JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Blood Meal-Based Compound. Good Choice as Iron Fertilizer for Organic Farming

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ABSTRACT: Prevention of iron chlorosis with Fe synthetic chelates is a widespread agronomical practice but implies high costs and environmental risks. Blood meal is one of the main fertilizers allowed to be used in organic farming. Through this work a novel blood meal fertilizer was audited. Measurements such as FTIR, Raman, electron paramagnetic resonance, and Mössbauer spectroscopy, UV−visible properties, stability against pH, and batch experiments were performed to characterize and assess the reactivity on soil constituents and agronomic soils. The spectroscopy findings give clear indications that Fe is in the ferric oxidation state, is hexacoordinated, and has a low-spin form suggesting a similar structure to hemin and hematin. A spectrophotometric method at 400 nm was validated to quantify blood meal concentration at low electrolyte concentrations. Batch experiments demonstrated high reactivity of blood meal fertilizer with soil constituents, mainly in the presence of calcium, where aggregation processes are predominant, and its ability to take Fe from synthetic Fe (hydr)oxides. The beneficial profile of blood meal by a providing nitrogen source together with the capability to keep the Fe bound to porphyrin organic compounds makes it a good candidate to be used as Fe fertilizer in organic farming.

KEYWORDS: *iron, fertilizers, organic farming, blood meal, EDDHA, spectroscopy, FTIR, Raman, Mössbauer, EPR, hemin, hematin, porphyrins*

INTRODUCTION

Iron (Fe) chlorosis in plants is an old problem occurring in areas of calcareous and/or alkaline soils.[1] The Fe chlorosis prevention/cure with Fe synthetic chelates analogous to ethylenediaminedi(2-hydroxyphenylacetic) acid (o,o-EDDHA) is a very widespread agronomical practice in vineyards and orchards.[2,3] Nevertheless, such an approach implies high costs and environmental risks.[2−4] Although the use of these synthetic fertilizers in organic farming would be legally allowed in the case of severe micronutrient deficiency, the recalcitrant nature of these synthetic products should be overcome by replacing them with effective and sustainable natural products.

Animal byproducts are materials of the livestock and food industries not intended for human consumption.[5] Every year, millions of tons of animal byproducts are produced worldwide.[6] The rendering industry processes nearly 25 million tons of raw materials per year in North America and about 15 million tons per year in the European Union.[6] Animal byproducts are protein-rich materials and, therefore, offer a high potential as N-fertilizers.[7] Some of these residues are commercially available as certified organic fertilizers. Their application as soil amendments is promoted as a beneficial agricultural practice.[8] Several studies have evaluated the chemical and biological changes produced in soil after the application of animal byproducts,[7,9] their potential to immobilize heavy metals,[10] and their potential as plant pathogen suppressors.[11]

Blood meal is mainly composed of globular proteins[12] containing around 20−30 g Fe kg^{-1}, as ferrous Fe (Fe^{2+}) complexed by the heme group of the hemoglobin molecule,[13] suggesting its use as an effective source of Fe for plants.[14] According to Mori,[15] the incorporation of Fe from the hemoglobin molecule into the root cells may follow a similar mechanism to the uptake of Fe in animal cells (endocytosis). Under field conditions, the application of blood meal (70 g per tree, equivalent to 180 mg of Fe per tree) alleviated Fe chlorosis symptoms of pear plants.[16] Blood meal is one of the main products allowed to be used in Europe as a fertilizer and

Received: January 1, 2013
Revised: April 5, 2013
Accepted: April 9, 2013
Published: April 9, 2013

soil conditioner in organic farming.[17] A solution culture study and a soil incubation study were conducted by Kalbasi and Shariatmadari[18] to investigate the use of poultry blood powder as an Fe source for plants. They demonstrated that Fe from blood powder was as effective as Fe from o,o-EDDHA/Fe in preventing Fe chlorosis of soybeans. Yield and Fe content of plants receiving blood powder were higher than the control and were not different from the plants receiving o,o-EDDHA/Fe. The stability of Fe in this blood meal was found to be high in neutral and acidic solutions. Blood powder appears to be a suitable Fe source for hydroponically grown plants and increases Fe availability when applied to soil. Recently we reported the high efficiency of an animal blood compound in the prevention of grapevine Fe deficiency.[19] Further studies focused on the understanding that the reactivity of blood meal in soils is limited. Adsorption studies on agronomic soils and on main soil constituents (organic matter, clay, carbonate, and Fe (hydr)oxides) should be conducted in parallel, as already done with the synthetic Fe fertilizers,[20−22] to know whether adsorption or leaching processes will be controlling the interaction mechanisms with soil constituents and, therefore, that environmental implications should be considered when applied in organic farming.

Through this work a novel blood meal fertilizer was audited following three steps: (a) structural composition, (b) validation of analytical methodology, and (c) reactivity with main soil constituents. Electroparamagnetic resonance and Fourier transform infrared (FTIR), Raman, and Mössbauer spectroscopy measurements were carried out to analyze Fe oxidation status as well as microenvironmental bonding (spin state, Fe coordination, Fe magnetic properties). The UV−visible spectra were used to validate an analytical methodology to determine the complexed Fe and, thus, to meet the legal requirements for its detection and quantification. Total Fe concentration from commercially available blood meal fertilizer was compared with that reported on the label. Blood meal stability against pH was determined, and batch experiments to evaluate the adsorption rate on soil constituents and agronomic soils were conducted with various pH values.

MATERIALS AND METHODS

Blood meal (kindly provided by FarproAgro, Modena, Italy) was a dark reddish blood powder containing an organic nitrogen content of min. 130 g N·kg^{-1} and an Fe content (as heme-Fe) of 5.5 g Fe·kg^{-1}. Red blood cells from centrifuged bovine whole blood (food grade quality) were hemolyzed and subsequently digested with an alkaline protease. The proteo-hemin fraction was then allowed to precipitate by means of acid addition. Neutralized and purified proteo-hemin was then spray dried to obtain a dark reddish powder. All reagents used in this work were of analytical grade. All aqueous solutions were prepared with Type I grade water.[23]

Spectroscopy. Mössbauer measurement was performed at room temperature with a ^{57}Co (Rh) source of ∼10^{9} Bq activity. The spectrometer (WissEl, Germany) was calibrated with α-Fe at room temperature. The spectrum evaluation was carried out with the assumption of Lorentzian line shape using the MOSSWIN 4.0 software,[24] and the parameters calculated for the spectral components were isomer shift (δ, mm·s^{-1}), quadrupole splitting (Δ, mm·s^{-1}), line width (full width at half-maximum, Γ, mm·s^{-1}), and partial resonant absorption areas (S_i, %). These principal parameters obtained from the spectrum provide information on the electron densities at the Mössbauer nuclei (indicating the valence state) and on the magnitude of any electric field gradients (providing valuable information about the coordination number of the resonant atom). The quantitative analytical information for the species can be obtained from the relative spectral areas.[25]

Electron paramagnetic resonance (EPR) spectroscopy measurements were carried out in the temperature range 160−300 K on 26 mg of blood meal powder, by the means of a Bruker ElexSys E500 X-band spectrometer equipped with a variable-temperature flow-through type insert, by applying evaporated liquid nitrogen as coolant in conjunction with a digital temperature and gas-flow control unit. The conditions of EPR measurements involved four scans, each with a sweep time of ∼168 s, with modulation frequency of 100 kHz, modulation amplitude of 5 G, microwave power of 10.37 mW, and microwave frequency of ∼9.34 GHz. The spectra (being proportional to the first derivative of the power of microwave absorption with respect to the applied magnetic field) were recorded in 4096 channels in the magnetic field range 250−11 750 G. Background correction of the as-measured spectra was carried out on the basis of a spectrum measured at 300 K under identical conditions but without the sample.

The FT-Raman spectrum was recorded by a Bruker Multi-RAM spectrometer equipped with a Ge-diode detector. The excitation source was a Nd^{3+}-YAG laser (1064 nm) with a power on the sample of about 1 mW (this very low power was chosen in order to induce no conformational variation nor thermal decomposition of the sample); the spectral resolution was 4 cm^{-1} and the number of scans was about 10 000 in order to achieve a good signal-to-noise ratio.

Infrared spectroscopy spectra were recorded on a Nicolet 5700 FTIR, equipped with a diamond attenuated total reflectance (ATR) accessory and a DTGS detector; the spectral resolution was 4 cm^{-1}. The ATR technique was chosen because of its nondestructive character.

The UV−vis spectra were recorded on a Varian Cary 100 spectrophotometer, using 10 mm quartz standard cuvettes, with a spectral band width (SBW) of 2.00 nm.

Total Iron Content. A solution of blood meal of 100 mg Fe L^{-1} was prepared by dissolving 1.815 ± 0.001 g of blood meal powder in 100 mL of ultrapure (Type 1) water.[23] Acid-hot digestion was used to make sure that blood meal compounds were destroyed and all Fe would remain in solution. Three volumes of blood meal stock solutions (2.5, 5, and 10 mL) were placed in 100 mL flasks. Volumes of 6 M HCl (2.5, 2.5, and 5.0 mL) and 30% H_2O_2 (2.5, 2.5, and 5.0 mL) solutions were added to blood meal aliquots, respectively. Solutions were left to react for one hour, and then digestion was completed by heating at 80 °C for 30 min. Acid-hot digestion and total Fe content were measured on both filtrated (by passing the solution through a 0.45 μm membrane filter) and nonfiltrated solutions. The Fe concentration was measured by flame atomic absorption spectroscopy (FAAS) at 248.3 nm with the specific hole cathode lamp on a SpectraAA-200 Varian spectrometer. Simultaneously, total Fe content was directly measured by FAAS on both filtered and unfiltered blood meal solutions but without further acid-hot digestion to skip this tedious step when a lot of samples were processed.

Spectroscopy Studies. A battery of experiments was carried out to validate an analytical methodology to quantify total Fe from blood meal product using UV−visible properties reported elsewhere.[26] The first attempt was thought to determine both the working Fe concentration and the more reliable wavelength. Several diluted solutions were prepared from a stock solution of 10 g product·L^{-1} to cover an Fe concentration up to 28 mg Fe·L^{-1} (0.28, 0.55, 1.10, 2.75, 5.50, 8.25, 11.0, and 27.5 mg Fe·L^{-1}). The pH of the resultant solutions and spectrum between 200 and 800 nm was recorded for each one. Although no precipitate was detected, the solutions were filtered through 0.45 μm membrane filters and the spectrum was recorded again.

A second attempt was designed to validate the spectrophotometric methodology by a calibration curve at a working wavelength. For that, several solutions of different blood meal concentrations were prepared covering Fe concentration from 0 to 1.1 mg Fe·L^{-1}. At the same time, another calibration curve was performed but filtering the solutions through 0.45 μm filters; in this case, the final Fe concentration was increased up to 2.0 mg Fe·L^{-1}. The pH for each solution was adjusted to 8.0 by adding HNO_3- or NaOH-diluted solutions. The Fe

concentration was measured by FAAS. The latter procedure was repeated but adjusting the ionic strength to 0.01 M $CaCl_2$ by adding the corresponding volume of the salt stock solution.

The last attempt was designed to assess the effect of pH on absorbance at the working wavelength, as an aggregation of porphyrins may be affecting the absorbance, as reported elsewhere.[26] In fact, blood meal solutions of 1.1 mg Fe·L^{-1} were prepared and pH ranged from 2 to 13. The spectrum was registered from 250 to 800 nm. Results from this experiment were compared with those obtained after addition of $CaCl_2$ to a final concentration of 0.01 M.

Blood Product Stability in Solution versus pH. Ten milliliters of blood meal solution (5.44 g product·L^{-1}) and 0.5 mL of 0.5 M $CaCl_2$ stock solution were added to a 25 mL volumetric flask and brought to the mark with ultrapure (Type 1) water.[23] The pH was adjusted from 2.0 to 12.0, with either HNO_3 or NaOH solutions as needed. Samples were transferred to plastic vessels, and the pH was checked again and corrected if needed for each solution. Solutions were shaken at room temperature for 72 h in an orbital shaker at 100 min^{-1}. The pH values were recorded, and the suspension was centrifuged at 10 000 min^{-1} for 10 min at 20 °C. The supernatant was filtered through 0.45 μm nylon membrane filters. Total dissolved Fe was directly determined by FAAS. Total Fe was measured on a solution containing only 10 mL of bovine blood solution (5.44 g·L^{-1}) and diluted up to 50 mL with ultrapure (Type 1) water[23] to be considered as a reference value.

Blood Meal Adsorption by Soils and Soil Materials. *Reagents.* All chemicals were analytical grade. The salt of $Fe(NO_3)_3·9H_2O$ from Merck was used for ferrihydrite and goethite synthesis. Diluted solutions of NaOH and HNO_3 were used for pH adjustment throughout the batch experiments and synthesis of two-line ferrihydrite and goethite. A sodium nitrate stock solution was prepared, and final desired ionic strength concentrations were obtained by taking the corresponding aliquots from the stock solution.

Soil Materials. Iron (Hydr)oxides. Two-line ferrihydrite was synthesized according to the procedure proposed by Schwertmann and Cornell.[27] Either 5 or 1 M NaOH solutions (depending on curve zone) were slowly added to 0.4 M $Fe(NO_3)_3$ up to a final pH of around 7.0.[28] The resulting sample suspension was allowed to age for 22 h at 20 °C in the dark and then was repeatedly washed with ultrapure (Type 1) water[23] until the conductivity was less than 10 μS·cm^{-1}. The aged suspension was freeze-dried and ground to obtain a reddish-brown powder. A similar procedure was followed for goethite synthesis.[28] In fact, NaOH volumes were slowly added on 0.4 M $Fe(NO_3)_3$ solution up to a final pH of 12. The resulting suspension was allowed to age for 72 h at 65 °C in the dark, and after several washes, the resulting dialyzed suspension was freeze-dried and ground to obtain a yellowish powder.

Total Fe content from the resulting ferrihydrite and goethite solids was determined by FAAS after the solid was dissolved in HNO_3. Structural characterization of both solids was analyzed by both X-ray diffraction and FTIR. Powder X-ray diffraction was measured with a Siemens D-5000 diffractometer. Brunauer–Emmett–Teller (BET) surface areas were measured by N_2 adsorption with a Micrometrics TriStar 300 instrument fitted with a SmartPrep 065 sample preparation system. Point zero of salt effect for each resulting solid was determined by titration using the method of three electrolyte concentrations.

Other Soil Materials. Acid peat was provided by Tolsa S.A. (Buyos, Lugo, Spain), with the following characteristics: pH (H_2O) = 4.0, oxidizable organic matter (g·kg^{-1}) = 854, total organic matter (dry ashed) (g·kg^{-1}) = 995, carbon in humic acids (g·kg^{-1}) = 302, carbon in fulvic acids (g·kg^{-1}) = 183, $N_{Kjeldahl}$ (g·kg^{-1}) = 14, C/N = 35.4, C.E.C. ($cmol_c$·kg^{-1}) = 150. Iron and Mn extracted by the Lindsay and Norvell method[29] were 295 (mg·L^{-1}) and 8.2 (mg·L^{-1}), respectively. Ca-montmorillonite Gonzalez County-Texas (STx-1) was commercially available from Clay Minerals Society Source (Clay Minerals Repository, Dept. of Geology, University of Missouri, Columbia, MO, USA). The $CaCO_3$ is an analytical reagent grade product (Sigma Aldrich).

Agronomic Soil. A Spanish calcareous soil was used as sorbent material as well. Main soil characteristics were as follows: textural class, sandy clay loam; pH (H_2O), 7.82; pH (KCl), 7.23; conductivity, 0.188 dS·m^{-1}; OM, 24.0 g·kg^{-1}; $N_{Kjeldahl}$, 1.40 g·kg^{-1}; total $CaCO_3$, 179 g·kg^{-1}; active $CaCO_3$, 52 g·kg^{-1}. Iron, Mn, Cu, and Zn extracted by the Soltanpour and Schwab method[31] were 26.7, 5.4, 47.1, and 27.4 mg·kg^{-1}, respectively.

Batch Experiments. All batch experiments were placed in a 50 cm^3 polypropylene centrifuge tube by mixing an aliquot (10 mL) of blood meal stock solution (4.35 g·L^{-1}) containing 20 mg Fe·L^{-1} and 0.01 M $NaNO_3$ with an aliquot (10 mL) of each soil material stock suspension with the following concentration: (1) 0.40 g of Ca-montmorillonite, (2) 0.40 g of acid pet, (3) 0.20 g of two-line ferrihydrite and goethite, (4) 1.60 g of $CaCO_3$, (5) 4.0 g of a agronomic soil. The pH values were measured and adjusted before by adding appropriate volumes of diluted HNO_3 or NaOH solutions and measured after interaction. Tubes were allowed to interact for 96 h in a controlled temperature room at 20 °C in the dark on an orbital shaker. After that, suspensions were centrifuged for 15 minutes at 12 000 min^{-1} at 20 °C and filtered through 0.45 μm membrane filters. Total dissolved Fe concentration was quantified by FAAS. Two blank solutions, soil material blank (by adding ultrapure Type 1 water instead of blood meal) and blood meal blank (by adding ultrapure Type 1 water instead of solid phase), were prepared in each experiment to identify color interference and to quantify total added blood meal concentration, respectively. Three replicates per each solid phase (five soil constituents plus one agronomic soil) and each pH point (six points from pH 5 to pH 10) were handled, resulting in 180 propylene tubes.

RESULTS AND DISCUSSION

Spectroscopy. *Mössbauer Spectroscopy.* The Mössbauer spectrum of the solid sample shows two symmetrical quadrupole doublets, suggesting two main chemical environments of Fe (see Figure 1). The Fe component (Fe_A, 40 ± 5% of the total Fe) with δ = 0.17 ± 0.01 mm·s^{-1}, Δ = 2.03 ± 0.01 mm·s^{-1}, and Γ = 0.34 ± 0.02 mm·s^{-1} can be associated with Fe(II)-hemoglobin-O_2 or Fe(III)-hemoglobin-H_2O complex.[25] The second component (Fe_B, 60 ± 5% of the total Fe) can represent a high-spin Fe^{3+} in a distorted octahedral O_6 environment. The Mössbauer parameters of Fe_B (δ = 0.34 ± 0.01 mm·s^{-1}, Δ = 0.66 ± 0.02 mm·s^{-1}, and Γ = 0.66 ± 0.04 mm·s^{-1}) are typical of $Fe_x(OH)_y(H_2O)_z$ oligomeric/polymeric compounds but could also represent μ-oxo-dimeric hemin compounds.[32]

Electron Paramagnetic Resonance. The EPR spectra of blood meal powder measured at temperatures of 160 and 300 K are shown in Figure 2. The origin of the main spectral features can be identified as follows. The peak at $g_a \approx 6.11$ can be

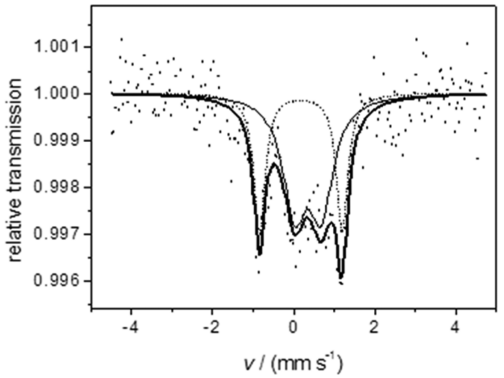

Figure 1. Mössbauer spectrum of the solid blood sample recorded at room temperature.

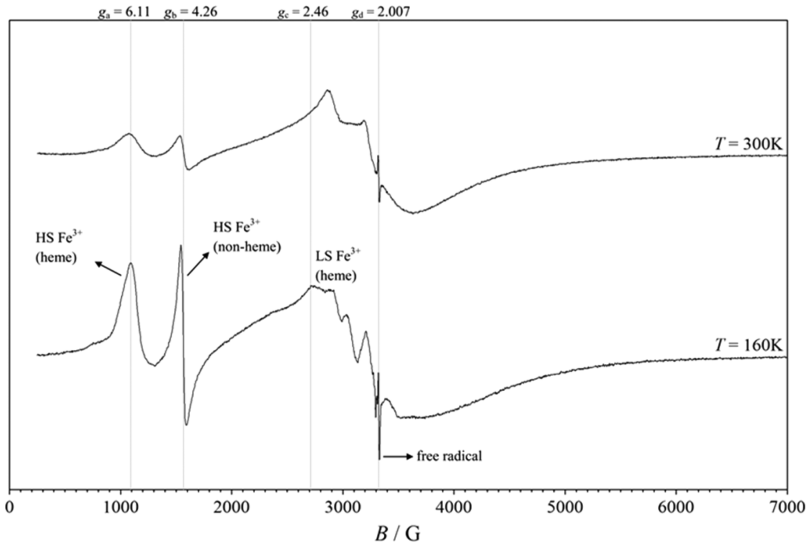

Figure 2. Background-corrected EPR spectra of the solid blood sample recorded at 160 and 300 K, presented for the relevant magnetic field range of 250−7000 G. In order to facilitate comparison of resonance field values, the magnetic field axes of the spectra were scaled together for the microwave frequency applied during the measurement performed at 300 K. The amplitude of the spectral peaks is drawn to the same scale in the two spectra. Specific resonance field values of the spectrum measured at 160 K are indicated by gray vertical lines and the corresponding apparent g values as follows. g_a = 6.11 belongs to the maximum of the EPR signal associated with high-spin Fe^{3+} in heme, g_b = 4.26 belongs to the zero crossing of the EPR signal associated with nonheme high-spin Fe^{3+}, g_c = 2.46 belongs to the maximum of the EPR signal for the range $g < g_b$, and g_d = 2.007 belongs to the maximum of absorption for the free radical signal. The relative error of the given g values is on the order of 0.1%.

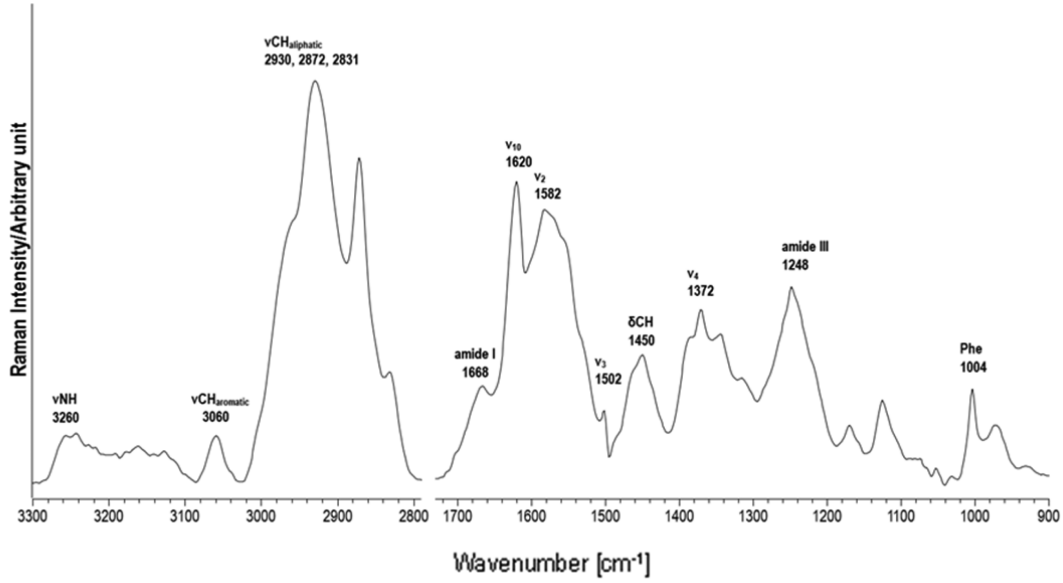

Figure 3. FT-Raman spectrum of blood meal in the 3300−900 cm^{-1} region with the attribution of the main bands discussed in the text.

attributed to high-spin Fe^{3+} in a crystal field of tetragonal symmetry.[33] It is a typical signal of ferric heme Fe in hemoglobin.[34,35] The peak at $g_b \approx 4.26$ refers to high-spin Fe^{3+} in a rhombic crystal field.[35] In tissues of blood and liver it was detected and attributed to ferric Fe in transferrin.[36,37] The narrow signal at $g_d \approx 2.007$ is associated with a free radical usually found in tissues such as blood, liver,[36,37] and brain,[38] where in the latter case it was identified as originating from neuromelanin. At the same time, base porphyrin could also result in a similar free radical signal.[31] The multiple-peak feature at around 3000 G can be attributed to low-spin ferric components, such as ferriheme.[34,39] The moderate spread of the corresponding g values and the peak at $g_c \approx 2.46$ may refer to Type III low-spin ferriheme centers[34,39] with the $(d_{xz},d_{yz})^4(d_{xy})^1$ heme-Fe electron configuration. The broad signal centered at $g \approx 2$ (over which the peaks of low-spin ferric components are superimposed) may be contributed to by superparamagnetic nanoparticles. The peak-to-peak width (i.e., $\Gamma_{pp} = B_{min} - B_{max}$ where B_{min} and B_{max} denote respectively the magnetic field belonging to the minimum and the maximum of the corresponding EPR signal) of this component increases with decreasing temperature from ∼0.71 kG (at T = 300 K) to ∼0.97 kG (at T = 160 K), presumably indicating the slowing down of the relaxation of the particles' magnetization with decreasing temperature.

Raman and FTIR Spectroscopy. Raman spectroscopy uses visible and UV excitation sources that enhance vibration of the porphyrin ring and allow studying the stereochemistry and

electronic properties of the Fe bound in the heme moiety.[40,41] However, other Raman spectroscopic techniques, such as SERS[42] and FT-Raman spectroscopy, proved to give useful information not only on the porphyrin ring but on the overall structure of blood meal. In Figure 3, the FT-Raman spectrum of blood meal in the region 3300−900 cm^{-1} is shown. The spectrum is dominated by the heme skeletal vibrational modes between 1650 and 1300 cm^{-1}: these bands can give useful information on the oxidation, spin, and coordination state of Fe bound in the porphyrin core. In particular, ν_4 is a marker band for oxidation state, ν_2 is sensitive to the spin state, and ν_{10} and ν_3 indicate both spin and coordination.[43] The Raman spectrum of blood meal gives a very clear indication that Fe is in the ferric oxidation state (ν_4 band at 1372 cm^{-1} and ν_3 band at 1502 cm^{-1}), hexacoordinated (ν_{10} band at 1620 cm^{-1} and ν_3), and with a low-spin form (ν_2 at 1582 cm^{-1} and ν_3). The presence of a hexacoordinated Fe(III) could suggest that Fe is coordinated by an OH^- ion and by a Cl^- (maybe OH^- at higher pH values) and that the blood meal structure is very similar to hemin (Cl^- coordinated) and hematin (OH^- coordinated). This hypothesis could be confirmed by a resonant Raman spectrum, where low-frequency bands of the Fe(III)−OH and Fe(III)−Cl modes could be more easily observed.

These results on Fe-porphyrin characterization can be explained by the strong hydrolytic and enzymatic reactions that bovine blood underwent during the industrial preparation of the fertilizer. Moreover, the FT-Raman spectrum suggested that part of the porphyrin core is surrounded by small amino acidic chains that gave rise to medium-intensity amide I and amide III bands (at 1668 and 1248 cm^{-1}) and to the 1004 cm^{-1} band, typical of the phenylalanine residues.

Total Iron Content. Similar Fe content from acid-hot-digested and nondigested solutions was found, suggesting that total Fe may be directly determined from blood meal solutions without further digestion procedures. No significant differences were found when total Fe from filtered (0.50 ± 0.04 %, expressed in terms of g Fe·100 g^{-1} product) and nonfiltered samples (0.48 ± 0.04 %) were compared at pH around 8.0. The presence of residues in the filter is not due to Fe precipitates, but to the precipitation of polycondensate compounds by aggregation of porphyrin units. Therefore, the total Fe concentration from experimental data, 0.49 ± 0.04 % (g Fe·100 g^{-1}), was slightly lower than that provided by the product manufacturer, 0.55 % chromatographically determined.

Spectrophotometric Studies. The pH values for all blood meal solutions were between 8.30 and 8.62. A broad peak with maximum absorbance around 400 nm was detected (Figure 4). An upper limit of the spectrophotometric method was detected, being around 1 mg Fe·L^{-1} (0.2 g blood meal·L^{-1}), for nonfiltered blood meal solutions (solid line in Figure 4). However, more than 50 % of the color intensity disappeared when it was filtered (dashed line in Figure 4). Although no Fe precipitation process was detected, a typical porphyrin aggregation process occurred as reported by Grenoble and Dickramer.[44] The resulting spectrum type may be related to a hemin-type spectrum. Lombardo et al.[26] reported that when the spectroscopic properties of a variety of hemin chlorides in low-polarity solvents were examined, two absorption peaks in the Soret region, one at about 360−380 nm (S band) and the other between 400 and 430 nm (S band, as mentioned above), could be readily identified (solid line in Figure 4). Additionally, spectrophotometric methods to estimate hemin by using a working wavelength of 400 nm were reported elsewhere.[45]

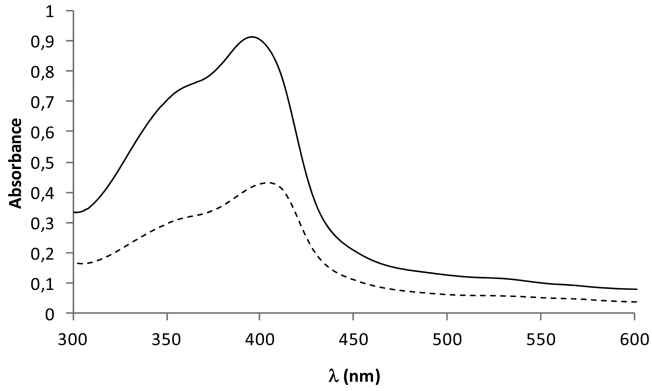

Figure 4. UV−vis spectrum of blood meal fertilizer (0.2 g·L^{-1}) solution. Solid line represents the nonfiltered solution and dashed line the same solution after filtering through a 0.45 μm membrane filter. The pH of the solution was 8.25.

Good linear fitting and comparable linear coefficients were reached for blood meal solutions regardless of filtration step (black diamonds and circles and pointed and solid lines in Figure 5). As expected from absorbance results, the upper Fe

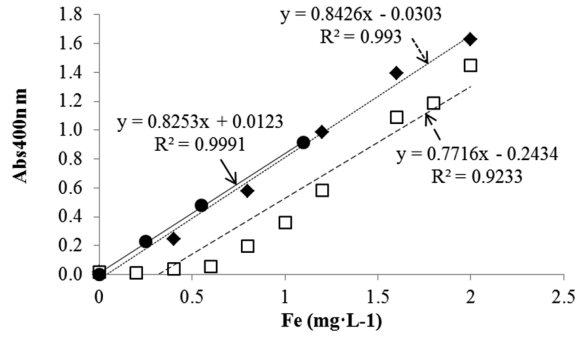

Figure 5. Spectrophotometric method for quantification of blood meal fertilizer (in terms of mg Fe·L^{-1}) by recording the absorbance value at 400 nm. Three calibration curves of blood meal product were arranged at desired concentrations by diluting from stock solutions. Black circles and solid line represent calibration curve and linear fitting without filtering, respectively. Black diamonds and pointed line represent calibration curve and linear fitting filtered through 0.45 μm, respectively. White squares and dashed line represent calibration curve and linear fitting at ionic strength 0.01 M $CaCl_2$ and filtered through 0.45 μm membrane filters, respectively. pH of all solutions was adjusted to 8.2 ± 0.2.

concentration limit might be increased up to around 2 mg·L^{-1} after the filtering step through 0.45 μm. In accordance with these results, the spectrophotometric method at 400 nm might be proposed as a simple analytical methodology to quantify blood meal concentration. However, when electrolyte concentration was increased up to 0.01 M $CaCl_2$, as majority cation in Mediterranean soils,[46] the lack of linearity in the calibration curve at low concentrations (up to 0.5 mg·L^{-1}) was evident (white squares and dashed line in Figure 5), limiting the proposed spectrophotometric methodology to be used at low electrolyte concentrations. Anyway, further analytical assays should be arranged to check whether this proposed analytical method could be used at different electrolyte levels. The ability of porphyrins to form molecular aggregates in solution was well established by White.[47] Porphyrins having negative charges at the periphery are considerably more basic and have much

greater tendency to aggregate than those having positive charges at the periphery.[48]

Blood Product Stability in Solution versus pH. The content of dissolved Fe versus pH is shown in Figure 6.

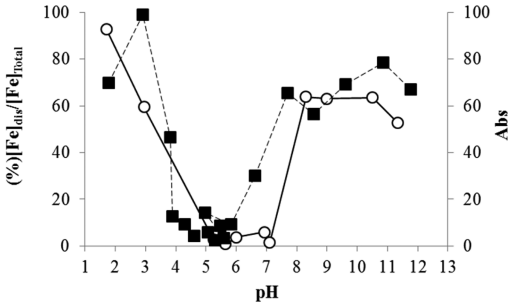

Figure 6. Percentage of soluble Fe recovered at different pH values in 10 mM Ca(II) solution (white circles and solid line) and in the absence of Ca (black squares and dashed line), respectively.

Solutions prepared with 0.01 M $CaCl_2$ (solid line and white circles) and without $CaCl_2$ (dashed line and black squares) were compared in order to assess the electrolyte effect on blood meal product. Dissolved Fe in solutions without $CaCl_2$ was estimated in terms of absorbance as explained above. Dark precipitates were detected for all solutions at pH values higher than 3 regardless of electrolyte concentrations. Almost all Fe remained in solution at a pH around 2 for both solutions. Around 60% of Fe was recovered at pH values higher than 7.0 for solutions without $CaCl_2$ and 8.0 for solutions with 0.01 M $CaCl_2$. To explain this fact, it should be kept in mind the different compounds formed along the pH interval. Hemin and hematin are the prototype molecules for hemoglobin. In both cases, ferric Fe is inserted at the center of a planar porphyrin ring so that the Fe(III) ion is in a site of essentially square planar symmetry with four nitrogen ligands.[44] In hemin, however, there is a chloride ion attached to the Fe atom, causing its displacement of 0.475 Å out of the plane of the ring. In hematin, the Cl^- ion is replaced by an OH^-; although the details are not as well determined as for hemin, the symmetry is very similar.[49] Thus, at pH values below 6.0, where chloride or nitrate anions are abundant, hemin compounds of low water solubility are predominant. By contrast, when the chloride anion is replaced by a hydroxide anion as the pH increases, a hematin compound is formed and the aggregation property is limited. Anyway, Fe remains bound to porphyrin organic compounds along a broad pH interval as hemin at acid pH's or hematin at basic pH's, preventing Fe release and precipitation as amorphous Fe (hydr)oxide.[44] Other common synthetic Fe fertilizers such as EDTA/Fe chelate present limited stability in calcareous soil, not recommended at pH's higher than 6.5.[1]

Blood Meal Adsorption on Soils. The BET surface areas of two-line ferrihydrite and goethite were 256.8 ± 1.1 and 59.9 ± 0.1 $m^2 \cdot g^{-1}$, respectively, in line with those reported by Villalobos and Antelo[50] and Iglesias et al.,[51] respectively. The structure and chemical composition of the resultant two-line ferrihydrite and goethite were confirmed by the XRD patterns (Figure 7A and B, respectively). Two broad peaks at about 2θ = 35° and 62° were found respectively for two-line ferrihydrite, in line with the results reported by Li et al.[52] No evidence of other Fe (hydr)oxides phases was detected with this technique.[29] Experimental point zero of salt effect for two-line ferrihydrite and goethite was calculated to be 7.9 ± 0.2 and 7.5 ± 0.3,

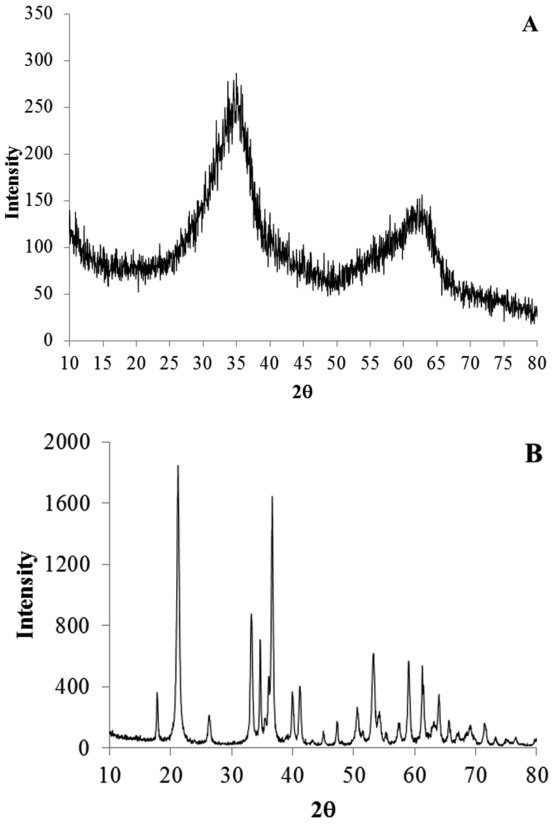

Figure 7. X-ray diffractograms for (A) two-line ferrihydrite and (B) goethite.

respectively. These results were in accordance with those compiled by Villalobos and Antelo.[50] FTIR spectra for two-line ferrihydrite and goethite are shown in Figure 8, and they can be

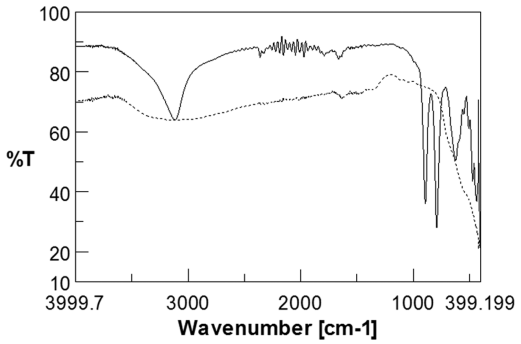

Figure 8. FTIR spectra of two-line ferrihydrite (dashed line) and goethite (solid line).

compared with those reported by Hausner et al.[53] and Tejedor−Tejedor et al.,[54] respectively. No clear evidence of large carbonate concentration may be argued from ATR-IR spectra information.

Concentrations of soil constituents were chosen following the concentrations recommended elsewhere.[21,22] Batch experiments were performed in a pH interval between 5 and 10 because, as commented above, aggregation processes out of this pH interval were detected in previous experiments. Total dissolved blood meal product in each soil constituent (in terms of percentage of dissolved Fe) is shown in Figure 9. No typical percentage of adsorption rate has been plotted because it would

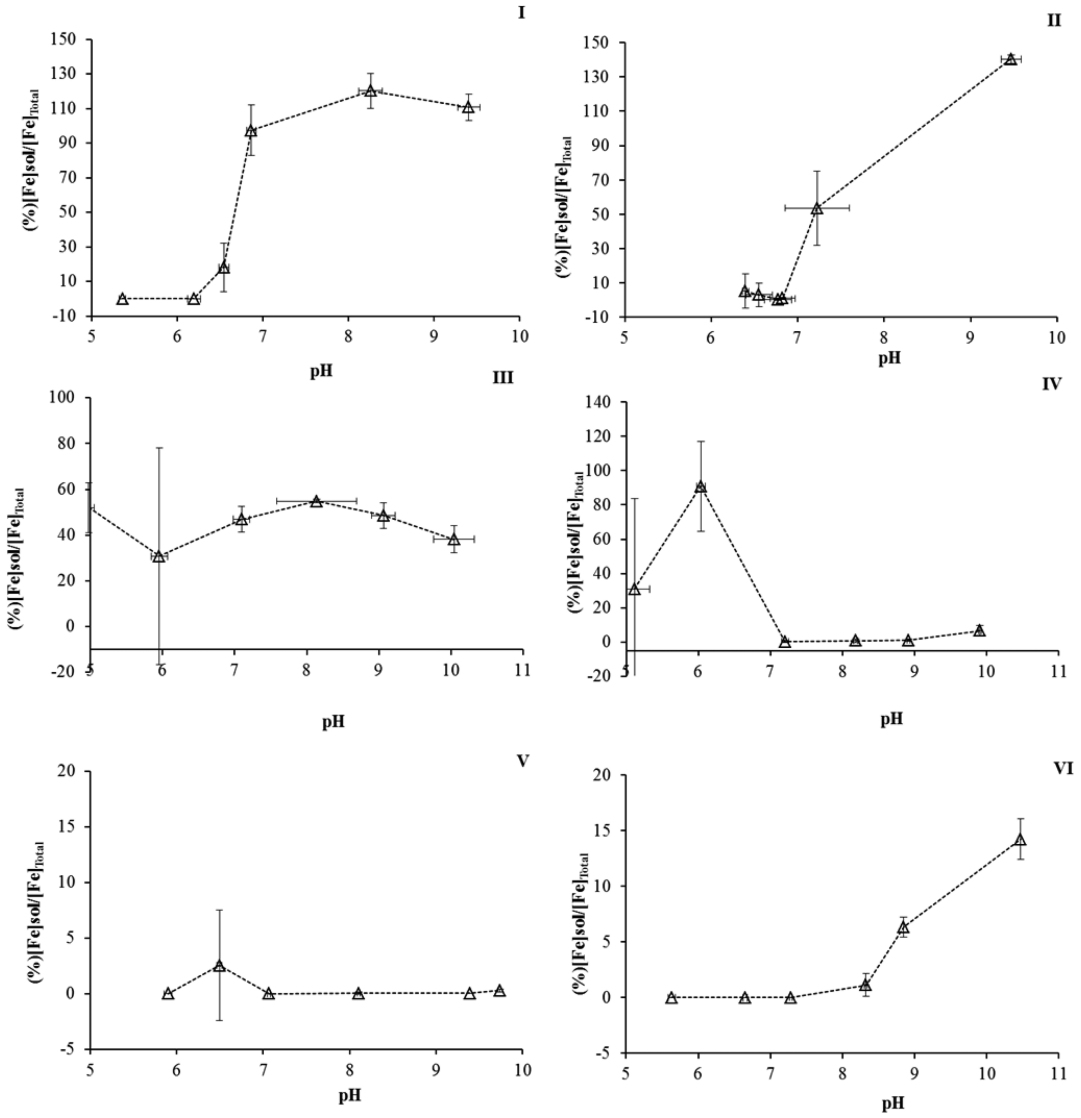

Figure 9. Blood meal adsorption as a function of pH on (I) 10 g·L^{-1} two-line ferrihydrite, (II) 10 g·L^{-1} goethite, (III) 20 g·L^{-1} acid peat, (IV) 20 g·L^{-1} Ca-montmorillonite, (V) 80 g·L^{-1} $CaCO_3$, and (VI) 200 g·L^{-1} agronomic soil. Total blood meal = 2.18 g·L^{-1}; electrolyte = 0.01 M $CaCl_2$. Error bars show confidence intervals at 95%.

not be rigorously true since adsorption/precipitation/aggregation processes might be simultaneously occurring, due to the hemin/hematin aggregation property explained above. All Fe remained in solution at pH's above 7 and above 9 when blood meal was interacted with two-line ferrihydrite and goethite, respectively. Maximum adsorption rates at acid pH's, where positive charges from two-line ferrihydrite and goethite are predominant on negative charges, confirm that blood meal product is negatively charged, as argued in the previous section, where the chloride anion in the hemin compound is directly bound to the central Fe. This finding is in line with that reported by Machado et al.,[55] where Fe(III) porphyrins charged negatively were strongly adsorbed on zinc hydroxides charged positively. Additionally, the interactions with Fe (hydr)oxides showed surprising results, as the blood meal product was able to dissolve Fe from native Fe (hydr)oxides at basic pH values: up to 20 % from two-line ferrihydrite at a pH around 8.0 and up to almost 40 % at pH 9.5 for goethite. This Fe solubilization from native oxides could be partially explained by the presence of either amino acid-type compounds or other types of organic residues from blood meal product that are able to solubilize Fe at alkaline pH's. On the other hand, no significant aggregates were detected at basic pH's due to the absence of cations such as Ca, which favors the presence of aggregates, and therefore, no Fe in solution was detected. The effect of Ca on blood meal compound aggregation is demonstrated when $CaCO_3$ and agronomic soil were used as sorbent materials. Regarding the interaction with peat, around 50% of Fe remained in solution along the pH interval. As observed from previous results, aggregation processes are predominant since no adsorption processes through ionic bonds between negative charges of organic matter and negative functional groups of blood meal would be expected.

Two different pH-dependent adsorption responses may be observed after interaction between clay and blood meal compound (see Figure 9, IV): large adsorption rate at basic pH's and either linear or low adsorption rate at acid or neutral pH's. Rice and Bergkvist[56] found a linear absorption response at acidic and basic pH's and a slight deviation, probably due to a gradual transition between protonated and deprotonated states,

dx.doi.org/10.1021/jf305563b | J. Agric. Food Chem. 2013, 61, 3995−4003

at intermediate pH's. They reported that porphyrin adsorption on clay occurs in a monolayer formation at low/high pH, while at slightly acidic/neutral pH, they possibly rearrange on the surface and/or form aggregates. In the same way, Weiss and Roloff[57] concluded that anionic porphyrin-type compounds may be adsorbed either on the edge surfaces or in interlayer sites.

Blood meal is mainly composed of globular proteins, which are mostly water-soluble and easily hydrolyzed by microorganisms.[12] Moreover, the beneficial profile of blood meal by providing a nitrogen source together with the capability to keep the Fe bound to porphyrin organic compounds makes it possible to be used as an Fe fertilizer in organic farming. However, the large retention rate observed with Ca soil materials and calcareous agronomic soil (with high Ca content) suggests taking into account a possible limitation in the use of this natural compound when the soil is highly rich in lime. Since aggregation processes appear to be predominant over free Fe precipitation, the effectiveness of the blood meal compound should be specifically assessed through plant cultures. In fact, the high ability of preventing Fe deficiency displayed by a similar animal blood compound[19] suggested that adsorption/aggregation processes at the root−soil interface may be modified by root activities. For example, the adverse effect of Ca on the solubility of Fe contained in blood meal compound (Figure 6) could be overcome by removing Ca as a result of root uptake.

In conclusion, a novel blood meal product was audited to be used as fertilizer. Spectroscopy studies have revealed the complexity of the Fe complexation in the blood meal product, although the ferric hexacoordinate form could be present. Regarding the analytical methodology, a spectrophotometric method at 400 nm was validated to quantify blood meal concentration at low electrolyte concentrations. The characteristics displayed by the compound indicate its good potential as Fe fertilizer in organic farming.

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Funding

This work has been partially supported by the European Science Foundation (ESF) within the framework of the ESF activity "The Functionality of Iron Minerals in Environmental Processes" (scholarship to M.D.F.).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors are very grateful to Prof. Raúl Fernández and Prof. Jaime Cuevas from Geology and Geochemistry Department of the University Autónoma of Madrid for the goethite and two-line ferrihydrite BET measurements and Prof. Juan J. Lucena and his research team from Agricultural Chemistry Department of the University Autónoma of Madrid for their support during iron (hydr)oxide synthesis. Many thanks to FarproAgro for kindly providing the blood meal product.

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