

Article

A Green and Simple Analytical Method for the Evaluation of the Effects of Zn Fertilization on Pecan Crops Using EDXRF

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Abstract

A simple and fast analytical method was developed and applied to assess the effect of two forms of zinc fertilization on a pecan tree cultivar in Uruguay: fertigation and foliar application with a specially formulated fertilizer. Zinc content was determined in 36 leaf samples from two crop cycles: 2020–2021 and 2021–2022. Fresh samples were dried, ground, and sieved. Analytical determinations were performed by flame atomic absorption spectrometry (FAAS, considered a standard method) and energy dispersive X-ray spectrometry (EDXRF, the proposed method). In the first case, sample preparation was carried out by microwave-assisted digestion using 4.5 mol L^{−1} HNO₃. In the second case, pellets (Φ 13 mm, 2–3 mm thick) were prepared by direct mechanical pressing. Figures of merit of both methodologies were adequate for the purpose of zinc monitoring. The results obtained from both methodologies were statistically compared and found to be equivalent (95% confidence level). Based on the principles of Green Analytical Chemistry, both procedures were evaluated using the Analytical Greenness Metric Approach (AGREE and AGREEprep) tools. It was concluded that EDXRF was notably greener than FAAS and can be postulated as an alternative to the standard method. The information emerging from the analyses aided decision-making at the agronomic level.

Keywords: zinc; *Carya illinoensis*; foliar analysis; fertilization monitoring; Green Analytical Chemistry; X-ray fluorescence spectrometry



Academic Editor: Jaroslava Svarc-Gajic

Received: 30 May 2025

Revised: 23 June 2025

Accepted: 8 July 2025

Published: 11 July 2025

Citation: Belluzzi Muiños, M.; Silva, J.; Conde, P.; Ibáñez, F.; Bühl, V.; Pistón, M. A Green and Simple Analytical Method for the Evaluation of the Effects of Zn Fertilization on Pecan Crops Using EDXRF. *Processes* **2025**, *13*, 2218. <https://doi.org/10.3390/pr13072218>

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1. Introduction

Pecan is the name given to the American walnut tree *Carya illinoensis* (Wangenh.) K. Koch, and pecan nuts are a growing consumer product. Pecan is native to northeastern Mexico and the southeastern United States, accounting for more than 90% of the global production of this crop. Smaller producers are Australia, Israel, and South Africa [1]. Uruguay began to be a small incipient producer, and there is an upward trend in their production increasing the cultivated area to around 1000 ha, driven by favorable climatic conditions and the growing international demand [2]. From a nutritional perspective, pecan nuts are considered a healthy food, with notable monounsaturated fatty acid content [3].

Pecan nut quality depends primarily on the genetic qualities of the variety, as well as crop management, including irrigation, pruning, and fertilization [4,5].

Pecan crop nutrition results can be managed in terms of increased fruit size, productivity, and quality. To ensure that the required quantity of micronutrients is achieved, several fertilizers are commonly used. They provide mainly nitrogen, phosphorus, and potassium but also contain Zn and Mn due to their importance in crop fertilization [6–11]. Traditional fertilization methods for this crop are direct soil irrigation (fertigation) and foliar irrigation. In calcareous soils with high carbonate and oxide content, which lead to the lower bioavailability of the target species, foliar fertilization is the most widely used method. It requires more expensive equipment, and its spraying does not reach the top third of the trees due to their height; this means that an entire tree cannot be fertilized [12].

The pecan tree is very sensitive to zinc deficiency, due to unbalanced soil pH levels, among other properties. The optimal range for this micronutrient's content in leaves is reported in the range of 50–100 mg kg^{−1}. The highest bioavailability of the metal occurs at pH values in the range of 5.0 to 7.0; therefore, under our conditions, zinc fertilization through fertigation would be both feasible and effective. Its deficiency is common in calcareous soils with pH values in the range of 7.0 to 8.6 [6–10].

On the other hand, excess of this element also causes problems for the healthy growth of the tree; a toxic level is considered when zinc concentration exceeds 300 mg kg^{−1}. When this occurs, critical metabolic processes involved in plant development can be affected [12].

Foliar analysis is widely used as a reliable tool for assessing the nutritional status of plant crops. The analysis should be carried out before each year's fertilization, at the end of spring, when the leaves reach full development, nutrient levels are relatively stable, and valid reference data are available to evaluate the nutritional status of the tree, as well as at the end of the cycle (in autumn), when the trees are close to losing their leaves [13].

For the determination of minerals in plant matrices, sample preparation using microwave-assisted digestion and subsequent analytical determination by flame atomic absorption spectrometry (FAAS) has been frequently reported as a reference methodology [14,15]. In this work, we proposed energy-dispersive X-ray spectrometry (EDXRF) as an alternative, simpler, sustainable, and environmentally friendly technique based on the principles of Green Analytical Chemistry.

In recent years, awareness of the environmental impact of human activities, including laboratory testing, has increased. The use of sustainability assessment criteria requires specialized tools. These criteria are based on the 12 principles of Green Analytical Chemistry (SIGNIFICANCE) that were transformed into a unified scale from 0 to 1 using the Analytical Greenness Metric Approach (AGREE and AGREEprep) tools [16–21].

Herein, a green analytical method, avoiding drastic sample preparation and the use of inflammable gas (i.e., acetylene), was developed and validated using EDXRF for Zn determination in pecan leaves, as a tool to assess the fertilization processes providing quick and reliable answers to the country's productive sector.

2. Materials and Methods

2.1. Samples' Origin and Preparation

A fertilization experiment was carried out on the pecan active germplasm bank established in 2010, at INIA "Las Brujas" (Estación Experimental Wilson Ferreira Aldunate, Canelones, Uruguay). Three treatments were applied with a specially formulated product containing Zn and Mn using four trees per treatment of a cultivar called Success: soil fertilization (fertigation), foliar fertilization, and unfertilized as a control. The experiment was repeated for two crop cycles: 2020–2021 and 2021–2022. For each crop cycle, the first application was carried out in early November and repeated every 15 or 20 days until the

end of February or the beginning of March, depending on the climate in each period. To minimize variability, sampling was restricted to fully developed leaves collected from the mid-portion of current-year shoots at mid-canopy height, following standard protocols for foliar analysis [12].

Thirty-six leaf samples were analyzed. The fresh samples were dried in a laboratory oven (Yamato DN93, Yamato, Tokyo, Japan) at 70 °C for 96 h. Once dried, the leaves were processed in a hammer mill (Willey, Thomas Scientific, Swedesboro, NJ, USA) and sieved through a 1 mm particle size mesh. The processed material was conditioned under low-humidity conditions until analysis. Zn determinations were carried out by flame atomic absorption spectrometry (FAAS), which is considered the standard method [22], and by energy-dispersive X-ray spectrometry (EDXRF), the new method.

2.2. Zinc Determination by FAAS

Sample preparation was carried out by weighing 300 mg of dried, ground, and sieved leaf material, adding 10.00 mL of 4.5 mol L⁻¹ HNO₃ (Merck, Darmstadt, Germany) to the reaction vessels (EasyPrep Plus®) and using microwave-assisted digestion (CEM Mars 6, USA). The operating condition program consisted of the following steps: heating to 200 °C for 15 min, holding at 200 °C for 15 min, and cooling to room temperature (power 400–1800 W). Reagent blanks were run simultaneously with the samples. Calibration standards (five concentration levels) up to 1.5 mg L⁻¹ and a blank were prepared from a 1000 mg L⁻¹ Zn commercial atomic absorption standard (Merck, Darmstadt, Germany) (Fluka, Buchs, Switzerland). Ultrapure water (Millipore Direct-Q 3 UV) was used to prepare the calibration standards and sample dilutions after digestion. The final volume of each solution was 25.00 mL; further dilutions were carried out if necessary. Analytical determinations were performed using flame atomic absorption spectrometry (FAAS-Perkin Elmer AAnalyst 200). The instrumental operating conditions involved an air-acetylene flame, a Perkin Elmer Lumina HCL lamp (I = 15 mA), and λ = 213.9 nm.

2.3. Zinc Determination by EDXRF

For Zn determination, pellets (Φ = 13 mm) with a 2–3 mm thickness were prepared directly from leaf samples (dried, ground, and sieved), 0.4 g of which was pressed at 20 bars for 3 min using a laboratory pneumatic press (Riken Seiki Co., Ltd., Shanghai, China). A Shimadzu 7200 EDXRF spectrometer (Shimadzu Corporation, Kyoto, Japan) was used, irradiating the samples at 40 KeV (Rh source) with a 5 mm collimator in an air atmosphere. A Certified Reference Material (CRM) of plant material (Embrapa-*Brachiaria Brizantha* cv., Brazil) was used for method calibration (fundamental parameter algorithm available in the software provided by Shimadzu-PCEDX-Pro™, Shimadzu Corporation, Kyoto, Japan) [23]. The approach of “fundamental parameters” to calibration in X-ray fluorescence is based on the theoretical relationship between measured X-ray intensities and the concentrations of elements in the sample. A standard reference material (SRM-NIST SRM1515 *Apple Leaves*, USA) was employed for matrix background correction and also to calibrate Zn intensity in a vegetable sample. The validation process, for both methods, was carried out following the recommendations of the Eurachem Guide; precision, trueness, detection, and quantification limits were evaluated (3 s and 10 s criteria). Trueness was assessed with recovery tests using the SRM [24]. To determine the acceptance criteria for precision and trueness, the AOAC recommendations were followed [25].

The statistical comparison of the results obtained applying FAAS and EDXR was performed by applying a mean comparison test (comparison of two experimental means) [25]. While fertilization treatments provided biologically relevant samples for method validation, statistical comparisons of field treatment effects were beyond this study’s scope. Complete

analytical data is available from the corresponding authors for agronomic researchers interested in treatment-specific analyses.

3. Results

3.1. Validation

Table 1 summarizes the results of validation for both analytical methods.

Table 1. Figures of merit of validation.

Parameter	FAAS	EDXRF
Linear range (mg L ^{−1})	0.049–1.5	Not applicable
Limit of detection (mg kg ^{−1}), <i>n</i> = 10	1.3	1.7
Limit of quantification (mg kg ^{−1}), <i>n</i> = 10	4.2	5.8
Intermediate precision (RSD%)	<7% (<i>n</i> = 5)	<13% (<i>n</i> = 10)
Zn * (mg kg ^{−1})	11.4–13.5	11.7–13.1
Trueness **	91.4–108.2 (<i>n</i> = 5)	94.0–105.2 (<i>n</i> = 7)

* Experimental values using SRM NIST SRM1515 Apple Leaves. Certified Zn concentration = (12.45 ± 0.45) mg kg^{−1}.

** Recovery (%).

3.2. Analytical Determinations and Statistical Comparison

Table 2 presents the obtained Zn levels, using both methods on real samples.

Table 2. Zn levels in analyzed samples.

Sample Code	AAS	EDXRF	Sample Code	AAS	EDXRF
1	67.2 ± 2.8	66.8 ± 3.3	19	198.2 ± 1.7	190.4 ± 5.9
2	68.5 ± 3.0	63.0 ± 3.6	20	186.7 ± 1.9	183.4 ± 6.3
3	73.1 ± 3.6	66.1 ± 1.9	21	154.1 ± 2.1	144.7 ± 6.8
4	70.0 ± 3.4	65.5 ± 2.3	22	182.9 ± 1.1	168 ± 14
5	68.8 ± 2.9	61.9 ± 2.9	23	152.8 ± 2.7	143 ± 19
6	62.0 ± 2.8	59.8 ± 4.3	24	186.0 ± 2.5	183 ± 20
7	68.2 ± 3.8	62.8 ± 2.5	25	105.7 ± 2.7	86.7 ± 7.1
8	48.9 ± 3.3	41.1 ± 3.7	26	107.1 ± 2.7	88.4 ± 3.3
9	42.1 ± 2.3	46.5 ± 5.4	27	107.2 ± 1.9	91.4 ± 3.1
10	74.1 ± 2.4	69.6 ± 3.3	28	130.9 ± 2.1	114.6 ± 5.3
11	65.9 ± 3.0	63.7 ± 3.0	29	96.2 ± 1.7	76.0 ± 3.6
12	62.5 ± 3.1	58.4 ± 1.3	30	100.4 ± 2.1	87.8 ± 3.6
13	144.2 ± 2.7	130 ± 11	31	76.8 ± 1.2	65.9 ± 1.8
14	181.0 ± 5.5	178.1 ± 6.2	32	75.5 ± 1.9	63.7 ± 1.5
15	146.3 ± 2.5	133.3 ± 4.6	33	85.65 ± 0.76	72.8 ± 2.2
16	140.0 ± 2.6	133.4 ± 4.2	34	108.0 ± 4.5	85.3 ± 2.8
17	200.1 ± 3.3	187.6 ± 9.1	35	129.8 ± 1.8	114.9 ± 3.3
18	187.1 ± 3.2	191.0 ± 5.4	36	73.3 ± 1.2	63.5 ± 1.2

Results (mg kg^{−1}): mean value ± *s* (standard deviation). The obtained data presented homogeneous variances and passed Student's test ($t_{\text{table}} = 1.99$; $t_{\text{calculated}} = 0.13$), meaning that the means obtained using both methodologies were statistically comparable ($p = 0.05$; $n = 36$) [26].

In addition, a graphic representation is presented in Figure 1. FAAS results are on the abscissa axis, and EDXRF results are on the ordinate axis. The function is $y = 0.9839 \times -7.2311$ ($R^2 = 0.9817$).

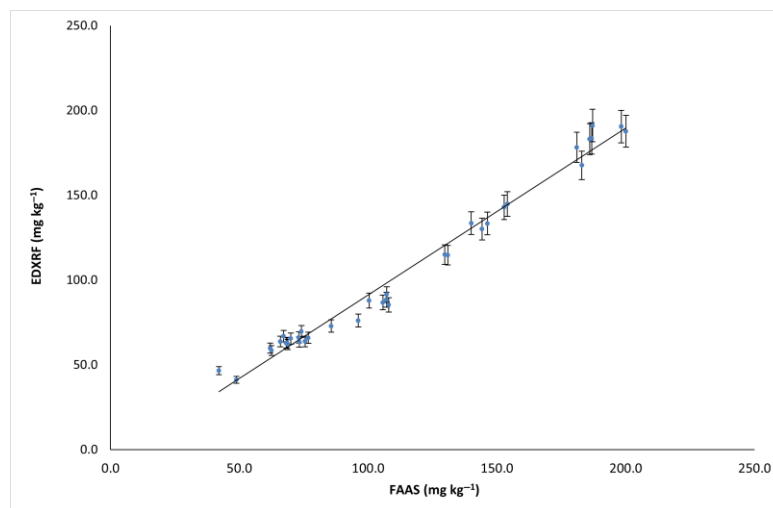


Figure 1. Graphical representation of results obtained by FAAS and EDXRF (n = 36).

Tables 3 and 4 present the statical comparison between different treatment cycles.

Table 3. Zinc concentration comparison by treatment—2020–2021 cycle.

Treatment	Method	n	Mean $\pm$ SD (mg kg ⁻¹)	CV (%)	Range	ANOVA	Tukey HSD
Control (T0)	AAS	4	63.8 $\pm$ 0.7 ^a	1.1	62.7–64.4	F = 1.02	All <i>p</i> > 0.05
	EDXRF	4	65.4 $\pm$ 1.5 ^a	2.3	63.0–66.8	<i>p</i> = 0.40	(NS)
Fertigation (T1)	AAS	4	60.5 $\pm$ 1.1 ^a	1.8	59.0–61.8	df = 2.9	
	EDXRF	4	56.4 $\pm$ 9.4 ^a	16.7	41.1–62.8		
Foliar (T2)	AAS	4	63.9 $\pm$ 2.6 ^a	4.1	61.4–67.6		
	EDXRF	4	59.6 $\pm$ 9.7 ^a	16.3	46.5–69.6		

df = degrees of freedom; the same superscript letter indicates no significative difference.

Table 4. Zinc concentration comparison by treatment—2021–2022 cycle.

Treatment	Method	n	Mean $\pm$ SD	CV%	Min–Max	ANOVA	Tukey HSD
T0 (Control)	AAS	8	136.8 $\pm$ 30.7 ^b	22.4	105.7–181.0	F = 4.25 (<i>p</i> = 0.023 *)	T1 > T0 *
	EDXRF	8	128.3 $\pm$ 32.1 ^b	25.0	86.7–178.1		T1 > T2 *
T1 (Fertigation)	AAS	8	162.1 $\pm$ 50.3 ^a	31.0	96.2–200.1	df = 2.21	
	EDXRF	8	151.9 $\pm$ 53.8 ^a	35.4	76.0–191.0		
T2 (Foliar)	AAS	8	129.6 $\pm$ 38.2 ^b	29.5	73.3–186.0		
	EDXRF	8	121.8 $\pm$ 42.6 ^b	35.0	63.5–183.1		

Different superscript letters within the row indicate significant differences (HSD Tukey–Kramer *p* ≤ 0.05); * indicates significant differences between treatments (*p* ≤ 0.05); df: degree of freedom.

3.3. Green Analytical Chemistry Approach

AGREE and AGREEprep are simple metrics tools for a greenness assessment that assigns a value on the 0–1 scale. The result of this evaluation is shown by a pictogram that indicates the final green performance score of the analytical methodology. The score is presented in the center of the pictogram: if the score is close to one, the color is dark green, and the method is greener.

The evaluation by applying the *Analytical Greenness Metric Approach* (AGREE and AGREEprep) methods yielded the results shown in the pictograms presented in Figure 2.



Figure 2. Pictograms for AGREE and AGREEprep.

4. Discussion

Overall, the results of the validation process show that the evaluated figures of merit were adequate for the stated objectives. The precision resulting from the EDXRF methodology is an exception, with $RSD < 13\%$, which is slightly higher than the AOAC recommendation. The hypothesis for this result was that the surfaces of the pellets prepared by pressing the dry, ground, and sieved samples (particle size $< 1\text{ mm}$) were not perfectly homogeneous; in this case, the higher percentage of the relative standard deviation is consistent. Despite this and considering that the objective of this work was to provide rapid analytical information for decision-making at the agronomic level, all the figures of merit were adequate for the proposed purpose. This problem can also be minimized by performing several measurements on both sides of the pellets and along the surface (aided with the camera that is included in the software) and averaging these measurements.

Once the foliar analysis results for the 2020–2021 experimental cycle were assessed, the local agronomic management at INIA Research Station made a strategic decision to increase the concentration of the zinc fertilizer applied. This adjustment aimed to optimize zinc uptake in pecan trees and address potential deficiencies that could impact growth and yield.

The subsequent foliar analysis from the 2021–2022 cycle revealed that zinc concentrations in the leaves were consistent with this intensified fertilization strategy, as confirmed by both analytical methodologies. This alignment highlights the role of zinc monitoring in adaptive agronomic decision-making, demonstrating that the analytical results provided valuable feedback for refining fertilization practices.

The proposed method was applied to analyze 36 samples, as previously described, and the results were compared with those obtained by FAAS. When matching, we expect both methods to be equivalent when the equation for a linear tendency is $y = x$.

Figure 1 shows the results obtained comparing EDXRF and FAAS and their correlation. An adequate correlation ($R^2 = 0.9817$) with a slope (0.9839) and intercept (-7.2311) was obtained. Thus, we considered both techniques as equivalent for this kind of analysis.

The observed Zn concentrations ($63\text{--}200\text{ mg kg}^{-1}$) exceeded the conventional optimal range ($50\text{--}100\text{ mg kg}^{-1}$) while remaining below toxicity thresholds. While no visual toxicity symptoms were observed, three agronomic considerations emerge from these elevated levels: First, pecan yield plateaus were reported above $\sim 80\text{ mg kg}^{-1}$ Zn [13], suggesting potential diminishing returns in our Year 2 samples, where means reached $142\text{--}178\text{ mg kg}^{-1}$. Second, chronic Zn elevation may induce imbalances in other micronutrients like Mn or Cu [8]. Third, the 35% increase in Zn fertilizer application during 2021–2022 resulted in only an 18% increase in leaf Zn concentration compared to controls, indicating potential resource inefficiency. These findings suggest that while our fertilization strategy avoided deficiency, future work could establish the cultivar specific response curves to optimize both economic and agronomic efficiency [12]. From an agronomic perspective (INIA Research Station), the data obtained through both analytical methods confirmed that zinc levels remained within operational limits for pecan cultivation in Uruguay ($50\text{--}300\text{ mg kg}^{-1}$). The results are shown in Tables 3 and 4. While treatment comparisons were not this study's primary aim, the results inform field decisions by verifying that (1) no samples reached toxicity thresholds, (2) foliar application showed marginally higher Zn uptake in Year 2 (consistent with increased dosage), and (3) both fertilization methods maintained concentrations above deficiency levels. These findings validate the suitability of EDXRF for routine monitoring, enabling timely adjustments without compromising crop safety.

The evaluation carried out from the Green Analytical Chemistry point of view showed for both metrics that the use of EDXRF yields notably greener results than FAAS. In *AGREE* (0.76 vs. 0.46), the greatest differences are found in two of the criteria: the degree of miniaturization and automation (criterion 5) and the origin and use of reagents (criterion 10). Although the sample quantity is the same in each case, the use of nonrenewable and hazardous reagents such as HNO_3 and acetylene in FAAS, as well as the semi-automation in EDXRF, are the main reasons why these differences favor the latter analytical technique. When considering *AGREEprep* (0.66 vs. 0.18), there are considerable differences in favor of EDXRF, including in criterion 3 (origin of reagents and their potential reuse), criterion 4 (waste generation), and criterion 10 (operator safety), and a smaller difference is observed in criterion 9 (analytical technique for analyte quantification). The differences in *AGREEprep* are basically explained by the very minimal sample preparation operation required by EDXRF, allowing a determination directly in the solid state. This result agrees with the fact that *AGREEprep* is a tool focused on the sample preparation step.

5. Conclusions

A classical analytical method for Zn determination using FAAS was compared with the use of EDXRF, avoiding a drastic sample treatment. Both methodologies were satisfactory for determining zinc content in the foliar analysis of pecan trees to evaluate different types of fertilization. Validation yielded reasonable performance parameters for achieving

reliable results, which was statistically supported. The results provide relevant information for decision-making to the production sector. Furthermore, it was demonstrated that Zn levels in the analyzed samples exceeded the optimal range but not the threshold for considering toxicity to the plant. The evaluation using Green Analytical Chemistry tools showed that the proposed method using EDXRF was greener than FAAS, being also more economic, faster, and more sustainable for this application. The results obtained using both methodologies were statistically equivalent (95% confidence level); thus, the proposed one can be postulated as an alternative to the productive agronomic authorities.

Author Contributions: M.B.M.: conceptualization, methodology, validation, formal analysis, investigation, writing—original draft preparation, and writing—review and editing. J.S.: methodology, formal analysis, writing—original draft preparation, and writing—review and editing. P.C.: methodology, formal analysis, writing—original draft preparation, and writing—review and editing. F.I.: conceptualization, investigation, resources, writing—original draft preparation, and writing—review and editing. V.B.: conceptualization, methodology, investigation, writing—original draft preparation, and writing—review and editing. M.P.: conceptualization, methodology, investigation, resources, writing—original draft preparation, and writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This research had no funding.

Data Availability Statement: Data will be made available on request.

Acknowledgments: INIA-Las Brujas and PEDECIBA-Química and CEFI of the Faculty of Chemistry and the Comisión Sectorial Científica (CSIC).

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Abbreviations

The following abbreviations are used in this manuscript:

FAAS	Flame Atomic Absorption Spectrometry
EDXRF	Energy Dispersive X-ray Spectrometry
AGREE	Analytical Greenness Metric Approach (also AGREEprep)
CRM	Certified Reference Material
SRM	Standard Reference Material
RSD	Relative Standard Deviation
INIA	Agricultural Research Institute
HCL	Hollow-Cathode Lamp
NIST	National Institute of Standards and Technology
AOAC	Association of Official Analytical Chemists

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