysis of Sulfur Compounds: An Investigation of the Relative Intensities Using Internal Calibration. *J. Synchrotron. Radiat.* **2010**, *17*, 683–688. [CrossRef] [PubMed]
- 55. Behyan, S.; Hu, Y.; Urquhart, S.G. Sulfur 1s Near-Edge X-ray Absorption Fine Structure (NEXAFS) of Thiol and Thioether Compounds. J. Chem. Phys. 2011, 134, 244304. [CrossRef] [PubMed]
- Pickering, I.J.; Barney, M.; Cotelesage, J.J.H.; Vogt, L.; Pushie, M.J.; Nissan, A.; Prince, R.C.; George, G.N. Chemical Sensitivity of the Sulfur K-Edge X-ray Absorption Spectra of Organic Disulfides. *J. Phys. Chem. A* 2016, 120, 7229–7286. [CrossRef] [PubMed]

- 57. Cairns, N.G.; Pasternak, M.; Wachter, A.; Cobbett, C.S.; Meyer, A.J. Maturation of Arabidopsis Seeds Is Dependent on Glutathione Biosynthesis within the Embryo. *Plant Physiol.* **2006**, *141*, 446–455. [CrossRef] [PubMed]
- 58. Mondal, S.; Pramanik, K.; Panda, D.; Dutta, D.; Karmakar, S.; Bose, B. Sulfur in Seeds: An Overview. *Plants* 2022, 11, 450. [CrossRef]
- 59. Hawkesford, M.J. Transporter Gene Families in Plants: The Sulphate Transporter Gene Family—Redundancy or Specialization? *Physiol. Plant* **2003**, *117*, 155–163. [CrossRef]
- 60. Glew, R.H.; Vanderjagt, D.J.; Lockett, C.; Grivetti, L.E.; Smith, G.C.; Pastuszyn, A.; Millson, M. Amino Acid, Fatty Acid, and Mineral Composition of 24 Indigenous Plants of Burkina Faso. *J. Food Compos. Anal.* **1997**, *10*, 205–217. [CrossRef]

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