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Direct analysis of plant leaves by EDXRF and LIBS: Microsampling strategies and cross-validation

Marcelo Braga Bueno Guerra[a], Andressa Adame[a], Eduardo de Almeida[a], Gabriel Gustinelli Arantes de Carvalho[a], Marcos Augusto Stolf Brasil[a], Dário Santos Jr[b], Francisco José Krug[a*]

[a]Centro de Energia Nuclear na Agricultura, Research Support Center, "Technology and Innovation for a Sustainable Agriculture", Universidade de São Paulo, Av. Centenário 303, 13416-000, Piracicaba - SP, Brazil.

[b]Departamento de Ciências Exatas e da Terra, Universidade Federal de São Paulo, Rua Prof. Artur Riedel, 275, 09972-270, Diadema - SP, Brazil.

*Corresponding author:

Prof. Francisco José Krug

Tel.: +55 19 3429 4774; fax: +55 19 3429 4774

E-mail address: fjkrug@cena.usp.br

Abstract

Microsampling strategies were evaluated for the direct analysis of dried sugar cane leaves by energy-dispersive X-ray fluorescence spectrometry (EDXRF) and laser-induced breakdown spectroscopy (LIBS). The analysis by EDXRF was carried out by irradiating each leaf fragment in its middle portion with a collimated 5 mm X-ray spot size during 50 s, allowing the determination of P, K, Ca, S, Fe, Mn and Si. EDXRF was also useful to conclude that 15 leaf fragments (37.5 % of the recommended sampling area) were enough for attaining a representative analytical response from the whole diagnostic leaf. Regarding LIBS, that employs a substantial smaller ablation area (*i.e.*, 750 μm laser spot size), sampling strategies were defined by taking into account the microchemical distribution of P, Ca, Mg, Fe, Mn, B and Si in 9 mm x 9 mm leaf fragment area. The proposed sampling protocol relied on the interrogation (rastering) of 3 equally spaced sampling lines in each leaf fragment with 48 accumulated laser pulses per line (Nd:YAG at 1064 nm, 5 ns, 10 Hz, 50 J cm^{-2}) perpendicularly to the leaf midrib. This strategy enabled the simultaneous determination of P, K, Ca, Mg, Fe, Cu, Mn, Zn, B and Si by LIBS. Cross-validation between LIBS and EDXRF for P, K, Ca, Fe, Mn and Si predicted mass fractions presented high linear correlation coefficients of up to 0.9778 (selecting 15 leaf fragments per diagnostic leaf from 10 different sugar cane varieties). Results provide insights into a novel and promising strategy for direct and fast plant nutrition diagnosis, fostering further studies for *in situ* analysis of fresh leaves, strengthening the implementation of Precision Agriculture and Green Chemistry concepts.

Keywords

LIBS, EDXRF, nutrients, foliar diagnosis, mapping, cross-validation, direct analysis.

Introduction

Foliar diagnosis has been used for almost a century[1-2] as a reliable tool to assess the nutritional status of vegetal crops.[3] To this end, the selection of the diagnostic leaf is relevant, as it is generally the most appropriate vegetal tissue for sampling, aiming at plant nutrition diagnosis based on macro- and micronutrients contents.[4-5] For sugar cane crop, the Top Visible Dewlap (TVD) leaf is the target plant tissue used for analysis.[5-6] Once collected in the field, the TVD leaves from a specific site are pooled in a composite sample and sent to the laboratory where they are washed, dried and homogenized.[5] The nutrient mass fractions are often determined by inductively coupled plasma optical emission spectrometry (ICP OES) after acid digestion of the ground samples.[3]

Alternatively, the elemental composition of the plant tissue can be determined by methods based on the direct analysis of solids.[3, 7-17] In these applications, the mostly applied analytical methods have been laser-induced breakdown spectroscopy (LIBS), laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), and energy-dispersive X-ray fluorescence spectrometry (EDXRF).

This research group has been recently involved in direct analysis of samples of agronomic and environmental interest by LIBS and EDXRF.[3, 18-19] The use of both methods has been proposed for the direct and simultaneous determination of P, K, Ca, Mg, S, Fe, Cu, Mn, Zn, B and Si in pelletized plant materials.[3, 11, 18, 20-22] From a practical point of view, the analysis of pelletized plant material is an elegant way to assess the nutritional status of crops, as it results in a substantial simplification of the analytical sequence when compared to methods requiring an *a priori* test sample decomposition step.[3, 22] The direct analysis of pellets can also improve trueness and

precision of measurements, and provides results in a faster way, as sampling throughput is improved.

An even simpler alternative is the direct analysis of plant leaves by LIBS[23-38] or EDXRF[39-40] without the need for the grinding and pelletizing steps. In spite of the LIBS and EDXRF attractive features in this context, most of the reported investigations have dealt with the qualitative or semi-quantitative determinations of nutrients and/or contaminants in fresh or dried leaves. Few studies have addressed the use of such methods for the direct quantification of macro- and micronutrients in leaves of vegetal crops of economic importance, namely sunflower, poplar, corn, orange and lemon.[41-44] It is also worth mentioning the lack of proposals of microsampling protocols for attaining representativeness when analyzing the inherently heterogeneous leaf samples. This purpose can be achieved by evaluating the spatial distribution of the nutrients over the diagnostic leaf by the construction of microchemical maps.

Frank et al.[43] applied EDXRF to the direct and simultaneous quantitative determinations of macro- (P, K, Ca, Mg and S) and micro- (Fe, Cu, Mn and Zn) nutrients in corn dried leaves. This study aimed at the comparison of results obtained from the analysis of unground leaf disks, ground leaf disks and ground whole leaf samples. Data from the analysis of the unground leaf blade disks by EDXRF statistically agreed with those from the ground material. In addition, it was recommended that influence of the midrib should be carefully taken into account, especially when nutrients mass fractions are near their critical levels.

Ma et al.[42] applied femtosecond LIBS to the direct analysis of dried poplar tree leaves by carrying out a calibration-free method for the quantitative determination of N, P, Ca and Fe, but no information regarding method validation and assessment of sampling strategies was provided.

Nevertheless, the effective and systematic implementation of the direct analysis of plant leaves requires prior elucidation of two meaningful points. Firstly, the evaluation of the most appropriate sampling strategy that overcomes the inherent heterogeneous elemental distribution over the diagnostic leaf.[45] This point is of paramount importance, as it addresses the compromise between analytical throughput and representativeness of the selected test portion. Secondly, the implementation of quality control and quality assurance procedures for the consolidation of these direct methods of analysis, making them as reliable as the well-established wet-based traditional approaches.

This work aimed at investigating the suitability of both EDXRF and LIBS for the direct analysis of dried sugar cane leaves for assessment of its nutritional status. For this purpose, leaves from 10 varieties of sugar cane were used. To the best of the authors' knowledge, this is the first study regarding the evaluation of both methods together for the direct analysis of plant leaves aiming at the proposal of appropriate microsampling strategies enabling direct foliar diagnosis. These efforts can provide insights into novel perspectives for *in situ* applications using both commercially-available portable LIBS and EDXRF systems.

Materials and methods

Samples

Sugar cane leaves were collected in Piracicaba, SP, Brazil, as recommended by McCray et al.[5] The following sugar cane varieties were used as laboratory samples: CTC 2, CTC 4, CTC 9, CTC 20, RB 92-579, RB 81-3250, RB 85-5156, RB 85-5453, RB 86-7515, SP 83-2847. These samples were chosen because they represent the most cultivated sugar cane varieties in the Brazilian South-Central region.[46] The middle third

portion of the TVD leaf was selected and sent to the laboratory. In the lab, leaf blades were washed with tap water and subsequently with deionized water. Thereafter, the midribs of all leaves were manually removed.[5] Samples were placed in paper bags and dried at 60 °C until constant weight. Sub-samples composed of leaf fragments (n = 40 per leaf) ranging from 1.5 – 2.0 cm length and *ca.* 1.5 cm wide were obtained from the dried middle-third leaves using a stainless steel scissor. Aiming at obtaining test samples with flat surfaces, the leaf fragments (n = 10 per round) were pressed between two paper disks into a stainless steel die set (ø = 31 mm) applying 3 ton during 3 s using a pneumatic press (Spex 3624B X-Press). After analysis by EDXRF and LIBS, all leaf fragments (sub-samples) from the 10 sugar cane varieties were ground in a cryogenic mill (Spex model 6800, USA) following the procedure described by Nunes et al.[7] The obtained composite samples were pelletized in a Spex model 3624B X-Press by transferring 0.5 g of the ground plant material to a 15 mm internal diameter die set and applying 8.0 t cm^{-2} during 5 min in order to obtain pellets (n = 10) of *ca.* 2 mm thickness.

EDXRF instrumentation

An energy dispersive X-ray fluorescence spectrometer (EDX 720, Shimadzu, Kyoto, Japan) furnished with a 50 W Rh anode X-ray tube, and a Si(Li) semiconductor detector with resolution lower than 155 eV at Mn Kα 5.90 keV was used. Leaf fragments (test samples) were placed onto a 16-sample turret located inside the irradiation chamber permitting automatic sample changing under pressure below 0.23 Torr. The monitored spectral region ranged from 1 to 40 keV. The collimated X-ray spot size was 5 mm.[18]

LIBS instrumentation

A Q-switched Nd:YAG laser (Brilliant, Quantel, France) at 1064 nm, which generates 5 ns pulses of up to (365 ± 3) mJ at 10 Hz and with a 6 mm diameter beam (quality factor $M^2 < 2$) was used. The laser pulses were focused through a convergent lens (Newport, USA) with 2.54 cm diameter and 20 cm focal length, placed at 50 cm from the laser output aperture. Test samples (*i.e*. leaf fragments) were fixed in a lab-made x-y automatic translation stage that permitted precise micrometric movements over the two axes in the orthogonal plane to the laser direction. The atmospheric air on the sample surface was displaced by flowing argon at 5.0 L min^{-1} directly at the beam incident point.[22] The radiation emitted by the plasma was collected using a telescope composed by 50 mm and 80 mm focal length fused silica lenses (LLA Instruments GmbH, Germany) and coupled to the entrance slit of a spectrometer (model ESA 3000, LLA Instruments) with Echelle optics and ICCD detector using an optical fiber (1.5 m, 600 μm core). The monitored spectral range was 200 – 780 nm with a resolving power ranging from 10 to 20 pm. The collection angle with respect to the laser optical axis was 25 °. The spectra data treatment was carried out by the software ESAWIN 3.22 of the same manufacturer (LLA) by activating the slit height tool. Three equally spaced sampling lines per leaf fragment were scanned by LIBS (rastering speed: 2.25 mm s^{-1}) with 48 accumulated laser pulses per line at 50 J cm^{-2} with 10 Hz repetition rate and 750 μm spot size (2 μs delay and 5.0 μs integration time gate).[47-48]

Elemental mapping

The elemental distribution in the leaf fragments was determined by the interrogation of 100 equally spaced sampling spots by LIBS. The emission spectra of

each sampling spot were gathered after the accumulation of 5 laser pulses at 50 J cm^{-2} using 750 μm spot size (2 μs delay and 5.0 μs integration time gate).[47-48]

Figure 1 summarizes the sampling protocol proposed for the direct analysis of sugar cane leaves by LIBS and EDXRF, as well as the strategy used for chemical mapping by LIBS involving the interrogation of 100 independent sampling spots in a 9 mm x 9 mm sampling grid.

Reference method for mass fraction determination

The reference method for P, K, Ca, Fe, Mn and Si determination in the pelletized plant materials from the 10 sugar cane varieties was based on direct analysis by EDXRF using the abovementioned instrumentation following a validated method recently published elsewhere.[18] The determined mass fractions were then used as reference to build both LIBS and EDXRF calibration models.

Cross-validation

EDXRF and LIBS calibration curves were built with the reference mass fractions and the corresponding signal intensities obtained from the analysis of 15 randomly chosen leaf fragments from the 10 sugar cane varieties.

Cross-validation was performed by correlating the P, K, Ca, Fe, Mn and Si mass fractions predicted by LIBS and EDXRF calibration models.

Results and discussion

Preliminary analysis

Performances of both EDXRF and LIBS for the direct analysis of dried leaves were initially investigated with a randomly chosen fragment of sugar cane leaf. The

fragment was obtained from the RB 86-7515 sugar cane variety, which is the most cultivated one in the Brazilian South-Central region.[46] Figure 2 shows the EDXRF (1 – 10 keV) and LIBS selected spectral regions obtained from the analysis of this leaf fragment, highlighting the main emission lines and characteristic X-rays of 5 macronutrients (P, K, Ca, Mg and S), 5 micronutrients (Fe, Cu, Mn, Zn and B) and a beneficial element (Si). It should be informed that sulfur was not detected by LIBS, since the most prominent lines are below the 200 – 780 nm spectral range of the optical emission spectrometer used herein. On the other hand, sulfur was properly detected by EDXRF, whereas magnesium, boron, copper and zinc were not. The detection of magnesium and boron by EDXRF was not feasible owing to their low fluorescent yields of 2.9 and 0.14 %,[49] respectively, and due to their expected concentration ranges in plant leaves (1 – 10 g kg^{-1} Mg and 10 – 200 mg kg^{-1} B).[47, 50] Similarly, copper (Kα 8.05 keV) and zinc (Kα 8.64 keV) were not detected in view of their low mass fractions ranges typically presented in sugar cane leaves (3.8 – 5.0 mg kg^{-1} Cu and 12.6 – 14.8 mg kg^{-1} Zn).[51]

These preliminary results revealed that both methods are equally important for the direct analysis of plant leaves for detecting the abovementioned 11 elements of interest, indicating that they can be used together aiming at plant nutrition diagnosis.

Evaluation of EDXRF measurement time

EDXRF measurement time was evaluated from 10 to 100 s (live time) by considering the coefficient of variation (CV) from five consecutive measurements carried out in one fragment of sugar cane leaf (RB 86-7515 variety). For 50 s, the CV of measurements ranged from 0.30 % for Ca to 6.2 % for Mn, and averaged 1.8 % regarding all elements, which can be considered appropriate for analytical purposes. No

substantial improvements in the measurement precision were observed with measurement times higher than 50 s.

Microsampling representativeness

Microsampling representativeness was firstly evaluated by EDXRF considering the RB 86-7515, SP 81-3250 and RB 85-5453 sugar cane varieties. In order to define the minimum number of fragments that represent the whole middle third leaf, the average characteristic P, K, Ca, S, Fe, Mn and Si Kα peak intensities from the analysis of the sets composed by 5, 10, 15 and 30 randomly chosen leaf fragments (representing 12.5, 25, 37.5 and 75 % of the whole diagnostic leaf area) were statistically compared with the average result from all 40 fragments (*i.e.*, 100 % diagnostic leaf area). Under these premises, the analytical response from 37.5 % (n = 15 fragments) of the leaf area was representative to the whole sample (Figure 3). The most prominent difference was observed for Ca in the SP 81-3250 variety, where the relative error was reduced from 16 % (for 5 fragments, 12.5 % diagnostic leaf area) to 3 % (for 15 fragments, 37.5 % diagnostic leaf area). The same trend was also observed for S, as the relative error diminished from 10 % (5 fragments, 12.5 % diagnostic leaf area) to 1.6 % (15 fragments, 37.5 % diagnostic leaf area). For the remaining evaluated elements, sampling 15 leaf fragments was appropriate for this intended purpose.

Another alternative to evaluate the sampling representativeness was based on the construction of microchemical maps of P, Ca, Mg, Fe, Mn, B and Si in 9 mm x 9 mm leaf fragment area by LIBS for assessing the spatial distribution of the analytes. The selected number of accumulated laser pulses (*i.e.*, 5 consecutive pulses) was defined as the threshold to perforate the leaf fragment at the experimental conditions set herein (50 J cm^{-2}, 750 μm spot size and 10 Hz). In addition, the microchemical maps were built

just for the elements whose emission signal intensities were detectable (*i.e.*, signal-to-noise ratio (SNR) > 3 for all sampling spots) under the abovementioned LIBS instrumental conditions. In general, a clear trend in the distribution of the detected elements did not manifest itself (Figure 4).

Data generated from the average emission intensities of the analytes from the 100 selected sampling spots (*i.e.*, the whole sampling grid in each leaf fragment) were statistically compared to the corresponding average emission intensities of 3 or 5 equally spaced sampling lines in the sampling grid. Each sampling line comprised 10 independent sampling spots (Figure 1), which were either parallel or perpendicular to the leaf midrib. Results for a leaf fragment from the RB 86-7515 variety are shown in Figure 5. From these data, the most appropriate microsampling strategy relied on the combination of the spectra generated from sampling lines perpendicularly to the leaf midrib, as the average emission signal intensities from 3 or 5 lines along this direction statistically agreed with the average values from the whole leaf fragment. This was statistically confirmed by applying the unpaired Student *t*-test at 95 % confidence level. The same behavior was observed for the other two leaf fragments mapped by LIBS from the RB 86-7515 sugar cane variety.

LIBS analysis

Based on the aforementioned findings, the rastering sampling strategy (*i.e.* laser ablation scanning mode) of three sampling lines along the perpendicular direction to the leaf midrib was chosen in order to improve analytical throughput and sensitivity. In this approach, the test sample continuously moves along the direction perpendicular to the leaf midrib by using an automatic translation stage. The rastering speed (2.25 mm s^{-1}) and the laser shooting (n = 48 accumulated laser pulses per sampling line, 10 Hz) were

experimentally synchronized permitting the detection of P, K, Ca, Mg, Fe, Cu, Mn, Zn, B and Si with appropriate sensitivities. Moreover, the analytical throughput is also an appealing feature of the proposed sampling strategy, as it takes less than half a minute to ablate three lines perpendicularly to the leaf midrib. It is noteworthy that this sampling approach provided a substantial improvement in the SNR for all analytes (up to ten fold better for Cu I 324.754 nm and K I 404.414 nm emission lines) as a consequence of the spectra accumulation (Figure 6).

Cross-validation

The cross-validation between LIBS and EDXRF for P, K, Ca, Fe, Mn and Si predicted mass fractions are shown in Figure 7. Mass fraction uncertainties were not provided because each result was obtained from the average of independent measurements performed in 15 leaf fragments, which are not authentic replicates of themselves. The linear correlation coefficients (r) ranged from 0.8704 (P) to 0.9778 (Ca) by considering the average spectra obtained from the analysis of 15 randomly selected leaf fragments from 10 sugar cane varieties.

Due to the inherent non-destructive characteristic of EDXRF, the same test sample was analyzed by both methods. Based on this possibility, the proposed analytical sequence was then defined by first interrogating the test sample by EDXRF and then by LIBS. Another attractive feature towards cross-validation between LIBS and EDXRF is related to their similar analyzed test portions. This was achieved by ablating three sampling lines with 48 accumulated laser pulses at 10 Hz (2.25 mm/s rastering speed), thus interrogating an equivalent area of 36 craters of 900 μm diameter (*ca.* 23 mm^2), which is comparable to the irradiated area of the selected 5 mm X-ray spot size (*ca.* 20 mm^2).

Additional Remarks

There are promising future perspectives on the LIBS and EDXRF association for the direct determination of nutrients in plant leaves, which can encourage researchers to evaluate both methods for *in-situ* analysis of fresh leaves in crops of economic importance. Notwithstanding, additional studies must be performed in order to propose appropriate calibration strategies, which may involve *e.g.* use of the fundamental parameters method[52] for the elemental determination by EDXRF. In addition, use of calibration curves from the analysis of pelletized test samples (for EDXRF and LIBS)[3, 18] can also be taken into account, after nutrients mass fractions determination by a validated reference method based on microwave-assisted acid digestion and ICP OES analysis.[3, 53] Other possibilities include matrix-matched calibration standards[54] and even certified reference materials.[3]

Therefore, after establishing the most appropriate calibration strategies, it is expected that *in situ* analysis will be more easily performed and controlled with portable LIBS[55] and/or EDXRF[18, 21] instruments. In addition, novel challenges may arise, which can be derived from the real-time determination of moisture content in the corresponding fresh leaves.

In spite of the high potential synergy between LIBS and EDXRF, it is worth mentioning that this combination is not mandatory, as suitable nutritional assessment data can be obtained with the individual use of these methods. For instance, phosphorus, recognized as one of the most limiting nutrient in terrestrial ecosystems,[56] can be determined by either EDXRF or LIBS in dried plant leaves.

Other relevant outcomes of using these simultaneous multielemental techniques include the utilization of pattern recognition tools, such as Principal Component Analysis (PCA),[57] Hierarchical Cluster Analysis (HCA),[58] k-Nearest Neighbor

(KNN),[59] and others.[60] In these cases, the discrimination between healthy and nutrient-deficient plants can be performed without requiring quantitative data.

Conclusions and perspectives

The proposed microsampling protocol for the direct determination of macro- (P, K, Ca, Mg, S), micro- (Fe, Cu, Mn, Zn and B) nutrients and Si provides important insights towards plant nutrition diagnosis fostering promising studies for *in situ* analysis of fresh leaves, strengthening the implementation of Precision Agriculture and Green Chemistry concepts. Another point that deserves special attention is the ability to perform cross-validation for P, K, Ca, Fe, Mn and Si, as these elements can be determined by either LIBS or EDXRF in the same test sample. Results presented herein may be helpful to motivate further studies towards method validation and trials with other crops of economic importance. Direct analysis of dried leaves of sugar cane by LIBS and EDXRF can be considered an advanced approach compared to the analysis of pelletized test samples by reducing the sample preparation steps to a minimum, thus minimizing the risk of systematic errors.

Acknowledgments

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP 2012/16203-5, 2010/16379-0) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq 309800/2011-0, 309679/2014-1) for financial support and grants. We also acknowledge B.S. Chem. Iolanda A. Rufini, Dr Lidiane C. Nunes and Dr Paulino F. Souza for sampling help. Dr José Lavres Jr and Dr Carlos Eduardo Faroni are thanked for discussions on plant nutrition and Prof. Elias A.G. Zagatto for critical comments and language improvement.

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FIGURE CAPTIONS

Figure 1 – Schematic overview of the proposed sampling protocol for LIBS and EDXRF direct analysis of sugar cane leaves. Leaf fragment depicts a 9 mm x 9 mm sampling grid (n = 100 sampling spots) used in the chemical mapping, as well as EDXRF and LIBS spot sizes. Ablation craters obtained after interrogation with LIBS by applying 5 consecutive laser pulses (50 J cm^{-2}, 10 Hz, 1064 nm) per site.

Figure 2 – EDXRF and LIBS selected spectral regions obtained from the analysis of a dried sugar cane leaf fragment (RB 86-7515 variety) under recommended experimental conditions. LIBS spectrum was obtained using the rastering microsampling strategy.

Figure 3 – Calcium and sulfur averaged relative errors from the analysis of leaf fragments (n = 5, 10, 15, 20 and 30). Error bars correspond to ± 1 standard deviation of relative errors from the analysis of 3 sugar cane varieties (RB 86-7515, SP 81-3250 and RB 85-5453). Data from 40 leaf fragments were the reference values used for estimating the relative errors.

Figure 4 – P, Ca, Mg, Fe, Mn, B and Si microchemical maps obtained by LIBS in the analysis of a dried sugar cane leaf fragment (RB 86-7515 variety). x = distance along the direction parallel to the leaf midrib; y = distance along the direction perpendicular to the leaf midrib.

Figure 5 – Comparison between P, Ca, Mg, Fe, Mn, B and Si reference data (average emission signal intensities from the whole leaf fragment) and average emission signal

intensities from 3 and 5 lines (a) parallel and (b) perpendicularly to the leaf midrib. Results from the analysis of one leaf fragment based on the average peak area of the following analytes emission lines: P I 213.618 nm, Ca II 315.887 nm, Mg I 277.983 nm, Fe II 259.940 nm, Mn II 259.373 nm, B I 249.772 nm and Si I 212.412 nm. Error bars correspond to ± 1 standard deviation from 3 or 5 average data.

Figure 6 – Influence of spectra accumulation on the Cu I 324.754 nm and the K I 404.414 nm emission lines in the analysis by LIBS.

Figure 7 – Correlation graphs between P, K, Ca, Fe, Mn and Si mass fractions predicted by LIBS (rastering approach) and EDXRF calibration models from the analysis of leaf fragments of 10 sugar cane varieties Data corresponding to the mean spectrum of 15 leaf fragments per sugar cane variety. Dotted lines represent 95 % confidence bands.

Figure 1

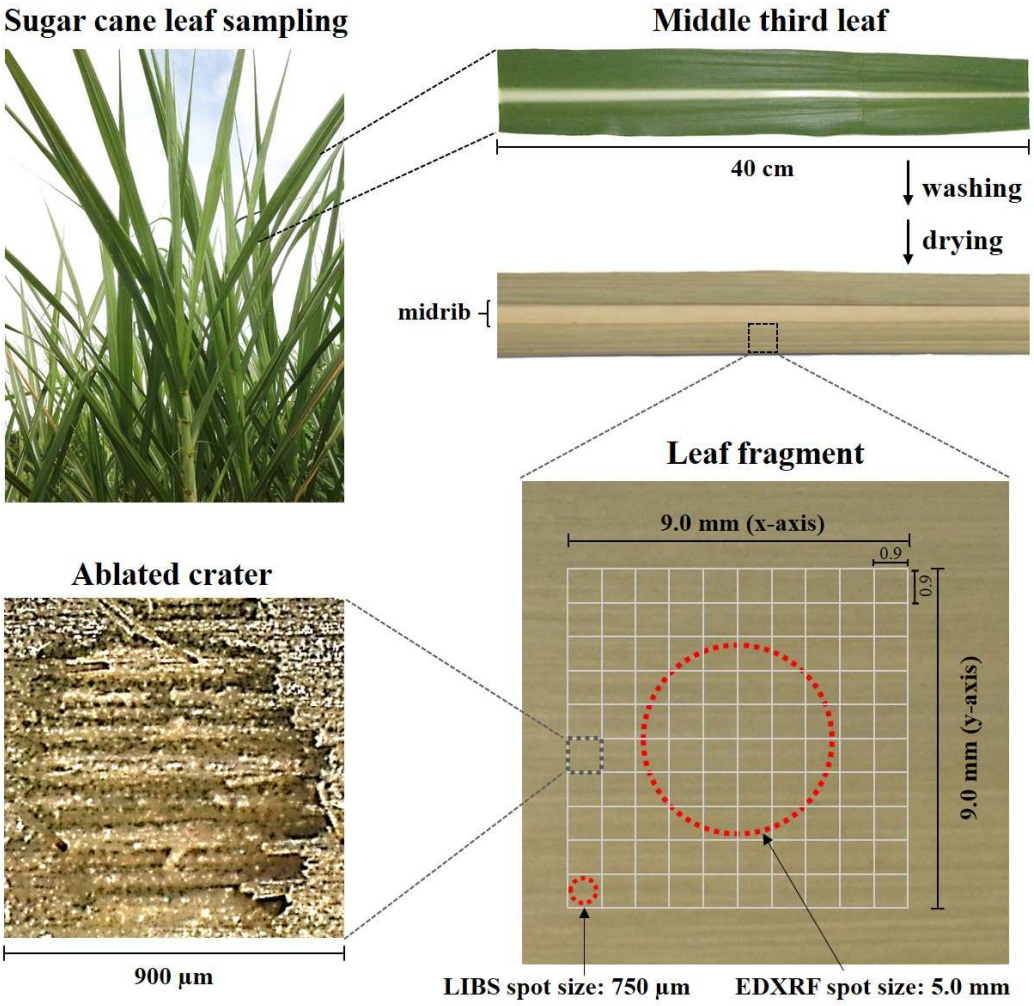


Figure 2

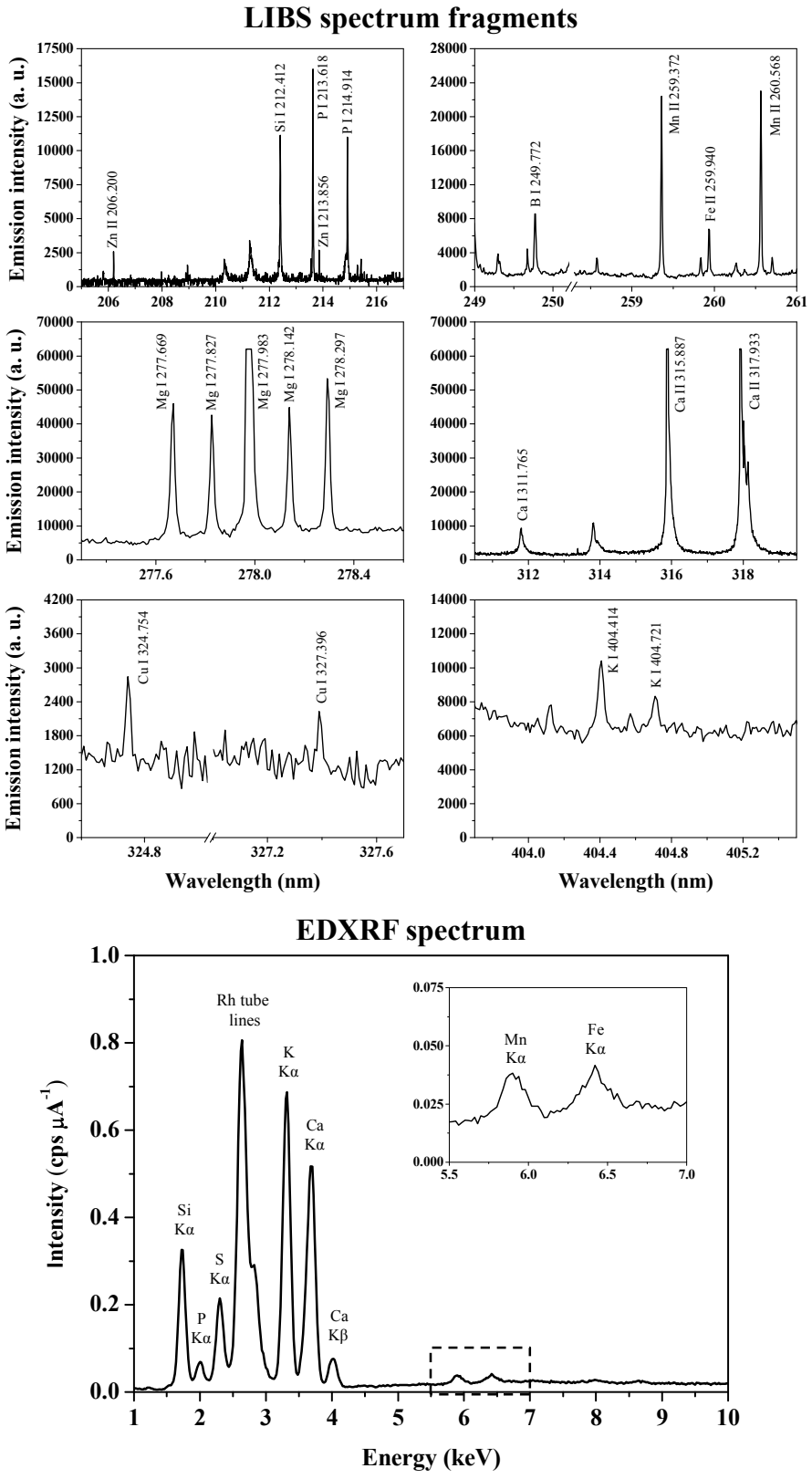


Figure 3

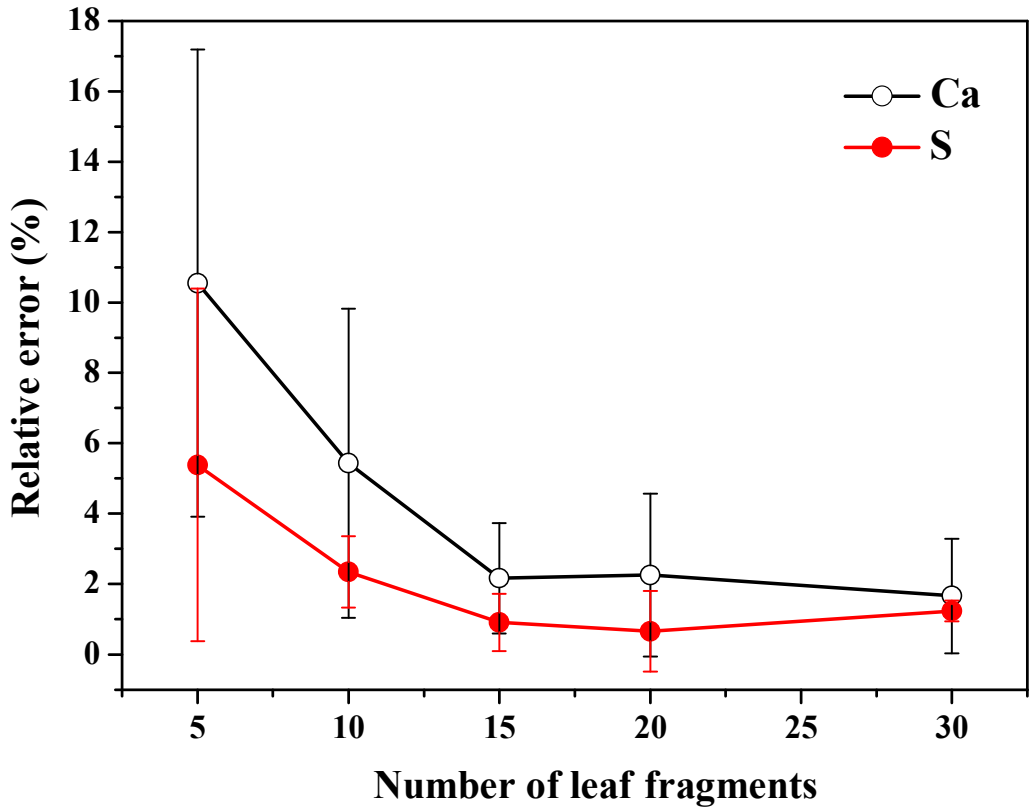
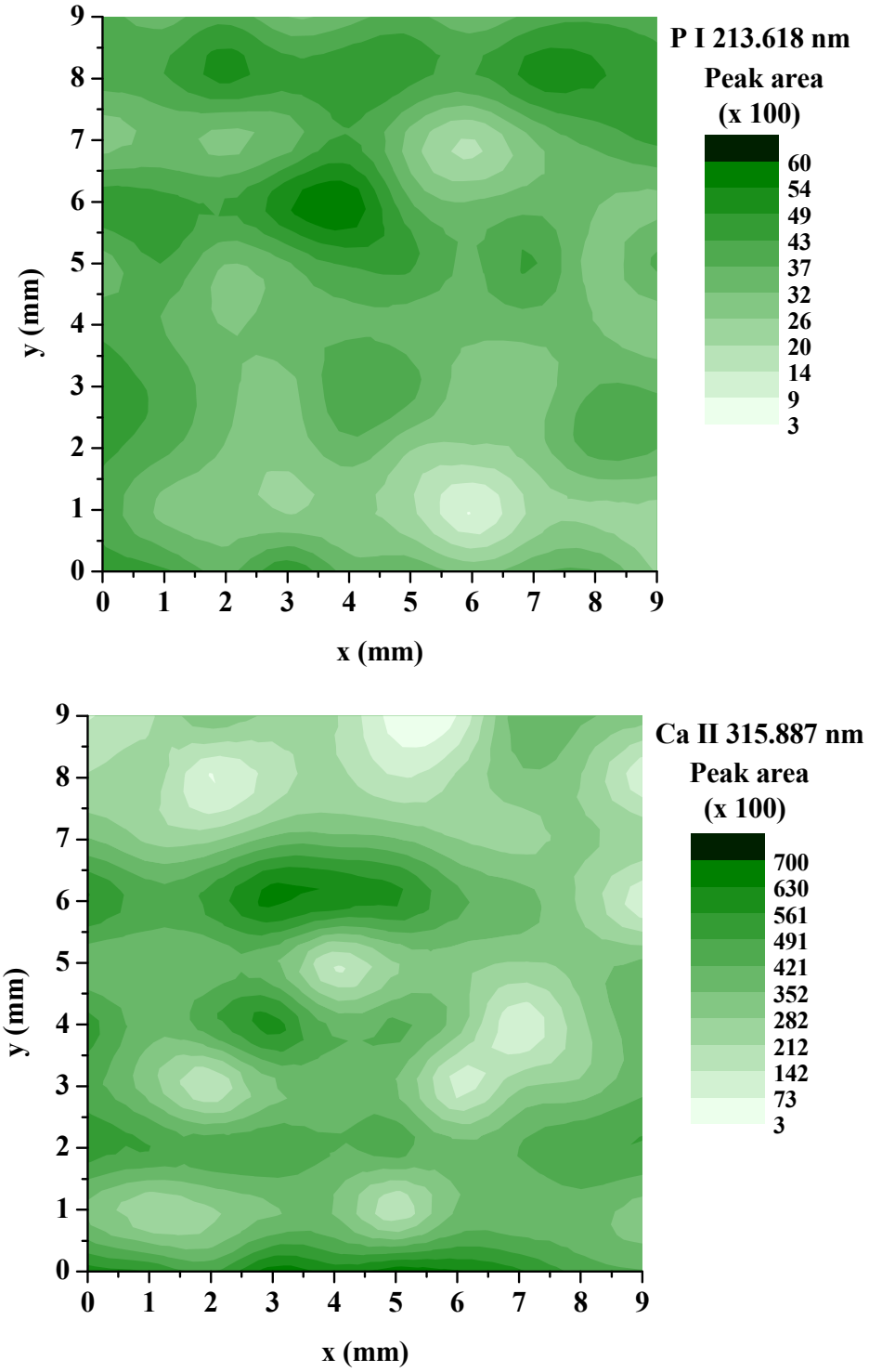


Figure 4



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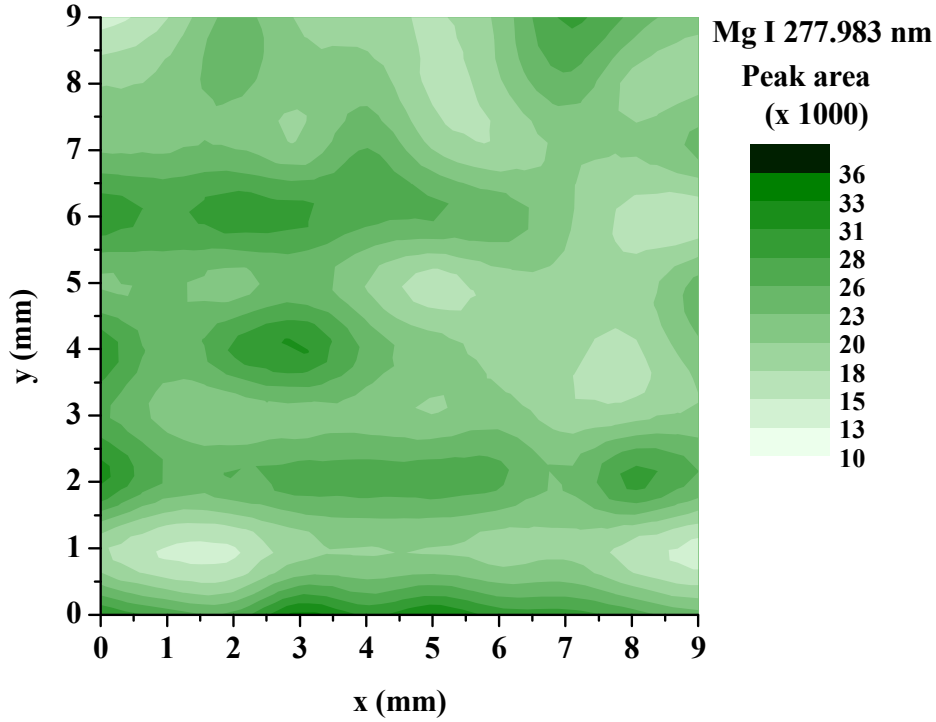

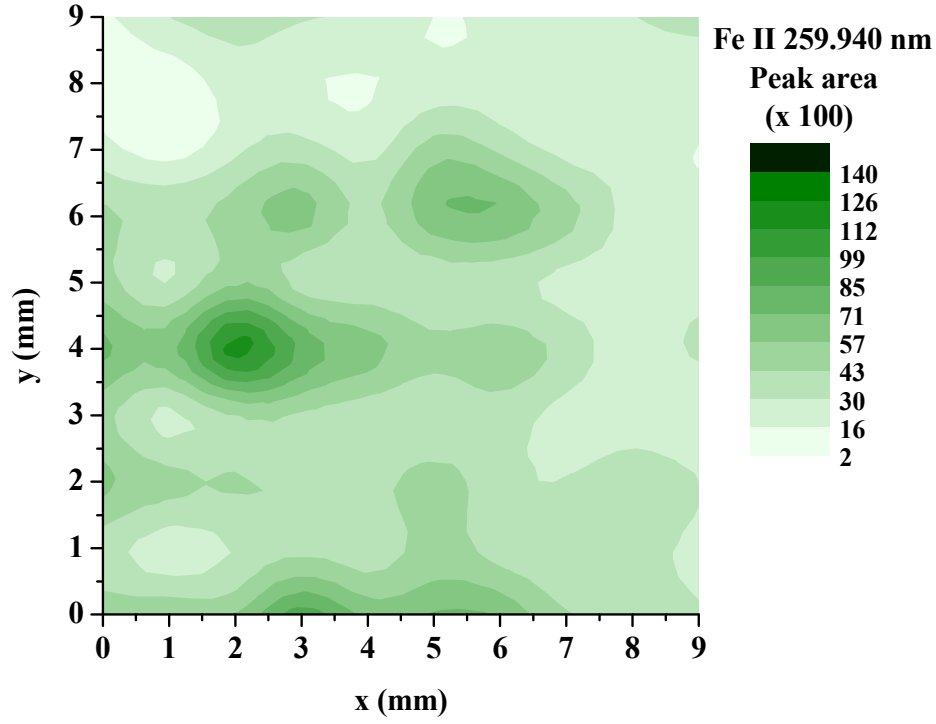

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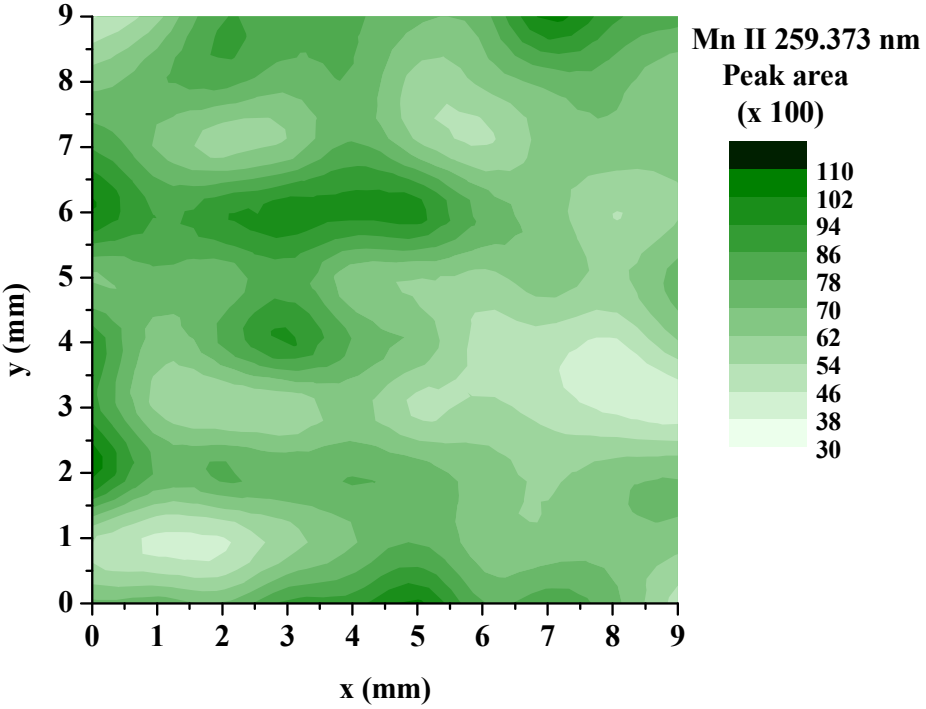

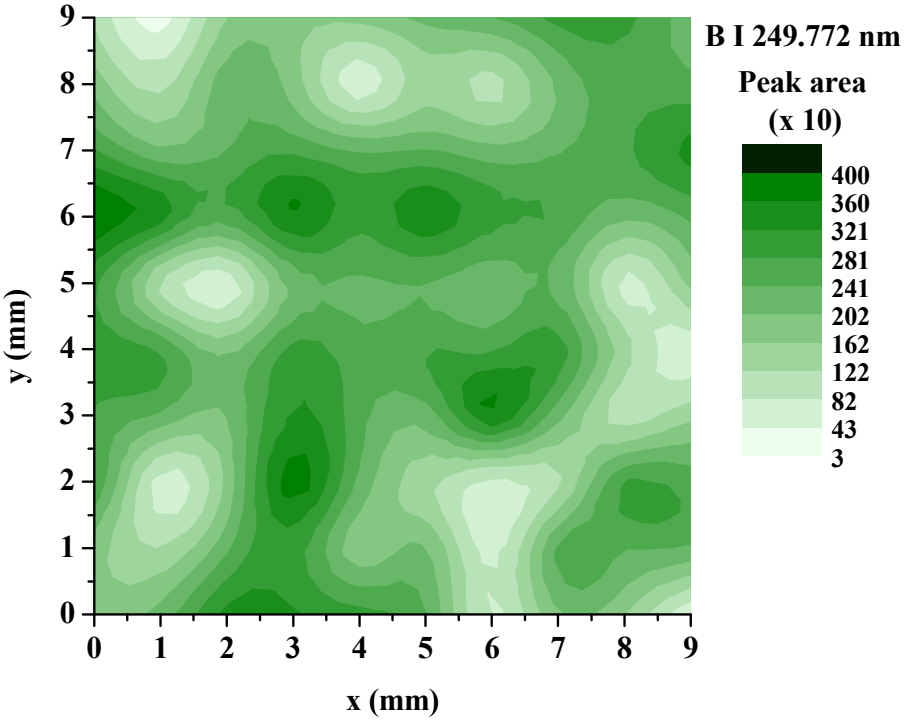

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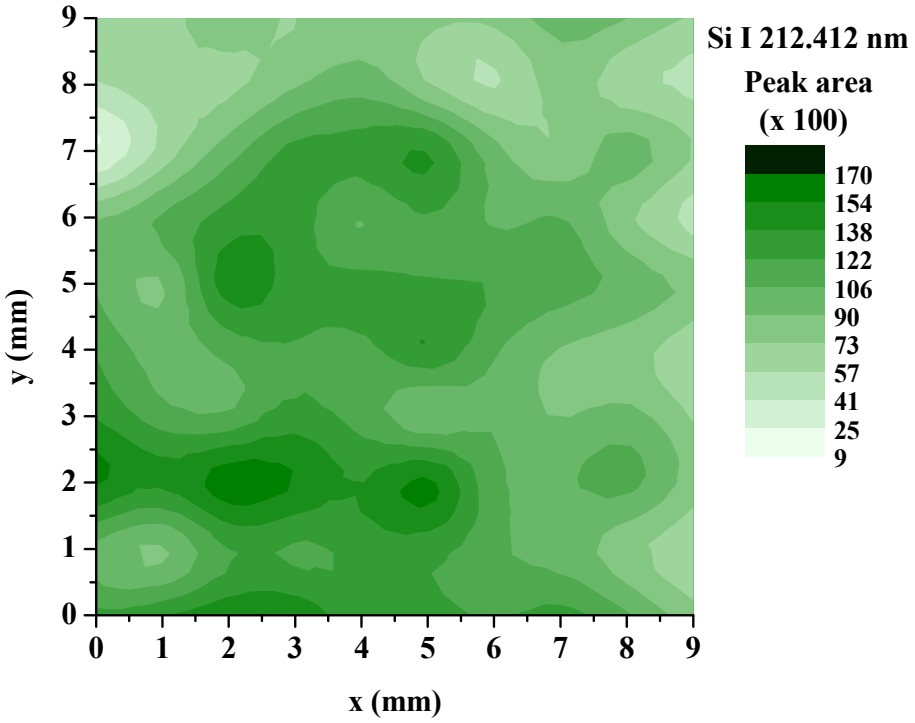


Figure 5

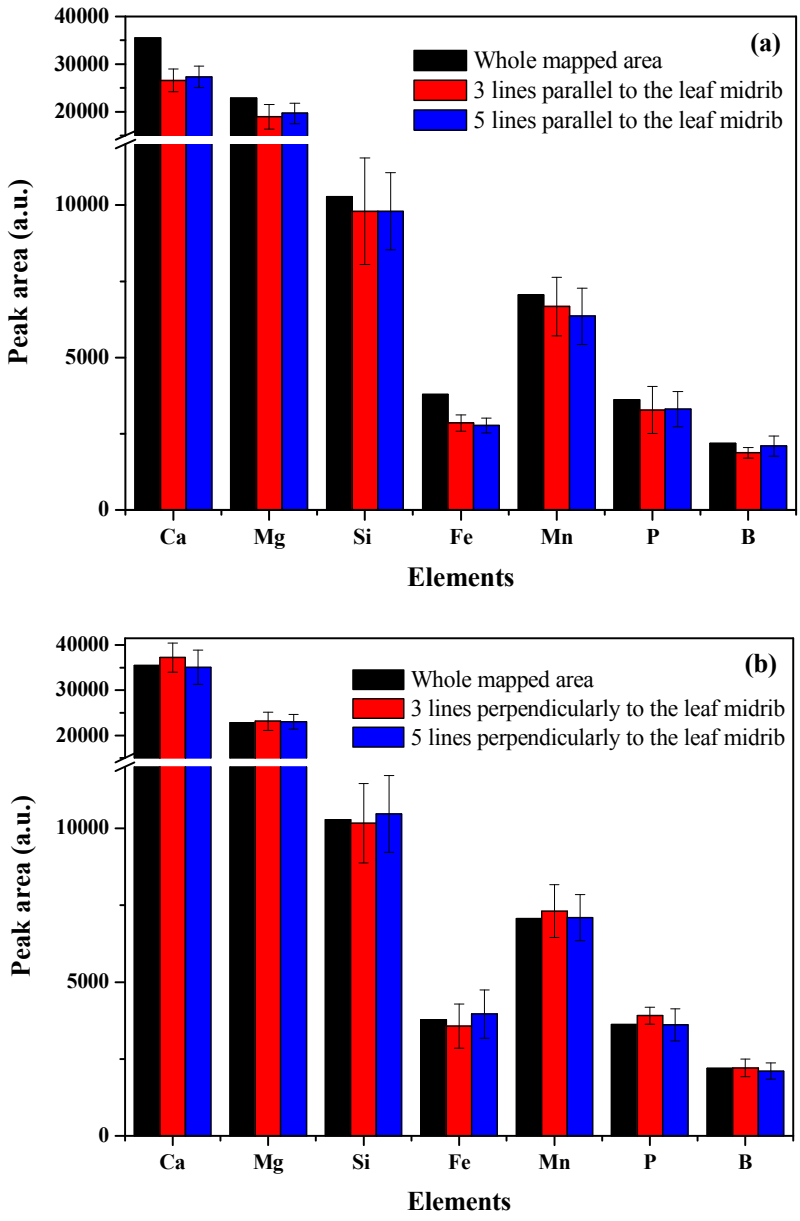


Figure 6

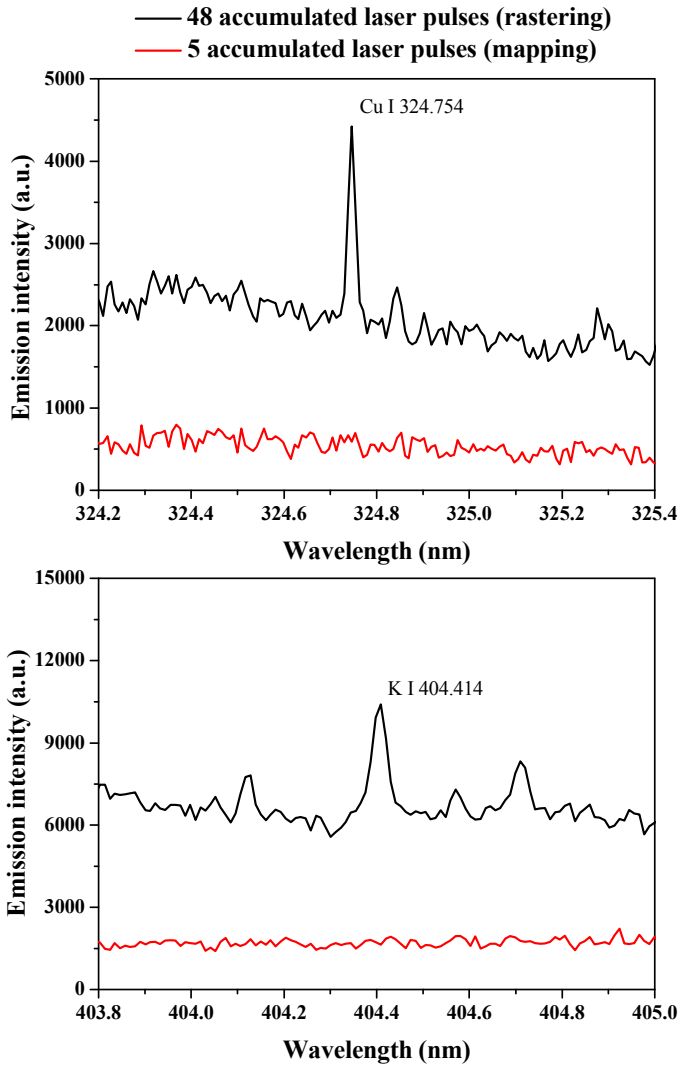
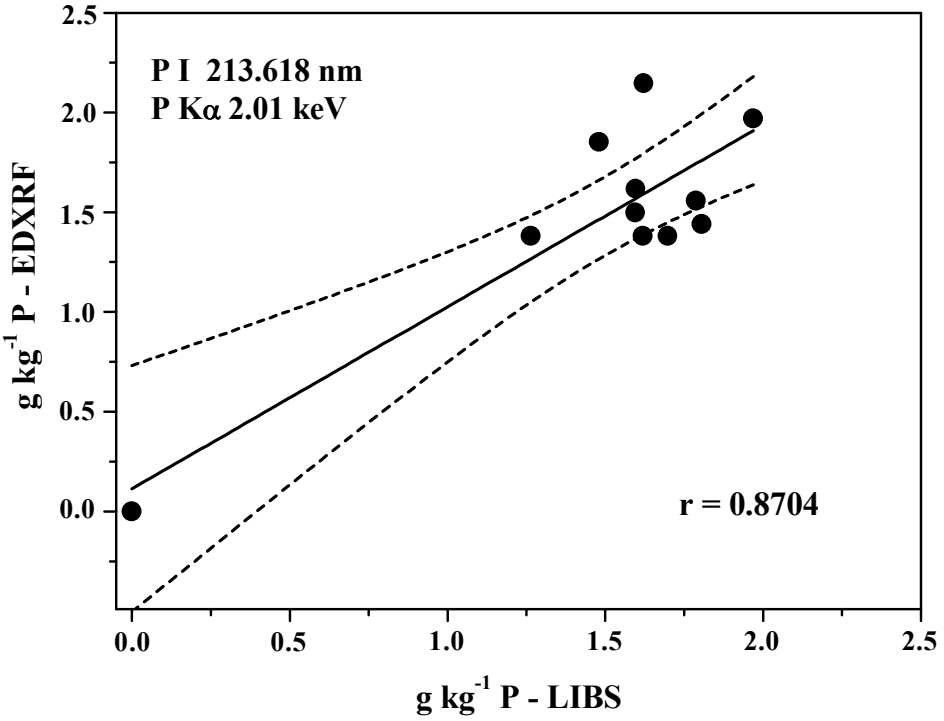
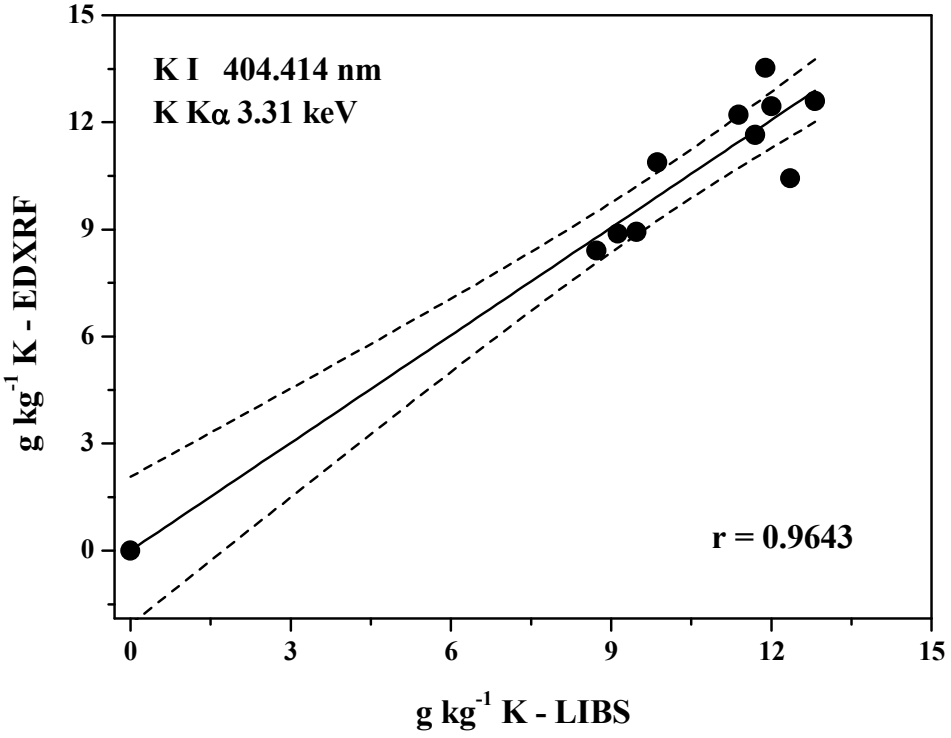
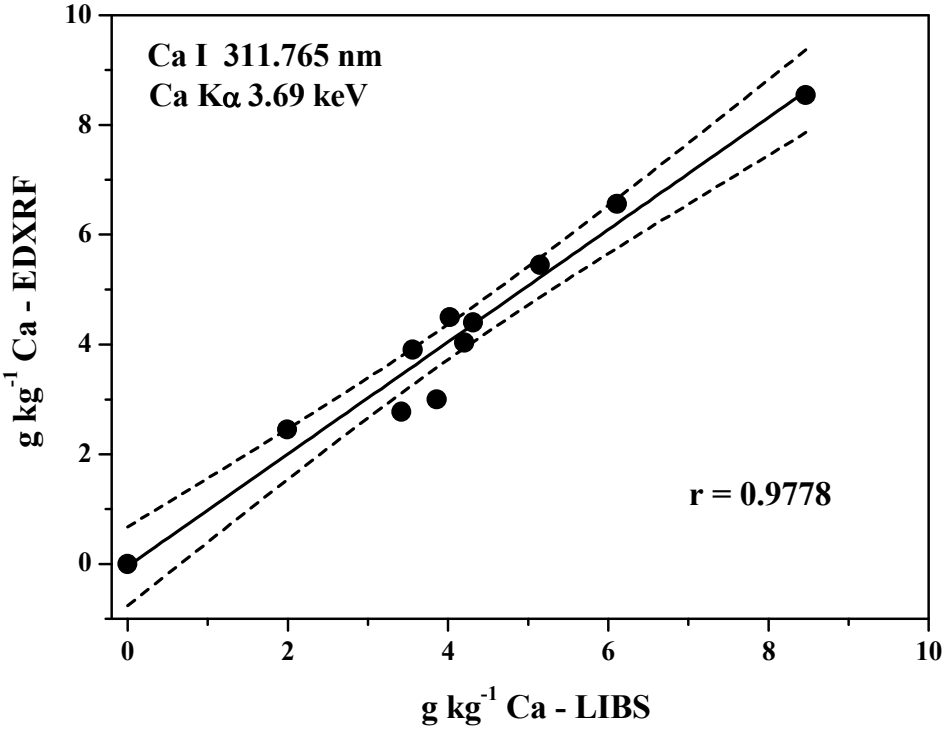
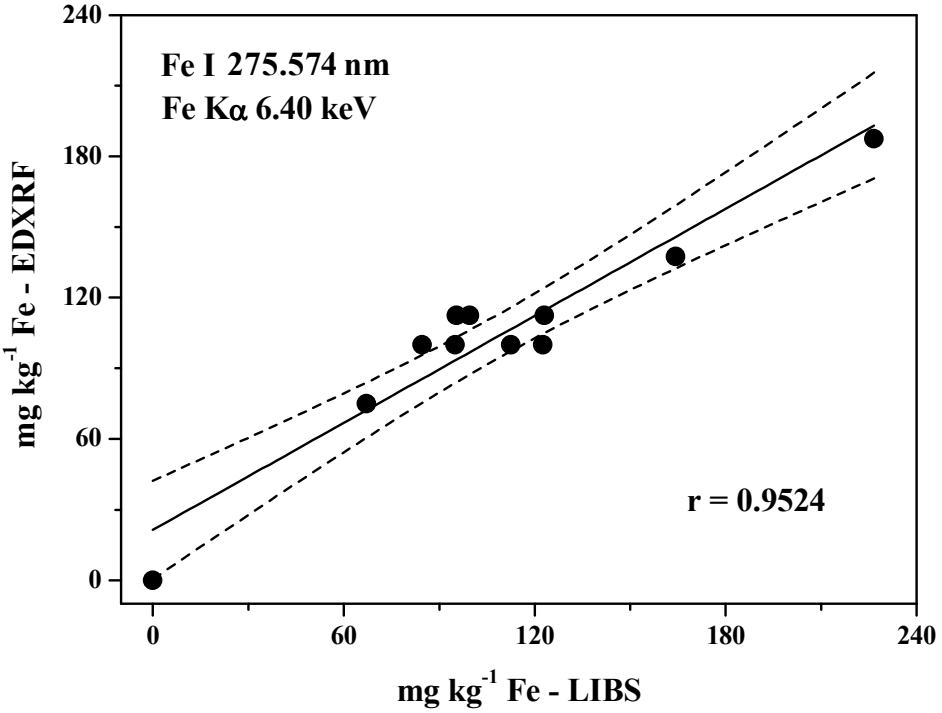


Figure 7

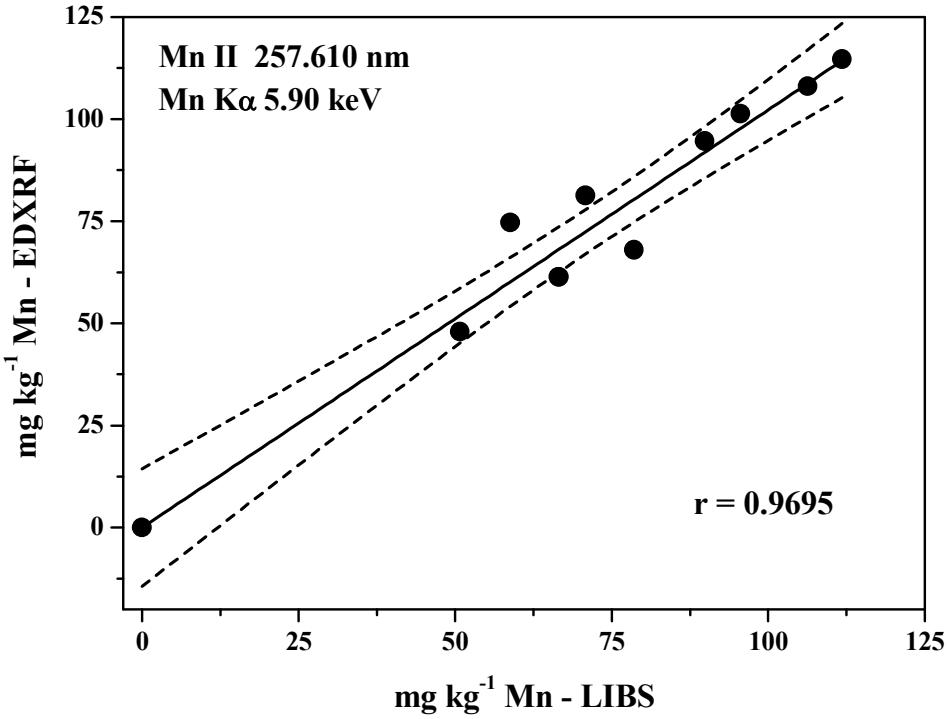


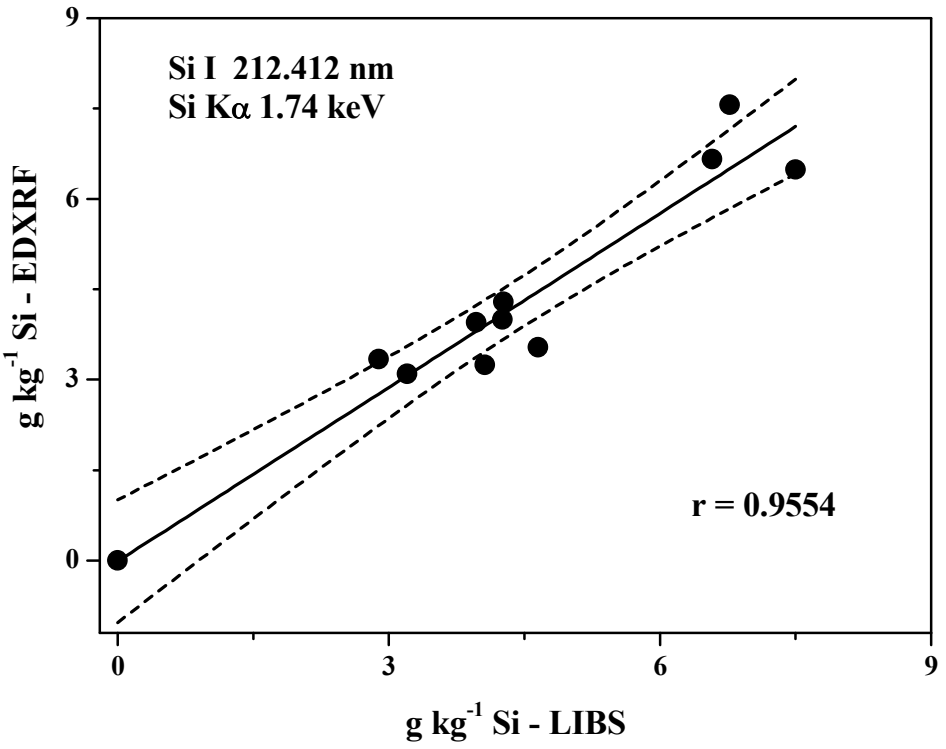






Continuation… Figure 7





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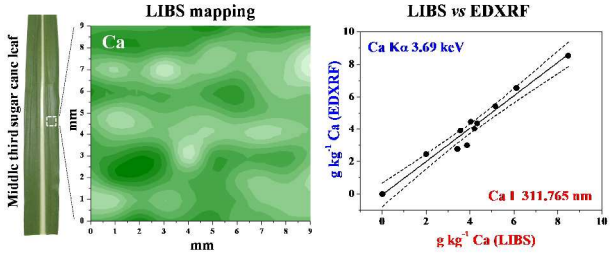

A novel strategy for direct analysis of dried leaves by EDXRF and LIBS aiming at plant nutrition diagnosis.