Engineering and Sustainability Journal, Volume 1, Issue 1, July (2026), e006

ISSN: XXXX-XXXX (Pending)

Contents lists available at Angola-Journals.com

Engineering and Sustainability Journal

journal homepage: angola-journals.com/index.php/esj

Extraction of Humic and Fulvic Acids from Leonardite for Agricultural Biostimulant Applications

Diogo Fabrício Campos; Neli Branco de Miranda; Dominicky Cristina Serrano; Aline Gonçalves Gehrke; Vanderleia Botton[1]

Department of Chemical Engineering/ University of Blumenau, Blumenau, Santa Catarina, Brazil

Abstract: Biostimulants have emerged as a promising strategy to support sustainable growth in global agribusiness. Brazil, as one of the world's leading agricultural producers, increasingly demands efficient and sustainable alternatives to enhance productivity. Among biostimulants, humic substances stand out due to their positive effects on soil properties and plant development. These compounds can be obtained from different mineral sources, and their origin influences their composition and effectiveness. Leonardite is recognized as one of the richest natural sources of humic substances and is widely used to produce agricultural biostimulants. In this study, the extraction of humic and fulvic acids from leonardite sourced was investigated, motivated by growing industrial interest in this material. The study included physicochemical characterization of the raw material and optimization of extraction conditions. A simplified sequential extraction approach was evaluated to investigate how operational parameters influence the selective recovery of humic substances. A 2^2 factorial design was applied to evaluate the effects of reagent concentration and extraction time. For fulvic acid extraction, the highest concentration (160.0 mg L^{-1}) was obtained at high acid concentration and short extraction time (30 min). For humic acid extraction, the maximum concentration (255.6 mg L^{-1}) was achieved under alkaline conditions after 3 h of extraction. FTIR analysis confirmed the presence of characteristic functional groups of humic substances in both the raw material and the extracted fractions. The results demonstrate that humic substances can be efficiently extracted under controlled conditions using a simple process. The short extraction times and operational simplicity highlight the potential for scale-up and industrial application, particularly in the production of agricultural biostimulants. Furthermore, the study contributes to the understanding of how extraction conditions influence the selective recovery of humic and fulvic fractions from leonardite.

Keywords: Biostimulants; Leonardite; Humic substances; Humic acids; Fulvic acids; Sustainable agriculture.

1. Introduction

The growing demand for sustainable agricultural practices has intensified the search for alternatives that enhance crop productivity while reducing environmental impacts. In this context, biostimulants have emerged as promising tools, defined as substances or microorganisms that stimulate natural processes in plants, improving nutrient uptake, stress tolerance, and crop quality (du Jardin, 2015). Among the different classes of biostimulants, humic substances have received considerable attention for their multifunctional roles in soil–plant systems (Nardi et al., 2021).

Humic substances are complex and heterogeneous organic materials formed during the decomposition and transformation of plant and animal residues (Maffia et al., 2025). They are commonly fractionated into humic acids (HA), fulvic acids (FA), and humin, based on their solubility behavior in alkaline and acidic media (Sheng et al., 2007). These fractions differ in molecular weight, functional group composition, and chemical reactivity, which directly influence their agronomic performance (Reemtsma et al., 2008). From a physiological perspective, humic and fulvic acids have been associated with enhanced root development, modulation of plant hormone-like activity, and improved nutrient transport and metabolism (Zandonadi et al., 2025).

The increasing use of humic substances in agriculture is linked to their role as soil conditioners and biostimulants, contributing to improved soil structure, cation exchange capacity, and microbial activity (Canellas et al., 2015; Ibraevaa et al., 2026). In particular, fulvic acids are known for their high solubility and chelating ability, facilitating nutrient availability, while humic acids are associated with long-term soil fertility and structural stability (Rosa et al., 2009; Barzgar et al., 2025).

Among the available sources of humic substances, leonardite stands out as one of the most relevant raw materials. Leonardite is an oxidized form of lignite rich in organic carbon, typically containing high concentrations of humic substances and exhibiting biological activity (Qian et al., 2015; Shah et al., 2018). Due to its degree of humification and chemical composition, leonardite is considered a suitable and economically viable source for large-scale extraction of humic and fulvic acids (Csicsor & Tombácz, 2022).

The extraction of humic substances from leonardite has been widely investigated using alkaline and acidic methods. Conventional approaches typically involve alkaline extraction (e.g., NaOH or KOH) followed by acid precipitation to separate humic and fulvic fractions (Jomhataikool et al., 2020; Machado et al., 2019; Sheng et al. (2007). Process parameters such as pH, extraction time, temperature, and solid–liquid ratio affect extraction efficiency and product characteristics (Allahkarami et al., 2025). More recently, studies have explored process intensification strategies and alternative extraction techniques aiming to improve yield and selectivity while reducing environmental impact (Sun et al., 2020).

Despite these advances, important challenges remain. Reported extraction yields vary widely depending on the source of leonardite and operating conditions, and understanding of the relationships among extraction conditions, selectivity, and the physicochemical properties of the extracted fractions remain limited. In addition, many studies focus on maximizing yield, while fewer works address process simplicity, scalability, and selective recovery of specific fractions, which are critical aspects for industrial applications.

The present study aims to evaluate the extraction of humic and fulvic acids from leonardite using a simplified solid–liquid extraction approach based on Sheng's methodology. The effects of process parameters, including reagent concentration and extraction time, were investigated to identify conditions that favor the recovery of humic substances. Furthermore, the study seeks to advance understanding of the relationship between extraction conditions and the selective solubilization of humic fractions, thereby supporting the development of efficient and scalable processes for agricultural applications.

---

[1] Corresponding author: Vanderleia Botton. E-mail: vanderleiabotton@furb.br

Received 28 May 2026; Received in revised form 10 June 2026; Accepted 26 June 2026; Published 4 July 2026.

2. Methodology / Materials and Methods

2.1. Materials

The leonardite used as the raw material in this study originated from the Cherkasy region of Ukraine, an area known for its significant leonardite deposits. The material was kindly provided by Tracia Holding. A commercial humic acid standard was purchased from Sigma-Aldrich (Brazil). All other reagents were of analytical grade and were used as received. A commercial humic acid standard was obtained from Sigma-Aldrich (Brazil). All other reagents were of analytical grade and used according to recommended purity specifications.

Experiments were carried out at the Laboratory of Chemical Analysis (LEQ), Department of Chemical Engineering, Regional University of Blumenau (FURB), Brazil. The leonardite sample was received in an air-dry state. This approach was adopted to prevent the loss of volatile compounds that may occur during thermal drying. This study was conducted according to the flowchart shown in Figure 1.

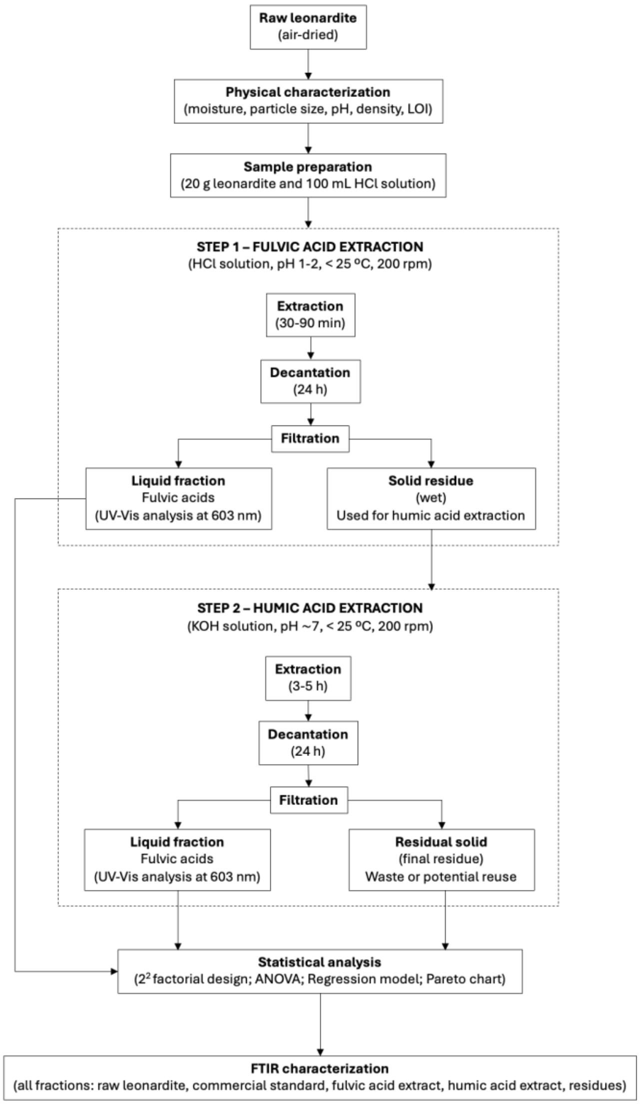

Figure 1. Process flow diagram for sequential extraction of fulvic and humic acids from leonardite, including acid and alkaline extraction steps, solid–liquid separation, and analytical characterization.

2.2. Physicochemical analysis of leonardite

Moisture content was determined using two methods: oven drying at 65 °C and infrared balance drying at 105 °C. Approximately 1 g of sample was used in each assay.

2.3. pH measurement

The pH was measured using a digital pH meter. A suspension was prepared by dispersing 10 g of leonardite in 100 mL of distilled water. The electrode was carefully immersed in the liquid phase, avoiding contact with settled solids.

2.4. Density determination

Density was determined using a calibrated pycnometer (19.94 mL) at 20 °C. A suspension of 10 g of leonardite in 100 mL of distilled water was used. Mass measurements were performed using an analytical balance.

2.5. Loss on ignition (LOI)

Loss on ignition was determined to estimate ash content and volatile compounds of leonardite. Samples were placed in porcelain crucibles and heated in a muffle furnace at 950 °C (heating rate: 20 °C min^{-1}) for 50 min. After cooling in a desiccator, samples were weighed.

2.6. Particle size distribution

Particle size analysis was performed using a laser diffraction analyzer (Mastersizer, 2000). The method is based on light scattering by dispersed particles. Ethanol and distilled water were used as dispersing media. The obscuration range was maintained between 10–30%, and approximately 20 g of sample was sufficient to reach this range.

2.7. Extraction of humic substances from leonardite

2.7.1. Experimental design

The concentration of humic and fulvic acids was determined by UV–Vis spectrophotometry at 603 nm using a calibration curve based on commercial standards, following the methodology of Sheng et al. (2007). The results were expressed as mg L^{-1}.

A 2^2 factorial design with triplicate central points was applied to evaluate the extraction of fulvic and humic acids (total of 7 experiments per design) following the standard design of experiments methodology. Two independent experimental designs were conducted: Design 1 (Fulvic acids extraction); Factors: HCl concentration (low/high) and extraction time (30–90 min); Design 2 (Humic acids extraction): Factors: KOH concentration (low/high) and extraction time (3–5 h); The response variable was the concentration of extracted acids (mg L^{-1}).

2.7.2. Fulvic acid extraction

Fulvic acid extraction was performed using 20 g of leonardite and 100 mL of HCl solution under agitation (200 rpm) at temperatures below 25 °C.

The pH of the system was adjusted to 1–2. After the extraction period (30–90 min), the mixture was allowed to settle for 24 h, then filtered. The supernatant was collected and used for further analysis.

2.7.3. Humic acid extraction

The solid residue obtained after fulvic acid extraction was used to extract humic acid. The process was carried out using 20 g of wet residue and 200 mL of KOH solution under agitation (200 rpm) at temperatures below 25 °C. The extraction time ranged from 3 to 5 h. After extraction, the mixture was allowed to settle for 24 h and then filtered. The supernatant was collected for analysis.

2.7.4. Analytical determination

Humic and fulvic acids were quantified by UV–Vis spectrophotometry at 603 nm, based on a modified method of Sheng et al. (2007), using a commercial standard (80% humic acid and 70% fulvic acid) for calibration.

2.7.5. FTIR analysis

Functional groups of leonardite, commercial humic acid standard, and extracted samples were characterized by Fourier Transform Infrared Spectroscopy (FTIR) using a VERTEX 70v spectrometer (Bruker).

2.7.6. Statistical Analysis

The values presented in the figures and tables of this work correspond to the means ± the standard deviation of the sample. The TIBCO Statistica® 14.1.0 software was used for the factorial design and its analysis. Statistical results, including ANOVA tables, estimated effects, Pareto charts, regression models, and response surface plots for humic and fulvic acid extraction, are provided in the Supplementary Material.

3. Results and Discussion

3.1. Physicochemical properties of leonardite

Leonardite was characterized as received, in its air-dried state (≈25 °C). The moisture content, determined in triplicate, was 35.19 ± 0.02% at 65 °C and 38.77 ± 0.15% at 105 °C.

The higher value observed at 105 °C suggests not only water removal but also the loss of volatile organic compounds, consistent with the thermal sensitivity of humic substances. Therefore, drying at lower temperatures is more suitable to preserve the chemical integrity of the material.

The density of the leonardite suspension was 1044 kg m⁻³, consistent with values reported for similar materials. Loss-on-ignition analysis indicated an ash content of 34.92 ± 1.39%, indicating a significant inorganic fraction. The remaining fraction is composed of organic matter, including humic substances such as humic acids, fulvic acids, and humin.

The measured pH of the leonardite suspension was 12.59, indicating an alkaline character, which may be associated with its mineral composition and the presence of basic functional groups. These properties are expected to influence the extraction behavior and efficiency of humic substances.

3.2. Particle size distribution

Particle size distributions obtained by laser diffraction (Figures 2 and 3) showed similar profiles across both dispersing media, indicating that the choice of dispersant had limited influence on particle-size measurements. The mean particle diameters were 484.6 µm in water and 453.2 µm in ethanol, confirming this similarity.

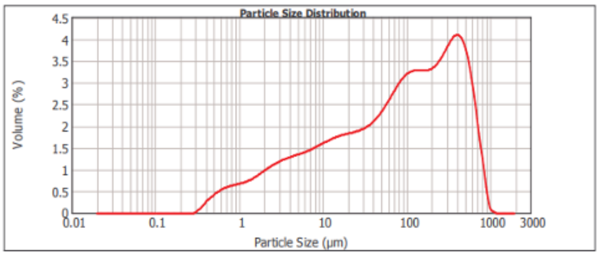

Figure 2. Particle size distribution of leonardite dispersed in water, obtained by laser diffraction analysis, showing the volume-based distribution of particle diameters.

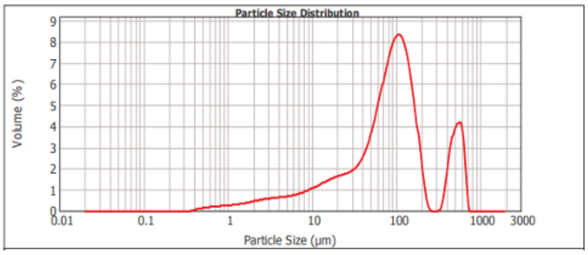

Figure 3. Particle size distribution of leonardite dispersed in ethanol, obtained by laser diffraction analysis, showing the volume-based distribution of particle diameters.

However, differences in the estimated specific surface area were observed, with higher values in water (0.754 m² g⁻¹) compared to ethanol (0.351 m² g⁻¹). This behavior may be attributed to the hygroscopic nature and swelling capacity of leonardite in aqueous media, which can increase particle dispersion and apparent surface area.

3.3. Extraction and fractionation of humic substances

The fractionation of leonardite was based on solubility differences: fulvic acids are soluble across all pH values, humic acids are soluble in alkaline media and precipitate under acidic conditions, and humin is insoluble over the entire pH range. This behavior enables sequential extraction using acid and alkaline solutions.

3.4. Fulvic acid extraction

The factorial design results for fulvic acid extraction are presented in Table 1. The complete statistical evaluation is presented in the Supplementary Material (Figures S1–S2 and Tables TS1–TS2).

Table 1: Experimental conditions and results for the 2² factorial designs used to optimize fulvic acid extraction from leonardite.

Trial	Independent variables (coded value)		AF (mg L⁻¹)
	HCl (mol/L)	t (min)	
1	0.07 (- 1)	30 (- 1)	111.8$^{\pm 4.7}$
2	0.13 (1)	30 (- 1)	160.1$^{\pm 1.2}$
3	0.07 (- 1)	90 (1)	122.2$^{\pm 1.8}$
4	0.13 (1)	90 (1)	99.7$^{\pm 1.8}$
5	0.1 (0)	60 (0)	158.4
6	0.1 (0)	60 (0)	156.8
7	0.1 (0)	60 (0)	155.1

Abbreviations: FA, fulvic acid; HCl, hydrochloric acid; t, extraction time. Note: FA concentration expressed as mg L⁻¹. The standard deviation at the central point was ± 1.7.

The highest concentration (160.1 mg L⁻¹) was obtained at high HCl concentration and short extraction time (30 min). The results indicate thatacid concentration had a positive effect, whereas prolonged extraction time had a negative effect on yield. Specifically, increasing the extraction time from 30 to 90 min at high acid concentration resulted in a decrease of approximately 38% in the fulvic acid concentration.

This behavior may be attributed to degradation or transformation of fulvic fractions under prolonged acidic conditions, possible re-association or precipitation phenomena, and changes in solubility equilibria over time. These findings suggest that short extraction times are critical for maximizing fulvic acid recovery, thereby improving process efficiency.

3.5. Humic acid extraction

The results for humic acid extraction (Table 2) show that the highest concentration (255.6 mg L⁻¹) was achieved at high KOH concentration and shorter extraction time (3 h). The complete statistical evaluation is presented in the Supplementary Material (Figures S3–S4 and Tables TS3–TS4).

Table 2: Experimental conditions and results for the 2² factorial design used to optimize humic acid extraction from leonardite.

Trial	Independent variables (coded value)		HA (mg L⁻¹)
	KOH (mol/L)	t (h)	
1	0.15 (- 1)	3 (- 1)	198.1$^{\pm 2.9}$
2	0.25 (1)	3 (- 1)	255.6$^{\pm 4.1}$
3	0.15 (- 1)	5 (1)	157.7$^{\pm 8.3}$
4	0.25 (1)	5 (1)	190.2$^{\pm 4.7}$
5	0.2 (0)	4 (0)	155.2
6	0.2 (0)	4 (0)	161.0
7	0.2 (0)	4 (0)	159.3

Abbreviations: HA, humic acid; KOH, potassium hydroxide; t, extraction time. Note: HA concentration expressed as mg L⁻¹. The standard deviation at the central point was ± 3.0.

In contrast to fulvic acid extraction, the extraction time showed a stronger influence. Increasing the extraction time from 3 to 5 h reduced yield, suggesting possible degradation or structural alteration of humic substances under prolonged alkaline conditions. This trend is consistent with previous reports indicating that excessive alkaline treatment may lead to breakdown of macromolecular structures, formation of soluble low-molecular-weight fractions, and reduced recovery of humic acids.

3.6. Comparison with literature

The obtained values were compared with literature data to evaluate process performance. Studies by Csicsor and Tombácz (2022) reported higher fulvic acid concentrations (310–400 mg L⁻¹), while the maximum value obtained in this study was 160 mg mL⁻¹. This discrepancy may be related to differences in raw material origin, extraction conditions, and analytical methods. The highest fulvic acid concentration was 160 mg L⁻¹, obtained under high acid concentration and short extraction time (30 min). The maximum humic acid concentration reached 255.6 mg L⁻¹ under alkaline conditions after 3 h of extraction. Similarly, Lima et al. (2017), using the same analytical method, reported 201 mg L⁻¹ for humic acids and 411 mg L⁻¹ for fulvic acids. In comparison, the present study demonstrates comparable efficiency for humic acid extraction.

3.7. FTIR Analysis

The FTIR spectra of the commercial humic acid standard, fulvic acid standard, and raw leonardite are presented in Figures 4–6. These spectra provide important insights into the functional groups present in the material and serve as a reference for evaluating structural changes after extraction.

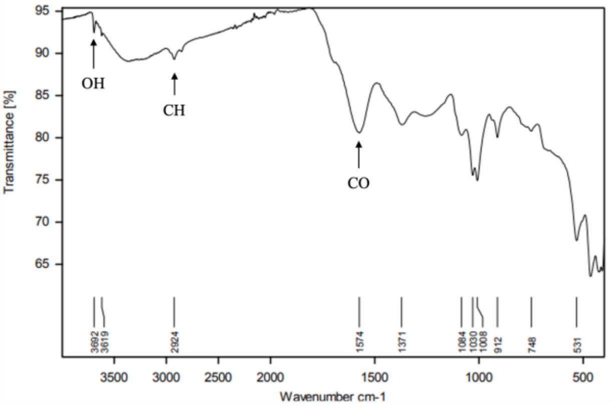

Figure 4. FTIR spectra of the commercial humic acid standard.

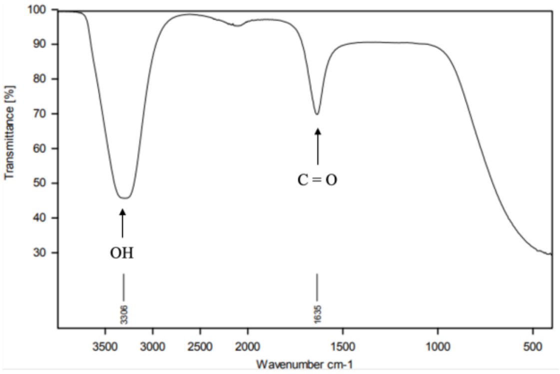

Figure 5. FTIR spectra of the commercial fulvic acid standard.

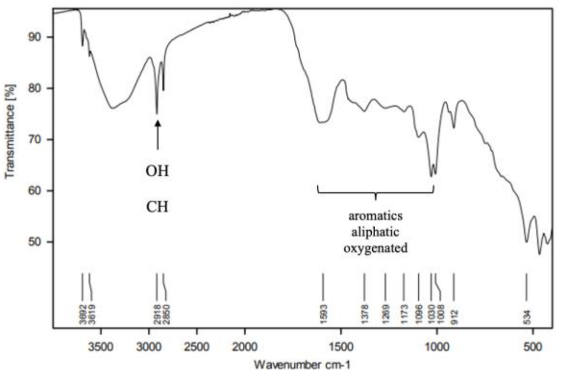

Figure 6. FTIR spectra of the raw leonardite.

The spectrum of the humic acid standard (Figure 4) shows a broad band in the region of 3692–3619 cm⁻¹, attributed to O–H stretching vibrations of hydroxyl groups from alcohols, phenols, and carboxylic acids, indicating the highly functionalized nature of humic substances. The bands observed at ~2924 cm⁻¹ are attributed to aliphatic C–H stretching, indicating the presence of hydrocarbon chains. The peaks at 1574 cm⁻¹ and in the region of 1371–1030 cm⁻¹ are related to amide groups and C–O stretching, suggesting contributions from nitrogen-containing structures and carbohydrate-like components.

The fulvic acid standard (Figure 5) exhibits characteristic bands at ~3306 cm⁻¹ (O–H/N–H stretching) and ~1635 cm⁻¹ (C=O stretching of conjugated carbonyl groups), indicating a structure rich in oxygenated functional groups, typical of low-molecular-weight humic fractions.

The spectrum of raw leonardite (Figure 6) presents similar features, confirming the presence of humic substances in the original material. Broad O–H bands, aliphatic C–H stretching (2918–2850 cm⁻¹), and signals in the region of 1593 cm⁻¹ and 1378–1030 cm⁻¹ indicate a complex organic matrix composed of aromatic, aliphatic, and oxygenated functional groups.

3.7.1. Effect of acid extraction - fulvic fraction

The FTIR spectrum of the solid residue after acid extraction (Figure 7) shows a reduction in the intensity of bands associated with oxygenated groups (O–H and C=O), suggesting the removal of more soluble fractions, particularly fulvic acids. This is consistent with the known solubility of fulvic acids over a wide pH range.

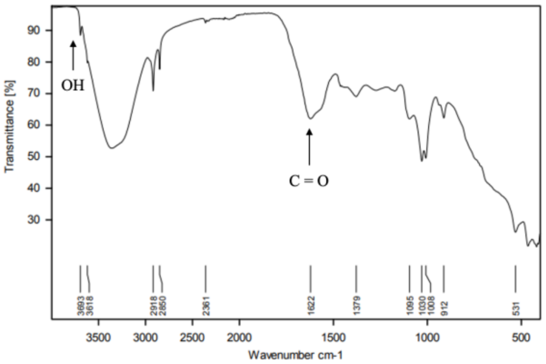

Figure 7. FTIR spectrum of the solid residue after acid extraction.

In contrast, the liquid fraction (Figure 8) exhibits prominent bands at ~3306 cm⁻¹ and ~1635 cm⁻¹, closely matching the fulvic acid standard. This confirms the successful extraction of fulvic acids and indicates that the extracted fraction is enriched in oxygenated and polar functional groups.

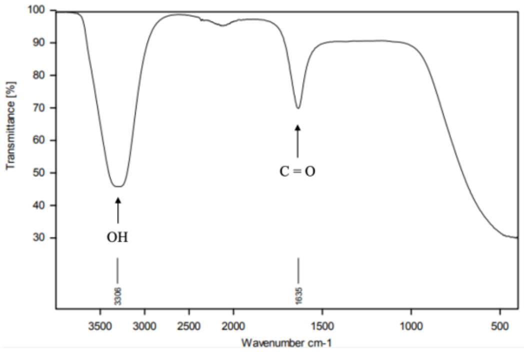

Figure 8. FTIR spectrum of the liquid fraction after acid extraction.

3.7.2. Effect of alkaline extraction - humic fraction

The FTIR spectrum of the solid residue after alkaline extraction (Figure 9) shows further reduction in functional group intensity, indicating the removal of humic substances from the solid matrix.

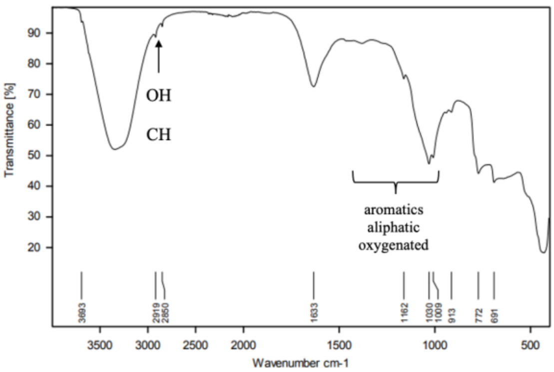

Figure 9. FTIR spectrum of the solid residue after alkaline extraction.

The liquid fraction obtained after alkaline extraction (Figure 10) presents characteristic bands at ~3311 cm⁻¹ (O–H stretching) and ~1636 cm⁻¹ (C=O stretching), consistent with the humic acid standard. The presence of these bands confirms the successful solubilization of humic acids under alkaline conditions.

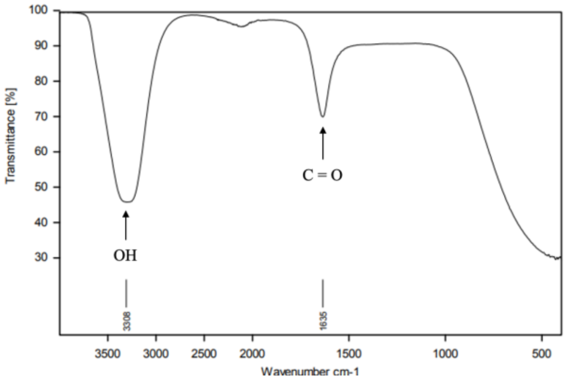

Figure 10. FTIR spectrum of the liquid fraction obtained after alkaline extraction.

3.8. Overall interpretation

The comparison among raw leonardite, extraction residues, and liquid fractions demonstrates a clear fractionation of humic substances by solubility. Acid extraction preferentially removes fulvic acids, while alkaline extraction promotes the solubilization of humic acids.

The broad O–H bands and the presence of C=O and C–O functional groups are consistent with the complex and heterogeneous structure of humic substances, which contain multiple oxygenated and aromatic moieties (Volkov et al., 2021; Petrov et al., 2017). These functional groups play a key role in the chemical reactivity and interaction capacity of humic materials, particularly through carboxylic and phenolic groups, as previously reported for leonardite-derived humic substances (Meng et al., 2017).

The FTIR results provide strong evidence that the proposed extraction methodology effectively separates humic fractions while preserving their main functional groups. The similarity between extracted fractions and commercial standards further supports the efficiency of the process.

The results demonstrate that both extraction stages are influenced by operational parameters, particularly reagent concentration and extraction time. Importantly, optimal conditions were achieved at relatively short extraction times, which represents an advantage for process scalability and industrial applications.

4. Conclusions

This study demonstrated the feasibility of extracting humic and fulvic acids from leonardite using a simple solid–liquid extraction approach based on Sheng's methodology. The results confirmed that leonardite is a suitable raw material for the recovery of humic substances, with its moisture content (~35–38%) and ash content (~35%).

The optimization of process parameters revealed that extraction efficiency is dependent on reagent concentration and extraction time. The highest fulvic acid concentration (160 mg L^{-1}) was obtained under high acid concentration and short extraction time (30 min), while the maximum humic acid concentration (255.6 mg L^{-1}) was achieved under alkaline conditions with an extraction time of 3 h. These results indicate that the process favors the solubilization of humic substances under specific operational conditions, with shorter extraction times contributing to process efficiency, particularly for fulvic acids.

Physicochemical characterization, including particle size distribution and surface area analysis, indicated that leonardite presents stable granulometric properties, while its interaction with aqueous media may enhance surface-related phenomena relevant to extraction.

From a technological perspective, the proposed process is operationally simple and potentially scalable, as it employs mild conditions and readily available reagents. The results also suggest that the extraction is selective, favoring the recovery of more soluble fractions of humic substances under the evaluated conditions.

Overall, this work contributes to the understanding of humic substance extraction from leonardite by demonstrating that targeted recovery can be achieved under optimized conditions with reduced processing time. These findings support the potential application of the extracted products in agricultural systems, particularly as biostimulants.

Future studies should focus on improving extraction yield through process intensification strategies, evaluating the variability among leonardite sources, and assessing the agronomic performance of the extracted humic substances under practical application conditions.

Acknowledgments

This work was supported by the Santa Catarina State Research Support Foundation (FAPESC), the National Council for Scientific and Technological Development (CNPq) through the Academic Master's and Doctoral Program for Innovation (MAI/DAI) and financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) – Finance Code 001.

References

Allahkarami, E., Allahkarami, E., Rezai, B., & Azadmehr, A. (2025). Advancing humic acids extraction procedures: A review of methods, innovations, and sustainability challenges. Separation and Purification Technology, 376, 133954. https://doi.org/10.1016/j.seppur.2025.133954

Barzgar, Z., Towfighi, H., Fathi-Gerdelidani, A., Shahbazi, K., Heidari, A., Etesami, H., Verrillo, M., Savy, D., & Spaccini, R. (2025). Molecular determinants of humic and fulvic acids in enhancing micronutrient availability in calcareous soils: a comparative study on Fe, Mn, Zn, and Cu dynamics. Scientific Reports, 15, 34142. doi: 10.1038/s41598-025-06678-3

Canellas, L. P., Olivares, F. L., Aguiar, N. O., Jones, D. L., Nebbioso, A., Mazzei, P., & Piccolo, A. (2015). Humic and fulvic acids as biostimulants in horticulture. Scientia Horticulturae, 196, 15-27. https://doi.org/10.1016/j.scienta.2015.09.013

Csicsor, A., & Tombácz, E. (2022). Screening of humic substances extracted from leonardite for free radical scavenging activity using DPPH method. Molecules, 27, 6334. https://doi.org/10.3390/molecules27196334

Du Jardin, P. (2015). Plant biostimulants: Definition, concept, main categories and regulation. Scientia Horticulturae, 196, 3-14. https://doi.org/10.1016/j.scienta.2015.09.021

Ibraevaa, Z. E., Kholiya, F., Kabdrakhmanova, A., Gaipbergenova, A. K., Kabdrakhmanova, S. K., Shaimardan, E., Davydova, V. V., & Maussumbayeva, A. M. (2026). Natural biostimulants for sustainable agriculture: the mechanisms and functions of humic substances for plant growth advancement. ACS Omega Article ASAP. https://doi.org/10.1021/acsomega.5c11781

Jomhataikool, B., Kuboon, S., Kraithong, W., & Eiad-Ua, A. (2020). Humic substance extraction from leonardite, lignite Mae Mho Mine by base-acid treatment process. Journal of Applied Sciences, 16, 26-32. https://doi.org/10.14416/j.appsci.2017.10.S04

Lima, L. S. M. S., Almeida, R., Quintaes, B. R., Bila, D. M., & Campos, J. C. (2017). Análises de metodologias de quantificação de substâncias húmicas em lixiviados de aterros de resíduos sólidos. Revista Ambiente & Água, 12, 87-98. https://doi.org/10.4136/ambi-agua.1972

Machado, W., Franchini, J. C., Guimarães, M. F., & Filho, J. T. (2019). Spectroscopic characterization of humic and fulvic acids in soil aggregates, Brazil. Heliyon, 6, e04078. https://doi.org/10.1016/j.heliyon.2020.e04078

Maffia, A., Oliva, M., Marra, F., Mallamaci, C., Nardi, S., & Muscolo, A. (2025). Humic substances: bridging ecology and agriculture for a greener future. Agronomy, 15, 410. https://doi.org/10.3390/agronomy15020410

Meng, F., Yuan, G., Wei, J., Bi, D., & Wang, H. (2017). Leonardite-derived humic substances are great adsorbents for cadmium. Environmental Science and Pollution Research, 24, 23006-23014. https://doi.org/10.1007/s11356-017-9947-8

Nardi, S., Schiavon, M., & Francioso, O. (2021). Chemical structure and biological activity of humic substances define their role as plant growth promoters. Molecules, 26, 2256. https://doi.org/10.3390/molecules26082256

Petrov, D., Tunega, D., Gerzabek, M. H., & Oostenbrink, O. (2017). Molecular dynamics simulations of the standard leonardite humic acid: microscopic analysis of the structure and dynamics. Environmental Science & Technology, 51, 5414-5424. https://doi.org/10.1021/acs.est.7b00266

Qian, S., Ding, W., Li, Y., Liu, G., Sun, J., & Ding, Q. (2015). Characterization of humic acids derived from leonardite using a solid-state NMR spectroscopy and effects of humic acids on growth and nutrient uptake of snap bean. Chemical Speciation & Bioavailability, 27, 156-161. https://doi.org/10.1080/09542299.2015.1118361

Reemtsma, T., These, A., Springer, A., & Linscheid, M. (2008). Differences in the molecular composition of fulvic acid size fractions detected by size-exclusion chromatography–on line Fourier transform ion cyclotron resonance (FTICR–) mass spectrometry. Water Research, 42, 63-72. https://doi.org/10.1016/j.watres.2007.06.063

Rosa, C. M. da., Castilhos, R. M. V., Vahl, L. C., Castilhos, D. D., Pinto, L. F. S., Oliveira, E. S., & Leal, O. dos A. (2009). Efeito de substâncias húmicas na cinética de absorção de potássio, crescimento de plantas e concentração de nutrientes em Phaseolus vulgaris L. Revista Brasileira De Ciência Do Solo, 33, 959-967. https://doi.org/10.1590/S0100-06832009000400020

Shah, Z. H., Rehman, H. M., Akhtar, T., Alsamadany, H., Hamooh, B. T., Mujtaba, T., Daur, I., Al Zahrani, Y., Alzahrani, H. A. S., Ali, S., Yang, S. H., & Chung, G. (2018). Humic substances: determining potential molecular regulatory processes in plants. Frontiers in Plant Science, 13 263. https://doi.org/10.3389/fpls.2018.00263

Sheng, G. P., Zhang, M. L., & Yu, H. Q. (2007). A rapid quantitative method for humic substances determination in natural waters. Analytica Chimica Acta, 592, 162-167. https://doi.org/10.1016/j.aca.2007.04.024

Sun, Q., Liu, J., Huo, L., Li, Y. C., Li, X., Xia, L., Zhou, Z., Zhang, M., & Li, B. (2020). Humic acids derived from leonardite to improve enzymatic activities and bioavailability of nutrients in a calcareous soil. International Journal of Agricultural and Biological Engineering, 13, 200-205. https://doi.org/10.25165/j.ijabe.20201303

Volkov, D. S., Rogova, O. B., & Proskurnin, M. A. (2021). Temperature dependences of IR spectra of humic substances of brown coal. Agronomy, 11, 1822. https://doi.org/10.3390/agronomy11091822

Zandonadi, D. B., Monda, H., Gralian, J., James, A., Lamar, R. T., & Santos, M. P. (2025). Humic acids as drivers of plant growth: regulating root development and photobiology through redox modulation. Chemical and Biological Technologies in Agriculture, 12, 71. https://doi.org/10.1186/s40538-025-00789-9

Rosa, C. M. da., Castilhos, R. M. V., Vahl, L. C., Castilhos, D. D., Pinto, L. F. S., Oliveira, E. S., & Leal, O. dos A. (2009). Efeito de substâncias húmicas na cinética de absorção de potássio, crescimento de plantas e concentração de nutrientes em Phaseolus vulgaris L. Revista Brasileira De Ciência Do Solo, 33, 959-967. https://doi.org/10.1590/S0100-06832009000400020

Shah, Z. H., Rehman, H. M., Akhtar, T., Alsamadany, H., Hamooh, B. T., Mujtaba, T., Daur, I., Al Zahrani, Y., Alzahrani, H. A. S., Ali, S., Yang, S. H., & Chung, G. (2018). Humic substances: determining potential molecular regulatory processes in plants. Frontiers in Plant Science, 13 263. https://doi.org/10.3389/fpls.2018.00263

Sheng, G. P., Zhang, M. L., & Yu, H. Q. (2007). A rapid quantitative method for humic substances determination in natural waters. Analytica Chimica Acta, 592, 162-167. https://doi.org/10.1016/j.aca.2007.04.024

Sun, Q., Liu, J., Huo, L., Li, Y. C., Li, X., Xia, L., Zhou, Z., Zhang, M., & Li, B. (2020). Humic acids derived from leonardite to improve enzymatic activities and bioavailability of nutrients in a calcareous soil. International Journal of Agricultural and Biological Engineering, 13, 200-205. https://doi.org/10.25165/j.ijabe.20201303

Volkov, D. S., Rogova, O. B., & Proskurnin, M. A. (2021). Temperature dependences of IR spectra of humic substances of brown coal. Agronomy, 11, 1822. https://doi.org/10.3390/agronomy11091822

Zandonadi, D. B., Monda, H., Gralian, J., James, A., Lamar, R. T., & Santos, M. P. (2025). Humic acids as drivers of plant growth: regulating root development and photobiology through redox modulation. Chemical and Biological Technologies in Agriculture, 12, 71. https://doi.org/10.1186/s40538-025-00789-9

SUPPLEMENTARY MATERIAL

Supplementary Information for the article "Extraction of Humic and Fulvic Acids from Leonardite for Agricultural Biostimulant Applications"

Table S1. Statistical analysis of effects at a 95% significance for fulvic acid extraction.

Factor	Effect	Standard Error	t(1)	p-value	-95% Cnf. Limit	+95% Cnf. Limit	Coef.	Standard Error Coef.	-95% Cnf. Limit	+95% Cnf. Limit
Average	137.73	0.62	220.81	0.00002	135.04	140.41	137.73	0.62	135.04	140.41
(1) HCl (mol/L)	12.90	1.65	7.82	0.01597	5.80	20.00	6.45	0.83	2.80	10.00
(2) t (min)	-25.00	1.65	-15.15	0.00433	-32.10	-17.90	-12.50	0.83	-16.05	-8.95
1 by 2	-35.40	1.65	-21.45	0.00217	-42.50	-28,30	-17.70	0.83	-21.25	-14.15

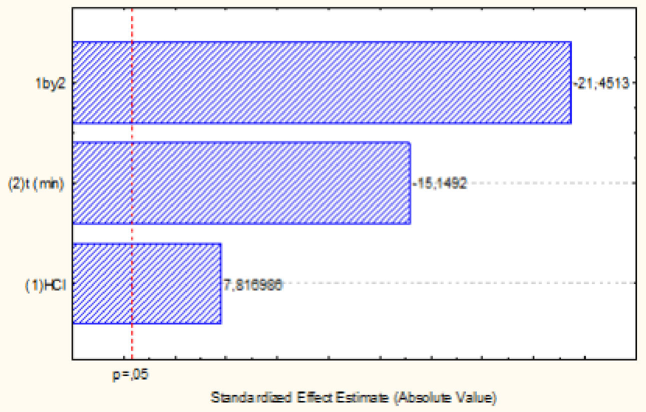

Figure S1. Pareto chart of standardized effects for fulvic acid extraction.

Table S2. Analysis of Variance (ANOVA) for the fulvic acid extraction.

Factor	Sum of squares	Degree of freedom	Mean square	F	p-value
(1) HCl (mol/L)	166.41	1	166.41	61.11	0.01597
(2) t (min)	625.00	1	625.00	229.50	0.00433
1 by 2	1253.16	1	1253.16	460.16	0.00217
Lack of fit	1902.86	1	1902.86	698.72	0.00143
Pure Error	5.45	2	2.72		
Total	3952.87	6			

The coded regression equations describing the extraction yields of fulvic acid (FA) are presented below:

Fulvic acid (FA):

$$FA = 137.73 + 7.82x - 15.15y - 21.45xy$$

where *x* and *y* represent the coded values of the independent variables evaluated in the experimental design.

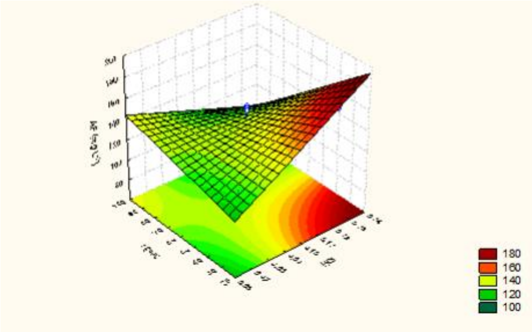

Figure S2. Response surface for the fulvic acid extraction.

Table S3. Statistical analysis of effects at a 95% significance for humic acid extraction.

Factor	**Effect**	**Standard Error**	**t(1)**	**p-value**	**-95% Cnf. Limit**	**+95% Cnf. Limit**	**Coef.**	**Standard Error Coef.**	**-95% Cnf. Limit**	**+95% Cnf. Limit**
Average	182.44	1.13	161.89	0.00004	177.60	187.30	182.44	1.13	177.60	187.30
(1) HCl (mol/L)	45.00	2.98	15.10	0.00436	32.20	57.83	22.50	1.49	16.09	28.91
(2) t (min)	-52.90	2.98	-17.74	0.00316	-65.73	-40.08	-26.45	1.49	-32.86	-20.04
1 by 2	-12.50	2.98	-4.20	0.05246	-25.33	0.33	-6.25	1.49	-12.66	0.16

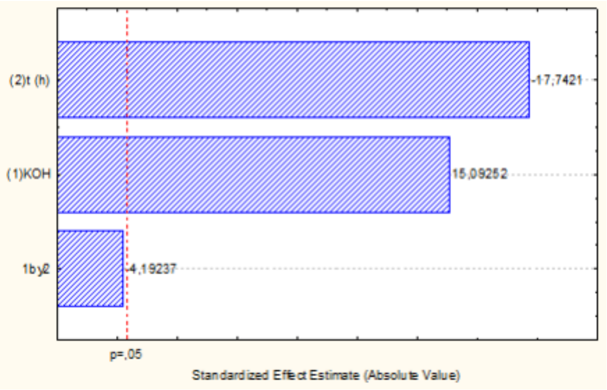

Figure S3. Pareto chart of standardized effects for humic acid extraction.

Table S4. Analysis of Variance (ANOVA) for the humic acid extraction.

Factor	**Sum of squares**	**Degree of freedom**	**Mean square**	**F**	**p-value**
(1) KOH (mol/L)	2025.00	1	2025.00	227.78	0.00436
(2) t (h)	2798.41	1	2798.41	314.78	0.00316
1 by 2	156.25	1	156.25	17.58	0.05246
Lack of fit	3009.62	1	3009.62	338.54	0.00294
Pure Error	17.78	2	8.89		
Total	8007.06	6			

The coded regression equations describing the extraction yields of humic acid (HA) are presented below:

Humic acid (HA):

$$HA = 182.44 + 15.09x - 17.74y$$

where x and y represent the coded values of the independent variables evaluated in the experimental design.

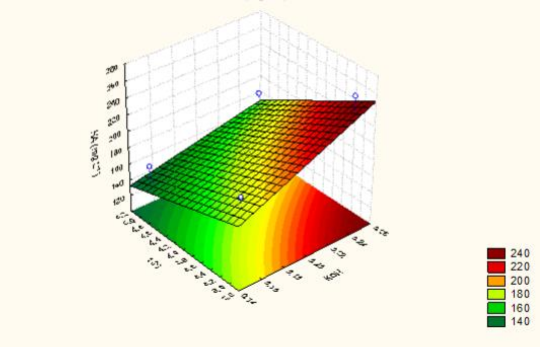

Figure S4. Response surface for the humic acid extraction.