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INTRODUCTION

In recent years, laser-induced breakdown spectroscopy (LIBS) has been considered a very promising analytical tool for qualitative and/or quantitative chemical analysis. LIBS is a method that uses a laser-generated plasma as the vaporization, atomization, and excitation source to determine the elemental composition of a sample by optical emission spectrometry (OES). The main advantages pointed out for LIBS comprise minimal or no sample preparation while increasing sample throughput and reduction of tedious and time-consuming sample preparation procedures; versatile sampling for all media, including both conducting and non-conducting materials; direct analysis of extremely hard materials that are difficult to get into solution (e.g., ceramics, glasses, and superconductors); analysis of microregions with a spatial resolving power better than 100 μm; direct analysis of aerosols or ambient air; and analysis in a hostile environment. Several reviews and recent textbooks describing the state-of-the-art of LIBS, reflect the growing interest of this technique in widely different areas such as agricultural, environmental, industrial, forensic, and clinical sciences.

In addition to the already mentioned features, an important advantage of LIBS over conventional spectrometric analytical methods is its ability to perform in situ elemental determinations using portable instruments. Because the plasma is formed by focused optical radiation, LIBS can also be used to interrogate samples remotely by stand-off analysis. Nevertheless, there is a lack of LIBS methods when compared to well-established atomic spectrometric methods such as ICP-OES (inductively coupled plasma optical emission spectrometry), GF-AAS (graphite furnace atomic absorption spectrometry), and ICP-MS (inductively coupled plasma mass spectrometry).

As in laser ablation ICP-MS, the analytical performance of LIBS for quantitative elemental determination depends strongly on the laser pulse characteristics (i.e., energy, duration, repetition rate, and wavelength), as well as on sample properties. In many cases, matrix effects also impair quantitative elemental analysis and, consequently, the evaluation of processes involved in laser–sample interaction is still necessary for LIBS development.

Recently, some authors investigated the use of femtosecond lasers in LIBS (fs-LIBS). Most works dealt with analysis of metallic samples and just a few contributions presented fs-LIBS applicability for the analysis of biological materials. For instance, Xu et al. demonstrated the feasibility of remote detection and differentiation of some similar agricultural-activity related bioaerosols using femtosecond filament-induced breakdown spectroscopy, showing the detection of molecular C2 and CN bands, as well as atomic and/or ionic lines from Si, C, Mg, Al, Na, Ca, Mn, Fe, Sr, and K in targets located 4.7 m away from the detection system.

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Samek et al.\textsuperscript{28} demonstrated the use of fs-LIBS for Fe determination in leaf samples and pointed out the potential of fs-LIBS as a technique for identification of storage and trafficking of iron ions within different plant compartments and in individual plant cells with minimal collateral damage and high spatial distribution.

Baudelet et al.\textsuperscript{29} used fs-LIBS to analyze bacterial samples, demonstrating that fs-LIBS reduced emission interferences from ambient air and increased the contrast (line peak to continuum ratio) for the detection of trace elements. The authors also observed a higher contrast between molecular and atomic emissions, implying a large concentration of molecular fragments in femtosecond laser-induced plasmas. In another study\textsuperscript{30} with fs-LIBS they also demonstrated that a kinetic study of the CN band head intensity allows the identification of the contribution of native CN molecular bonds from the biological medium. In addition, the possibility of discriminating microbiological samples from the correlation of Na, Mg, P, K, Ca, and Fe line emissions was shown.\textsuperscript{31}

Xu et al.\textsuperscript{32} reported the remote time-resolved filament-induced breakdown spectroscopy (FIBS) of biological materials (i.e., egg white and yeast powder) with targets located 3.5 m away from the detection system. Elemental lines and band emissions from Ca, Na, CN, and NH\textsubscript{2} were used to identify the biological species. It was concluded that FIBS can be a good alternative for remotely detecting and identifying biological species when combined with time-resolved measurements. However, in view of the complexity of biological systems, additional effort is necessary for practical applications of this technique in remote analysis of potentially hazardous biological agents.

Assion et al.\textsuperscript{33} used fs-LIBS for Ca analysis of biological samples with high spatial resolution, and a method was proposed for in situ investigation in the outer epidermal wall of a sunflower seedling stem with axial resolution of about 100 nm.

As emphasized by Russo\textsuperscript{34}, it is still early to decide on the use of ultrashort pulse lasers for LIBS, mainly because there are few analytical applications with no more than preliminary evaluations. However, the data available in the literature suggest that the smaller heat-affected zone, lower ablation threshold, finer depth resolution, lack of laser–plasma interaction, faster broadband-background decay, and matrix-independent sampling are the most attractive features of fs-LIBS. The aim of this work was to evaluate LIBS for the elemental analysis of animal tissues by using pellets of certified reference materials. We have studied the temporal behavior of emission lines and evaluated the detection ability of elements by using a femtosecond laser system.

**EXPERIMENTAL SETUP**

Laser-Induced Breakdown Spectroscopy Instrumentation. A schematic diagram of the LIBS system is shown in Fig. 1. Experiments were carried out with ultrashort pulses generated by a Ti:Sapphire chirped-pulse amplification (CPA) laser system (Coherent Mira-Seed pumping a Quantronix Odin amplifier). The 40 fs pulses, centered at 800 nm with 40 nm bandwidth, were generated at 90 Hz repetition rate with a maximum energy of (840 ± 20) μJ, in an 8 mm diameter beam with a beam quality factor $M^2 = 1.6$. Laser pulses were focused on sample pellets by a 7.5 cm focal length converging lens. The lens-to-sample distance was smaller than the lens focal length so that the laser intensity at the sample surface was below the air breakdown threshold, and it was adjusted to assure a high signal-to-noise ratio and the lowest relative standard deviation of measurements between successive sampling spots. To have a fresh spot for each laser shot, the pellets were fixed to a two-axis computer-controlled translation stage that performed 3 mm amplitude sinusoidal movement in each axis in the plane orthogonal to the laser propagation direction; adjusting each axis sinusoidal frequency independently, the laser etched Lissajous figures in the sample surface, covering a square area of 9 mm\textsuperscript{2}. Plasma emission was collected by a telescope composed of two fused silica lenses. A short focal length lens (5 cm) maximized the plasma emission collection solid angle, and a 12.5 cm focal length lens injected the collected light in the 600 μm core spectrometer fiber, matching its numerical aperture. The telescope and the laser beam optical axes were apart by 45 degrees.

A model ESA 3000 spectrometer (LLA Instruments GmbH, Germany) equipped with Echelle optics and a focal length of 25 cm with aperture of 1:10 and a 24.5 × 24.5 mm\textsuperscript{2} flat image plane was used. This system is a compromise that offers maximum resolution in the wavelength range between 200 and 780 nm with resolving power ranging from 10 000 to 20 000. The linear dispersion per pixel ranges from 5 pm at 200 nm to 19 pm at 780 nm. The wavelength calibration was checked by using Hg and Zn atomic lines from electrodeless discharge lamps (EDL II System, Perkin Elmer, Germany). The detector is an intensified charge-coupled device (ICCD) camera, comprised of a Kodak KAF 1001 CCD array of 1024 × 1024 pixels full frame (24 × 24 μm\textsuperscript{2}) and a microchannel plate image intensifier of 25 mm diameter coupled to a UV-enhanced photocathode. The image signals are digitalized in dynamic
range of 16 bits and further processed by an industrial computer. The features of using Echelle spectrometers equipped with an ICCD for analysis by LIBS methods can be found elsewhere.\textsuperscript{35} The dark current of the ICCD was automatically subtracted from the measured spectral data. For all measurements presented in this work, the plasma emission spectra were measured with a 200 ns integration time and 500 pulses were accumulated in 5.6 s (90 Hz measurements). The delay time after the laser pulse was evaluated by recording the emission intensity from different atomic and ionic lines.

**Samples and Certified Reference Materials.** All certified reference materials were lyophilized and cryogenically ground for 2 min by a cryogenic mill with a laboratory self-container liquid nitrogen bath model MA-775 (Marconi, Brazil). The potentialities of using cryogenic grinding are described elsewhere.\textsuperscript{36–38} The following certified reference materials were used for evaluation of fs-LIBS for elemental determinations: Oyster Tissue (National Institute of Standards & Technology, NIST 1566b), Bovine Liver (NIST 1577b), Cod Muscle (Community Bureau of Reference, BCR-CRM 422), Pig Kidney (BCR-CRM 186), Dogfish Liver (National Research Council Canada, NRCC Dolt-3), and Lobster Hepatopancreas (NRCC Tort-2). Pellets were prepared by transferring 2 g of powdered material to a 31 mm die set and applying a 2 ton/cm\textsuperscript{2} pressure. The pellets were approximately 3 mm thick and 31 mm in diameter. Binder agents were not required. At least three spectra of each sample were collected in different test portions of the pellet.

**RESULTS AND DISCUSSION**

**Experimental Conditions.** The lens-to-sample distance (LTSD) was optimized in order to obtain the highest emission intensity signals. The net emissions at the defined wavelengths were obtained by subtracting the background from the peak intensity of the line. The laser irradiance on the sample surface depends on LTSD affecting the emission line intensities and the mass of the ablated test portion.\textsuperscript{1} The LTSD was chosen to be between 1 and 2 mm, shorter than the focal length of the focusing lens. Under this condition, the ultrashort pulses are focused inside the material bulk and all the pulse energy is deposited in the sample. This optical arrangement provided a calculated beam radius on the sample surface of roughly 55 μm and laser fluence and irradiance of approximately 8 J/cm\textsuperscript{2} and 2 × 10\textsuperscript{14} W/cm\textsuperscript{2}, respectively, at the maximum pulse energy of 840 μJ.

Figures 2A and 2B show a typical temporal evolution of a fragment of LIBS spectra from 60 to 140 ns delay time after the laser pulse with a 200 ns integration time. Due to the transient nature of laser-induced plasmas, the population of the various species present in the plume rapidly evolve with time and position.\textsuperscript{39} At 60 ns delay time, there are only a few contributions of the continuum emission due to Bremsstrahlung processes, which involves collisions of electrons with ions and atoms (i.e., free–free emission) and recombination of electrons with ions (i.e., free–bound emission).\textsuperscript{39} After 60 ns the continuum emission rapidly decreases as a consequence of the plasma expansion and cooling, and the ionic lines become progressively narrower due to the decrease of the Stark broadening effect. At 140 ns, ion lines are weak while the atomic lines present a slower decay on emission intensity. This fast plasma time evolution agrees with the data previously reported by Eland et al.\textsuperscript{40} when using fs-LIBS for analysis of steel and glass and with Baudelet et al.,\textsuperscript{29} who recently compared nanosecond and femtosecond LIBS for bacteria analysis. As a compromise, in order to provide high signal-to-background ratios (SBR), an 80 ns delay time was chosen for further measurements.

The excitation temperature of the plasma was estimated through Boltzmann plots\textsuperscript{1} using Fe(I) line emission from a plasma induced in a dogfish liver reference material. At 80 ns delay time the spatially averaged value of the excitation temperature was estimated as (5860 ± 230) K. Although the temperature measurements analyzed by a Boltzmann plot presented a low coefficient of variation (approximately 4%, \( n = 3 \)), it should be mentioned that the method is accompanied by errors related to the transition probabilities. Le Drogo et al.\textsuperscript{59} estimated that the relative error of the excitation temperature, measured by a Boltzmann plot, was 20% by using atomic iron lines.

The excitation temperature of the femtosecond laser-induced plasma in an animal tissue was lower than the temperatures generally obtained by nanosecond laser-induced plasma (i.e., 8000–12 000 K). The lower temperature of fs-LIBS compared

![Temporal evolution of the plasma spectrum of lobster hepatopancreas (NRCC Tort 2).](image-url)
to ns-LIBS has been reported.\textsuperscript{29,39} In the femtosecond regime, the absorbed laser energy is fully deposited in the sample and no further laser–plasma interaction takes place.\textsuperscript{39} Thus, immediately after the laser shot, the plasma can only cool down, as no other source of energy is supplied to the plasma. According to Baudelet et al.,\textsuperscript{29} because the continuum emission is related to temperature, the weak continuum emission in the femtosecond regime confirms the lower temperature of plasma induced by femtosecond lasers. Due to the lower plasma temperature, emission lines are not superimposed by continuum, are less broadened than in the nanosecond regime, and can be detected from biological tissues with a higher signal-to-background ratio. Additionally, the higher temperatures observed in nanosecond plasmas when compared to femtosecond ones are a direct consequence of the higher energy deposited in the material, which is typically two orders of magnitude greater in ns pulses. This has to be studied further in future works.

The emission line intensities in LIBS depend on many factors, including the ambient gas atmosphere.\textsuperscript{41} Figure 3 shows that the line emissions obtained under argon flow at 0.5 L/min were significantly more intense than in the presence of air. Tognoni et al.,\textsuperscript{11} reviewed the influence of the ambient gas on the LIBS plume in several applications. Kim et al.,\textsuperscript{42} observed the signal increase and longer plasma lifetime in argon atmosphere and explained this phenomenon with the smaller conductivity and specific heat of argon gas with respect to the corresponding air values. According to the authors,\textsuperscript{42} such differences in the thermal properties result not only in a plasma of higher temperature, leading to a stronger emission, but also in a slower cooling of the plasma, implying a longer emission period. In addition, the argon environment also minimizes the formation of stable compounds such as oxides, which will certainly reduce the emissions from the excited analytes. Wisbrun et al.,\textsuperscript{43} pointed out that, in lower ionization potential atmosphere, the plasma is easily produced and its final temperature is expected to be higher. In addition, the cooling processes of the excited atoms in the plasma are subjected to gas masses. When the surrounding atmosphere is heavier, the collisional translational energy transfer is less effective, and the plasma lifetime is longer. In general, the relative line emission intensities are higher as the masses are heavier and when the ionization potential is lower.

**Elemental Analysis.** Pellets produced from certified reference materials were analyzed by the described LIBS system by applying 500 consecutive laser shots in 3 different areas at the sample surface under argon atmosphere (argon flowing at 0.5 L/min). Data were based on cumulative spectra \((n = 500)\) by using 80 ns delay time and 200 ns integration time. Figure 4 presents an example of a spectrum of plasmas induced in the pellet surface of oyster tissue in the range from 200 to 780 nm. An important feature of femtosecond ablation is the intense emission from CN molecular bands observed in the 370–390 nm region for the pulse energies used in the present work (up to 840 \(\mu\)J). The ablation in the femtosecond regime generally produces more “native” molecular species than in the nanosecond regime and this property of fs-LIBS can be used for the characterization of organic and biological samples.\textsuperscript{29}

Figures 5 and 6 show the calibration curves for calcium and copper, respectively. Although most certified values are based on a minimum sample mass from 150 to 250 mg, it was observed that they can be used for analytical calibration purposes. The calculated coefficient of variation of the line intensities observed in the plasma induced in each pellet of the certified reference materials varied from 10 to 22% \((n = 3)\),
which are relatively low taking into account that the estimated ablated test portion for each 500 pulses was lower than 50 μg. The horizontal bars in the X-values indicate the uncertainties of the certified mass fractions (at 95% confidence level).

In principle, it can be suggested that the main reason for the relatively high coefficients of variation of the results is the microheterogeneity of the analytes in the sample pellet. In this sense, it must be observed that the uncertainties associated with

![Fig. 5](image-url)

**Fig. 5.** Analytical calibration curve at Ca(II) 393.366 nm with reference materials: NIST 1577b: (116 ± 4) μg g⁻¹; BCR 186: 295 μg g⁻¹; BCR 422: 330 μg g⁻¹; NIST 1566b: (838 ± 20) μg g⁻¹. Delay time: 80 ns; integration time: 200 ns. Vertical bars are standard deviations (1 s) of three measurements from 500 laser shots each, and horizontal bars are the confidence interval of certified mass fractions. (*) Reference values only.

![Fig. 6](image-url)

**Fig. 6.** Analytical calibration curve at Cu I 324.754 nm using reference materials: NRCC Dolt-3: (31.2 ± 1.0) μg g⁻¹; NIST 1566b: (71.6 ± 1.6) μg g⁻¹; NRCC Tort-2: (106 ± 10) μg g⁻¹; NIST 1577b: (160 ± 8) μg g⁻¹. Delay time: 80 ns; integration time: 200 ns. Vertical bars are standard deviations (1 s) of three measurements from 500 laser shots each, and horizontal bars are the confidence interval of certified mass fractions.
the certified mass fractions of most reference materials is lower than 2–4% at a 95% confidence level, but for test portions of 150 mg, at least. However, other causes of variation must be taken into account, because there are not only changes in sample homogeneity. For instance, it must be considered that the amount of vaporized sample mass may also change from crater to crater, which can be a result from deviations in laser pulse energy. In the present situation the uncertainty due to laser energy variation was less than 5% and in most measurements the uncertainties due to counting statistics were lower than 1%.

Notwithstanding, it is clear that additional investigations must be done in the near future taking into account the inhomogeneity of analyte distribution in different biological tissues. In this way, the LIBS technique seems suitable to investigate the micro-homogeneity of certified reference materials for calibration towards direct microanalysis of biologic materials.

Table I shows the detection limits estimated from data obtained from different certified reference materials. The detection limits were based on the standard deviation of the background (BG) noise, measured in the surroundings of the emission line of interest by selecting a spectral window (0.5 nm) at approximately constant BG, i.e., when no contribution of the analytical line was noticed. Limits of detection were calculated according to IUPAC recommendation, assuming that the standard deviation of BG was equivalent to the standard deviation of the blank. Except for Al, Sr, and Zn, which can be determined only in toxic levels, detection limits were appropriate for the other detected elements. Strontium is a non-essential element but can be found in human and animal tissues. In the present paper, it is was decided to point out the analytical capability of fs-LIBS for Sr detection because there is a probability that this element will in the future be found to have more significant roles in the nutrition of humans and animals.

With respect to Zn within normal expected concentrations in biological tissues, it is clear that the detection limit is not appropriate, i.e., the experimental setup used herein does not allow the determination of this analyte. For practical purposes, at present, LA-ICP-MS is the most appropriate method for direct analysis of liver and kidney tissues; the detection limit reported by Feldmann et al. (20 µg kg⁻¹ Zn) is far better than that shown in Table I. The question that arises is how to improve fs-LIBS detection limits. In principle, an increase in the pulse energy could lead to an increase in ablated mass. Besides, the ionizations are more important in LIBS than the mass ablated and due to the highly nonlinear processes involved in fs-LIBS the ionization could grow nonlinearly with the pulse energy (more ionization per ablated mass), improving the LODs.

A close look at all analytes in Table I, and following the recommendations of the Eurachem Guide for method validation, i.e., if the proposed method fits for the purpose, it can be observed that the proposed method, if applied for animal tissues such as liver, for example, is valid for the determination of Na, K, Ca, Mg, P, Fe, and Cu, but not for Zn and Sr. When compared to other techniques, there is no doubt that the relative detection limits obtained by LIBS are not yet comparable to those observed by ICP-MS, ICP-OES, and GF-AAS. In spite of this observation, fs-LIBS can fit for the purpose for the determination of important elements in animal nutrition.

CONCLUSION

Although spectra of fs-LIBS are very well resolved and presented a very low background emission, allowing accumulation of signals, efforts must be made to improve detection limits for the determination of Al, Sr, and Zn. It is important to point out that the small amount of ablated mass can be an advantage when analyzing unique samples, where the material removal should be as minimal as possible. Moreover, these features are useful for the development of quantitative methods for analysis of animal tissues and probably could be applied for characterization of malignant tissue cells by LIBS or for imaging analysis of elements throughout an entire organism, allowing studies of bioavailability, transport process, and contamination and monitoring of environmental risks.

It seems clear that even with the above-mentioned advantages, widespread use of femtosecond lasers in LIBS cannot be expected soon due to the actual cost and complexity of chirped-pulsed amplification laser systems. However, as was well emphasized by Gurevich and Hergenröder, the trends in other fields of application of these lasers (e.g., physical, medical, or material processing) probably will change this perspective in the near future. In addition, according to them the optimal experimental parameters for fs-LIBS implementation have not yet been found and, consequently, there is a large need for methodical experimental optimization.

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