

Identification of Cu in *Crassostrea virginica* Tissue Using the Technique of Laser-Induced Breakdown Spectroscopy (LIBS)

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Abstract

The analysis of metals in aquatic organisms is of great importance due to the health problems they can cause to be consumed by human beings. In this study, the Laser-Induced Plasma Spectroscopy (LIBS) technique is evaluated as an alternative method to identify Cu in *Crassostrea virginica* oysters' tissue. It focuses on the characterization of oysters caught a natural bank and the identification of different Cu concentrations. To carry out experimentation, oyster samples were collected in autumn (October 2017) and spring (May 2018) from San Andres Lagoon, Aldama, Tamaulipas. A single pulse Nd:YAG laser (1064 nm) was used, and tissue was contaminated with 0, 2, 10, 20, 50, and 100 µg/g of Cu. In tissue were identified atomic lines for Ca, Cr, Mg, Mn, Na, N, O, and H. However, the intensities of emission lines for autumn samples were greater than spring samples. Cu emission lines at 324.6, 327.1, 510.3, 515.1, and 521.5 nm were found for contaminated pills. The intensity of emission lines showed a linear increase with the concentration; whereby, they can be used as calibration curves to quantify Cu concentrations in oyster tissue.

Keywords

LIBS, *Crassostrea virginica*, Heavy Metals

1. Introduction

Chemical pollutants negatively impact aquatic ecosystems; it is common to find human diseases such as digestive disorders and poisonings derived from consuming products contaminated by heavy metals [1]. Therefore, it is essential to identify and evaluate the concentration of heavy metals in aquatic organisms pe-

riodically. Among the organisms most vulnerable to aquatic contamination are mollusks [2]. They are sessile and filtering organisms exposed to the accumulation of heavy metals, promoting the biomagnification that affects human beings. An aquatic ecosystem can be characterized through the analysis of heavy metals in mollusks tissue. Oysters are indicators of bioaccumulation due they are sessile organisms and their location in the trophic chain [3]. On the other end, consuming oysters provides an essential source of nutrients [4].

A variety of techniques such as atomic absorption spectroscopy (AAS) [5] [6] [7], wavelength dispersive X-ray fluorescence (WDXRF) [8], and inductively coupled plasma mass spectrometry (ICP-MS) [9] [10] [11] are commonly used to analyze oyster tissue. However, many of these techniques are not readily translatable into field instruments [12] due to prolonged and complicated sample preparation steps that produce chemical residues. Thus, classical techniques are far from low cost and being implemented in real-time [13].

The laser-induced breakdown spectroscopy (LIBS) is an atomic emission spectroscopy technique capable of determining any material's elemental composition in real-time. Focusing the energy of a laser pulse on the sample surface produces plasma that gives information about the material composition [14]. Spectral features such as emission lines, peak intensity, and integrated intensity determine the elemental concentration of the target or discriminate one material from another through their unique spectral signatures [15]. This technique has the advantages of not needing sample preparation; likewise, it could analyze samples in any state of matter. Also, LIBS analysis is relatively fast since, with a laser shot (20 ns), it is possible to obtain the sample's emission spectrum [16].

LIBS has proved to be a powerful technique for elemental analysis of shells [17] and mollusk tissue [12], and the identification of metals in fresh fish [5]-[18]. However, there are no recent studies about the content of copper in *C. virginica* organisms from San Andres lagoon; this may be due to the complexity of transportation, processing, and materials necessary to analyze heavy metals. In this paper, the natural *Crassostrea virginica* tissue and copper contaminated tissue are analyzed with the Laser-Induced Breakdown Spectroscopy technique.

2. Study Area

Figure 1 shows the sampling site, which corresponds to a natural oyster bank, located at 22°42'50.82"N and 97°51'44.79"W at the mouth of the Rio Tigre near the San Andres Lagoon, Aldama, Tamaulipas, Mexico. San Andres Lagoon is a region of fishing and shrimp farms located on the western Gulf of México in the Tamaulipas State (22°32'N - 22°47'N and 97°41'W - 97°54'W). It communicates with the Gulf of Mexico through the mouth of Chavarría and receives contributions from Tiger and Barberena rivers. The lagoon is a nursery and growing zone; its biodiversity includes mollusks, crustaceans, fish, turtles, birds, sea-grasses, and mangroves [19]. Due to its physical characteristics, it exposes changes in lagoon depth due to rain and tides. Previous research has been reported metal

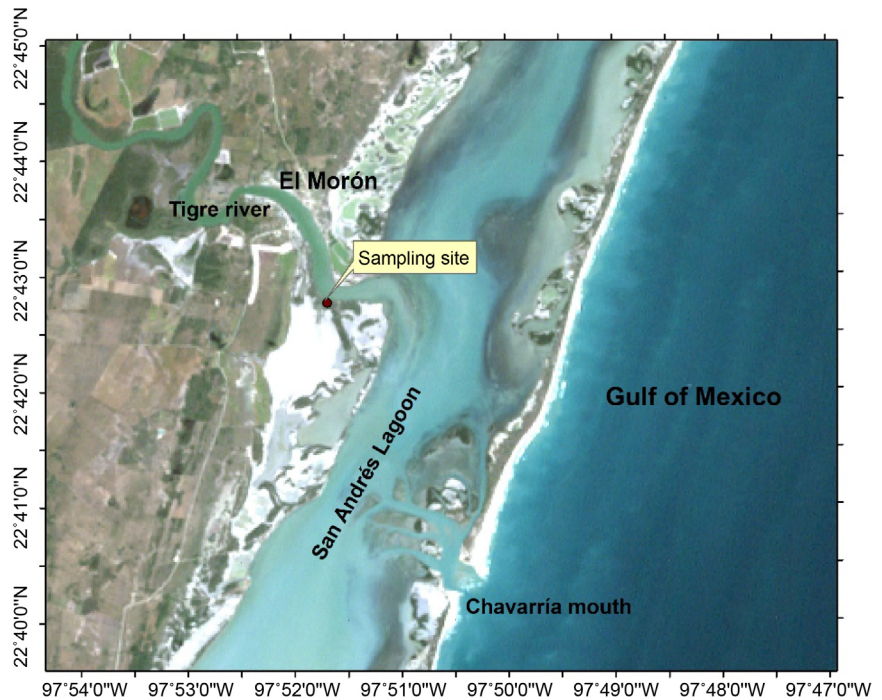


Figure 1. Sampling site in the San Andres Lagoon, Aldama, Tamaulipas, Mexico.

concentrations in water, sediment, and *C. virginica* oyster tissue [20]. The commercial fishery of *Crassostrea virginica* oysters is a highly relevant productive activity in Southern Tamaulipas.

3. Experimental Details

Figure 2 shows the LIBS experimental setup employed in all the experiments described in this work. The samples were ablated with an Nd-YAG laser (Quantel Q-smart 450), delivering 200 mJ/pulse at a wavelength of 1064 nm with a pulse duration of 10 ns FWHM and rate repetition of 4 Hz. The light emitted by the plasma was collected using an optical fiber (with diameter 200 μm) connected to an Ocean Optics Spectrometer USB4000 with a spectral range of 200 - 900 nm. Besides, the installation includes a delay device and photodetector that allows delaying the spectrum's time of capture. The spectrometer's capture time is microseconds after the laser trigger occurring; the delay time used was 2 ms.

For the equipment calibration and to find the lines associated with the Copper element, 50 spectra were taken from a Copper standard with 99.99% purity of the Kurt J. Lesker brand. **Figure 3** shows the highest intensity peaks in the 300 to 550 nm range. For the identification of the average rise of each line, a Gaussian function was fitted, and Cu I peaks were located at 324.6, 327.1, 510.3, 515.1, and 521.5 nm, which coincide with the NIST database [21].

4. Sample Preparation

C. virginica organisms were collected in October 2017 and May 2018. Thirty

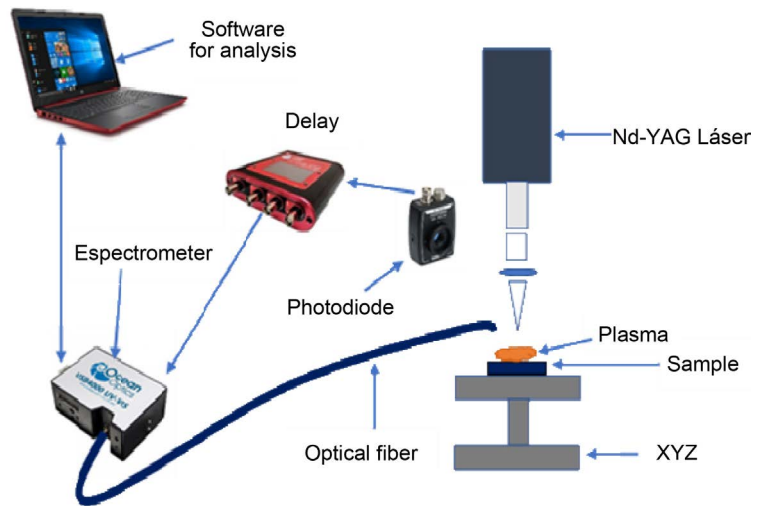


Figure 2. LIBS experimental setup.

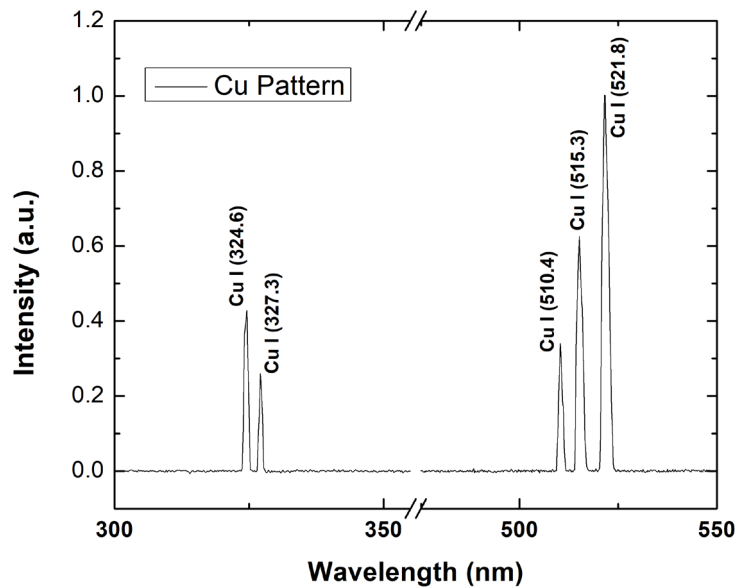


Figure 3. LIBS spectrum of Cu pattern.

oysters of different sizes were manually caught at 0.5 m depth in the river substrate for each sampling. Each specimen was washed with distilled water, removing the remains of sediment, epibionts, and predators. The weight of each organism was determined with an electronic balance (precision 0.1 g), and measures of length, width, and thickness were obtained with a digital vernier (Mitutoyo). The condition index (CI) was calculated according to the method proposed in [22]. The tissues and shells of 30 specimens were used, soft tissues were dried and dry weight was then determined. The CI was calculated as: $CI = (W1 \times 1000)/W2$, where $W1$ is the dry weight of soft tissue (g) and $W2$ is the dry weight of the shell (g). The index reflects the oyster's physiological condition; that is, it allows determining how environmental parameters affect growth and its gametogenic activity.

Oysters were chipped using a spatula, and the tissue was extracted. Tissue was placed in a glass container and dried in a muffle at 70 °C for 24 hours to remove the water content. The tissue was ground in a glass mortar and sieved with a 0.0029" mesh (#200). Two pills (P_{May} , P_{Oct}) of 1 g, 18 mm diameter and 3 mm thickness were obtained with the sieved tissue pressed to 10 tons for 2 minutes with a hydraulic press (Hydra).

Fifty laser shots were applied to each pellet to obtain LIBS spectra of samples. The spectrometer software averaged the resulting 50 spectra, bringing the average spectrum. Each spectrum's Emission lines were fitted to a Gaussian function, and the peaks were compared with the NIST database.

To identify the concentration at which Cu is detected using the LIBS technique, the tissue of *C. virginica* was contaminated with Cupric Nitrate $\text{Cu}(\text{NO}_3)_2$ from Baker Analyzed A. C. S. Reagent Pm 232.590S. This analysis was performed with oysters captured in May 2018. Five work standards were performed corresponding to concentrations 2, 10, 20, 50, and 100 $\mu\text{g/g}$ of Cu. One gram of oyster tissue was contaminated with 1 ml of each Cu concentration. Samples were dried (24 hours, 70 °C) and pressed (10 tons) to obtain pills P_2 , P_{10} , P_{20} , P_{50} and P_{100} with 2, 10, 20, 50 and 100 $\mu\text{g/g}$ of $\text{Cu}(\text{NO}_3)_2$, respectively. In the experiments, the P_{May} pill was used as a blank. Fifty laser shots were applied to each contaminated pellet and the blank. To identify the emission lines, the procedure described previously was followed.

5. Results

For oysters captured in October 2017, the average length was 71 ± 7 mm, the average width was 38 ± 4 mm, and the thickness was 22 ± 3 mm. Otherwise, oysters collected in May 2018 had an average length of 68 ± 11 mm, the average width was 38 ± 5 mm, and the thickness was 26 ± 5 mm. These results indicate that the oysters analyzed were adults. Meanwhile, the condition index was 18.2 and 24.6 for October and May, respectively.

Figure 4 shows the LIBS spectra corresponding to the analysis of an oyster sample ablated at frequencies of 1, 2, 4, 10, and 20 Hz to obtain the spectrum's lowest background. At frequencies of 1, 2, and 4 Hz, there is a low background, and at higher frequencies, the intensity of the background increases, and intensity saturation occurs. In LIBS experiment is common to have a noticeable continuum background, which typically occurs due to Bremsstrahlung and recombination radiation. In general, the low background is very important when spectra LIBS are used for material identification [23]. The 4 Hz frequency was selected due to the spectra have the lowest background and the highest emission intensities.

Figure 5 shows the characteristic LIBS spectra for pills P_{May} , P_{Oct} . The LIBS spectra are complex due to multiple elements present in the tissue of oysters. In the P_{May} spectrum, lines associated with Ca, Cr, Mn, N, Mg, Na, O and H are present. Almost all of the P_{Oct} spectrum lines are present in the P_{May} spectrum,

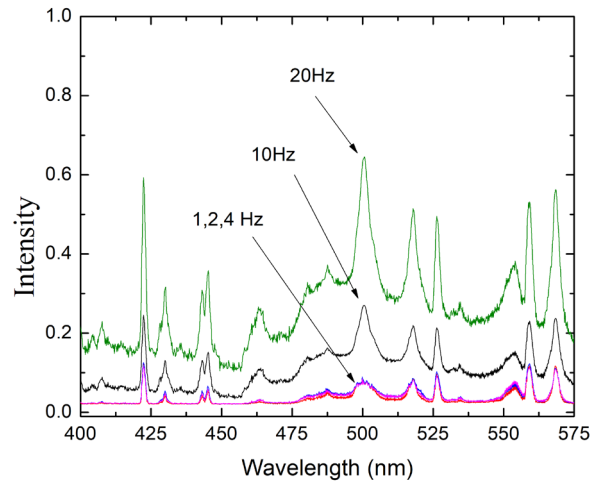


Figure 4. LIBS spectra of oyster sample irradiated with different laser frequency.

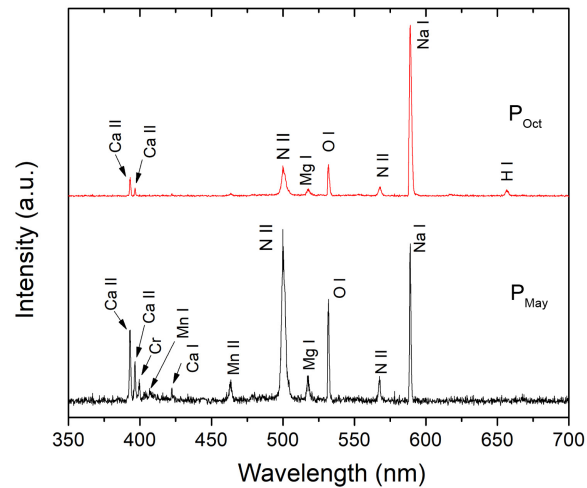


Figure 5. LIBS spectra for oyster samples P_{Oct} and P_{May} .

but with lesser intensity. Such is the case of the Na and Mg lines, both elements belonging to the oyster's natural content [24] [25]. The Na/Mg ratio was calculated to observe the relationship of intensities between samplings. For the October sampling, the rate was 0.68, and for the May sampling, the value was 0.94. These results show that oysters caught in May have a higher amount of Sodium than Magnesium. The list of emission lines is presented in **Table 1**.

Figure 6 shows a LIBS spectrum of five oyster tissue pills contaminated with 2, 10, 20, 50, and 100 $\mu\text{g/g}$ Cu and the uncontaminated blank. Fifty different spectra were taken from each sample, and an average spectrum was generated for each tablet analyzed. The wavelength range where the Copper lines appear is highlighted with red rectangles.

All emission lines presented in **Table 1** were also found in the samples contaminated with Cu. Also, the emission line on 531.8 nm (O I) was observed in the uncontaminated sample. Contaminated samples show a peak at 777 nm

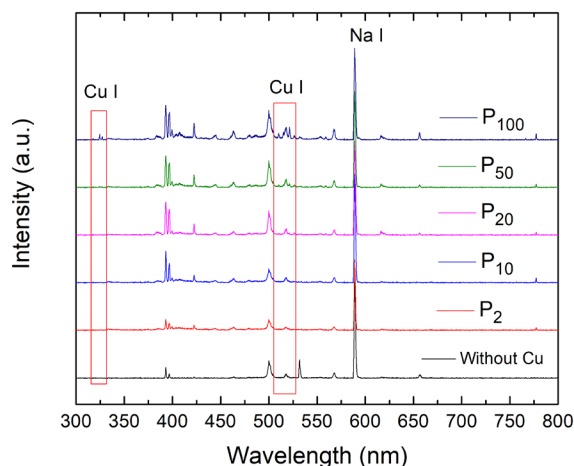


Figure 6. LIBS spectra for oyster tissue contaminated with Cu.

Table 1. Elemental emission lines used in the spectral fingerprint of the oysters.

Peak	Central Wavelength (nm)	Element (NIST)
1	393.14	Ca II (393.3)
2	396.5	Ca II (396.8)
3	399.58	Cr I (399.08, 400.13)
4	407.15	Mn I (407.9)
5	422.37	Ca (422.7)
6	463.4	Mn II (463.9)
7	500.52	N II (500.4)
8	517.63	Mg I (518.42)
9	531.8	O I (532.4)
10	567.46	N II (567.8)
11	589.1	Na I (589.5)
12	656.69	H I (656.26)

associated with the Oxygen added through the Cupric Nitrate $\text{Cu}(\text{NO}_3)_2$.

Figure 7 shows the LIBS spectrum of *C. virginica* tissue contaminated with Cu in the region a) 320 - 332 and b) 505 - 530 nm. The Cu emission lines in the contaminated pill with 50 $\mu\text{g/g}$ begin to be distinguished; however, the intensity of Cu lines for 100 $\mu\text{g/g}$ pill is clearly distinguishable.

Figure 8 shows the intensities of Cu emission lines as a function of its concentration, which results from applying linear regression to data, and the correlation coefficient (R). All Cu lines increase in intensity associated with copper contamination, and significant correlations greater or equal to 0.95 ($p \leq 0.05$); however, the emission line at 521.5 nm has the highest correlation (0.97) concerning the regression line.

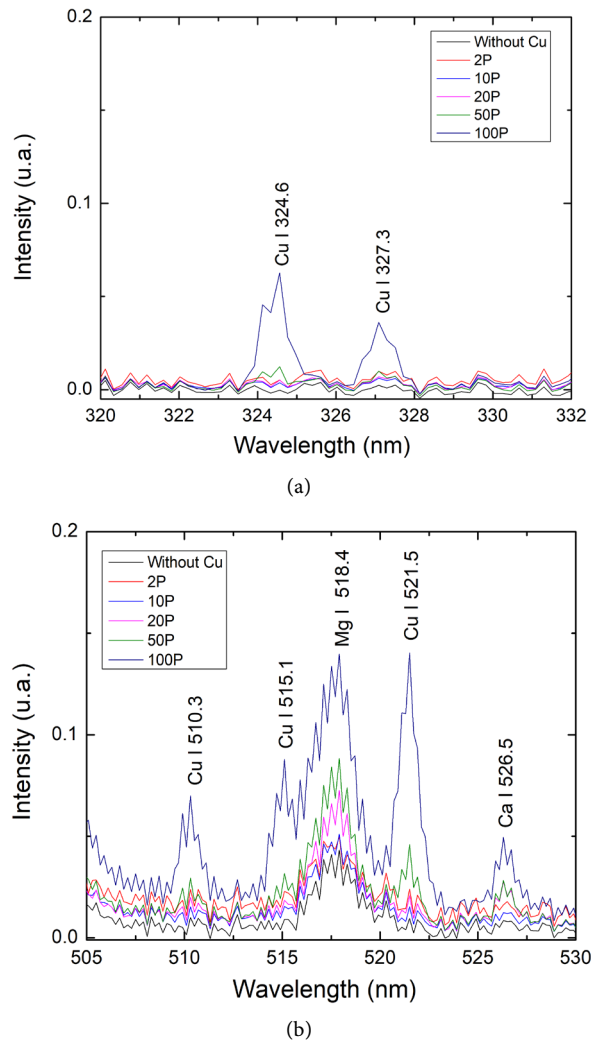


Figure 7. LIBS spectrum region (a) 320 - 332 nm, and (b) 505 - 530 nm of *C. virginica* pills contaminated with Cu.

6. Discussion

Organisms caught during spring (May 2018) had an average length of 68 ± 11 mm. In autumn (October 2017), the average length was 71 ± 7 mm. These results indicate that the organisms were commercial-sized adults who reproduced at least once [26]. On the other end, the condition index is essential to evaluate the nutritional status of bivalves and their commercial quality and determine different contaminants or diseases. The gametogenic cycle or food availability may explain the low physiological condition index [27].

In our study, emission lines for Ca, Cr, Mn, N, Mg, Na, and H were identified in oyster pellets analyzed. These partially agreed with the lines presented in [12]. They collected *C. virginica* organisms at six sample sites from two coastal estuarine reserves: 4 locations in Grand Bay, Mississippi in March 2011, and 2 points in Apalachicola Bay, Florida, in February of the same year. Using LIBS spectral analysis, they identified the lines of Al, C, Ca, H, K, Mg, Na, Si, Sr, and Zn and

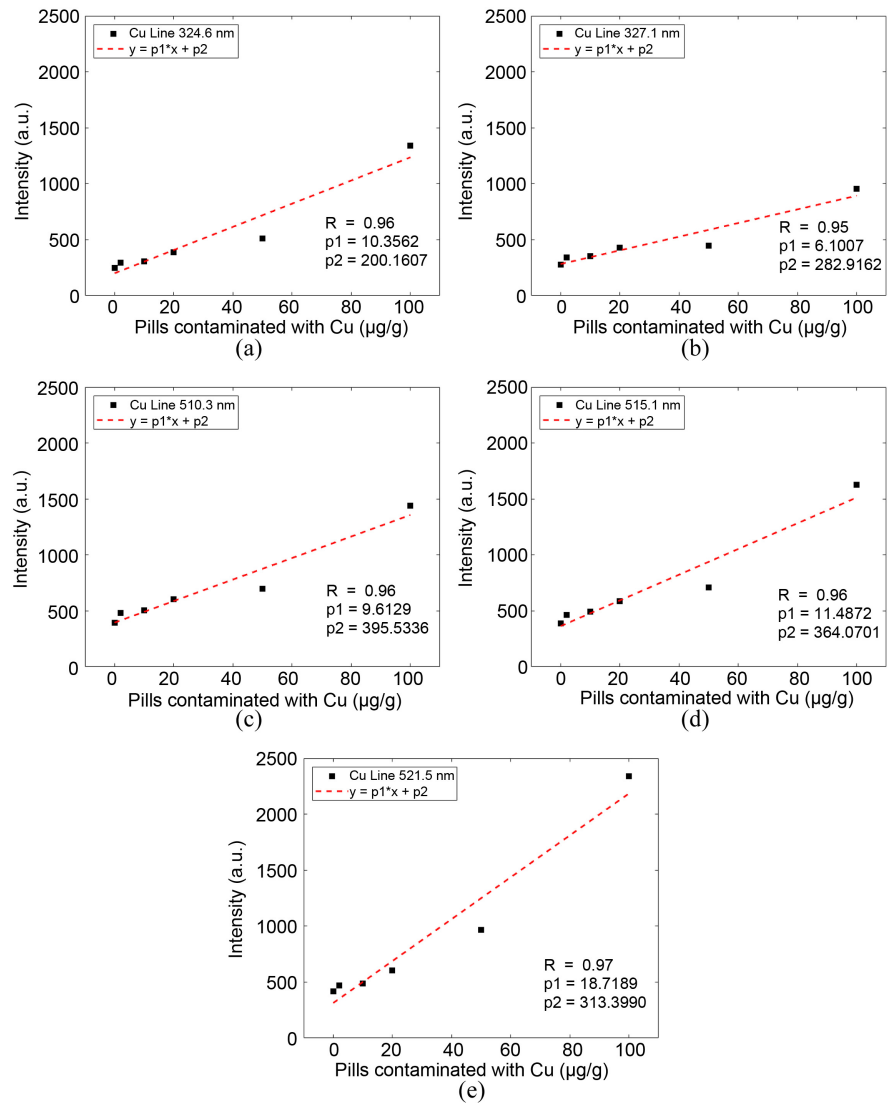


Figure 8. Intensities of Cu emission lines: (a) 324.6 nm, (b) 327.1 nm, (c) 510.3 nm, (d) 515.1 nm, and (e) 521.5 nm; for concentrations 0, 2, 10, 20, 50, and 100 $\mu\text{g/g}$.

made intra-site and inter-site clusters of the oysters according to their geographical origin. Furthermore, our samples also showed lines associated with Cr and Mn. From the nutritional point of view [28], traces of the major essential nutrients Ca, Na, and Mg, and ultra-essential nutrients such as Cr and Mn are present in the oysters' tissue analyzed in this research.

Based on the NIST database, Al I has emission lines at 394.4 and 396.2 nm, very close to those of Ca II (396.3 nm, 396.8 nm); however, they vary in intensity is likely no aluminum present in our samples. Our spectra do not contain emission lines lesser than 380 nm due to the spectrometer technical characteristics, so it was impossible to identify C, Si, and Zn.

The emission line at 521.5 nm presented the highest intensity when increasing the Cu concentration. Furthermore, when applying the least-squares method, a correlation coefficient of 0.97 was found. These results open the possibility of

using the method proposed in [18] to quantify Cu concentrations utilizing LIBS. Our results agree with [18] in which the Cu emission lines show an increase when the Cu concentration is greater than or equal to 100 µg/g.

7. Conclusions

Our results show that through LIBS technique can identify Cu concentrations in oyster tissue. The intensity of emission lines 324.6, 327.1, 510.3, 515.1, and 521.5 nm present a linear behavior associated with the Cu concentration. We consider it convenient to repeat the experiment with a higher number of Cu concentrations to improve the linear fit. On the other hand, identifying the detection limit continues to be an open problem, for which it would be desirable to explore the capacity of the technique to identify Cu concentrations lower than 50 µg/g. Also, it is necessary to determine the calibration curve to estimate Cu concentration, and results must be validated using techniques such as ICP or atomic absorption.

The oyster *Crassostrea virginica* is a natural resource of economic importance in the study area, so its characterization is relevant. Hence, the LIBS technique can carry out continuous monitoring of natural banks or oyster crops at different times of the year. Our results show that the applied method can identify seasonal differences; however, the causes were not determined. The methodology applied to tissue samples can be used to analyze other aquatic organisms (mollusks, crustaceans, fish, turtles), even considering other heavy metals.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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