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Chemical Evaluation of HBED/Fe^{3+} and the Novel HJB/Fe^{3+} Chelates as Fertilizers to Alleviate Iron Chlorosis

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Iron chelates such as ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic) acid (*o*,*o*-EDDHA) and their analogues are the most efficient soil fertilizers to treat iron chlorosis in plants growing in calcareous soil. A new chelating agent, HJB (*N*,*N*′-bis(2-hydroxy-5-methylphenyl)ethylendiamine-*N*,*N*′-diacetic acid) may be an alternative to *o*,*o*-EDDHA since its synthesis yields a purer product, but its chemical behavior and efficiency as chlorosis corrector should be evaluated. In this research, a known analogous HBED (*N*,*N*′-bis(2-hydroxyphenyl)ethylendiamine-*N*,*N*′-diacetic acid) has also been considered. First, an ion-pair high performance liquid chromatography (HPLC) method has been tested for the HJB/Fe^{3+} and HBED/Fe^{3+} determination. The ability of HJB and HBED to maintain Fe in solution has been compared with respect to *o*,*o*-EDDHA. Theoretical modelization for HBED and HJB in agronomic conditions has been done after the determination of the protonation and Ca(II), Mg(II), Fe(III), and Cu(II) stability constants for HJB. Also, batch interaction experiments with soils and soil materials have been conducted. According to our results, HJB/Fe^{3+} and HBED/Fe^{3+} present high stability, even when competing cations (Cu^{2+}, Ca^{2+}) are present, and have low reactivity with soils and soil components. The chelating agent HJB dissolves a higher amount of Fe than *o*,*o*-EDDHA, and it seems as effective as *o*,*o*-EDDHA in keeping Fe in solution. These results indicate that these chelates may be very efficient products to correct Fe chlorosis, and additional plant experiments should demonstrate plants' ability to assimilate Fe from HJB/Fe^{3+} and HBED/Fe^{3+}.

KEYWORDS: Iron; chelates; fertilizers; HBED; HJB; *o*,*o*-EDDHA; HPLC; speciation

INTRODUCTION

Amino polycarboxylic acids are recognized as highly effective Fe chelators. Ethylenediaminedi-(2-hydroxyphenylacetic) acid (*o*,*o*-EDDHA) is widely used to control iron chlorosis. This is a nutritional disorder in plants characterized by a significant decrease of chlorophyll in leaves, which, among other causes, results from the low availability of Fe in calcareous soils (*1*). *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HBED) also forms highly stable Fe(III) chelates and is of great interest for the removal of iron from test animals and therefore is of concern for the treatment of Cooley's anemia (*2*). Both *o*,*o*-EDDHA and HBED are hexadentate ligands that have two phenolic groups instead of two of the carboxylates of EDTA. They contain the same set of donor groups (two phenolate donors, two amino-nitrogen donors, and two carboxylate donors) and therefore show very high affinities for Fe(III), with stability constants for *o*,*o*-EDDHA of $\sim 10^{35}$ (*3*) and HBED of $\sim 10^{39}$ (*2*). However, the presence of two chiral carbon atoms (those next to carboxylate groups) in the EDDHA structure leads to the formation of *meso* and *rac* forms of the chelates, which have different stability constants because of changes in coordination geometry (*2*, *4*). In HBED, no chiral carbons occur; therefore, it does not present optical isomers.

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The industrial synthesis of commercial EDDHA yields a mixture of regioisomers (*o*,*o*-EDDHA, *o*,*p*-EDDHA, and *p*,*p*-EDDHA) and polycondensation products in variable amounts (*5*−*7*). Taking into account that the two *p*-hydroxyphenyl groups of *p*,*p*-EDDHA are sterically impeded to bind Fe(III) (*3*) and that oligomeric EDDHA-like compounds have a limited value as Fe fertilizers (*7*), the Fe chelated content in commercial EDDHA formulations is limited to 6% chelated Fe. However, the synthesis of HBED may yield a product with higher Fe content, more than 9% (*8*), but as far as we know, it has never been used in agriculture since it has been considered inappropriate for providing Fe to plants due to its very high Fe(III) stability constant. The chelating agent *N*,*N*′-bis(2-hydroxy-5-methylbenzyl)ethylenediamine-*N*,*N*′-diacetic acid (HJB) is related to HBED but with methyl groups bound to the phenolates in the *para* position (see **Figure 1**). HJB has the same donor groups and a structure similar to that of HBED. The synthesis of HJB produces also a purer product than Fe-EDDHA because no optical isomers or other byproducts are formed (*8*). A high affinity for Fe(III) is expected, but the presence of the methyl substituent in the phenolic rings may affect the chelating ability of HJB compared to that of HBED, similar to the effect observed on EDDH5MA when compared to *o*,*o*-EDDHA (*3*), the latter being more effective than EDDH5MA for binding Fe(III).

The efficacy of a Fe chelate to remediate Fe chlorosis is determined by (a) its ability to maintain Fe in solution, without

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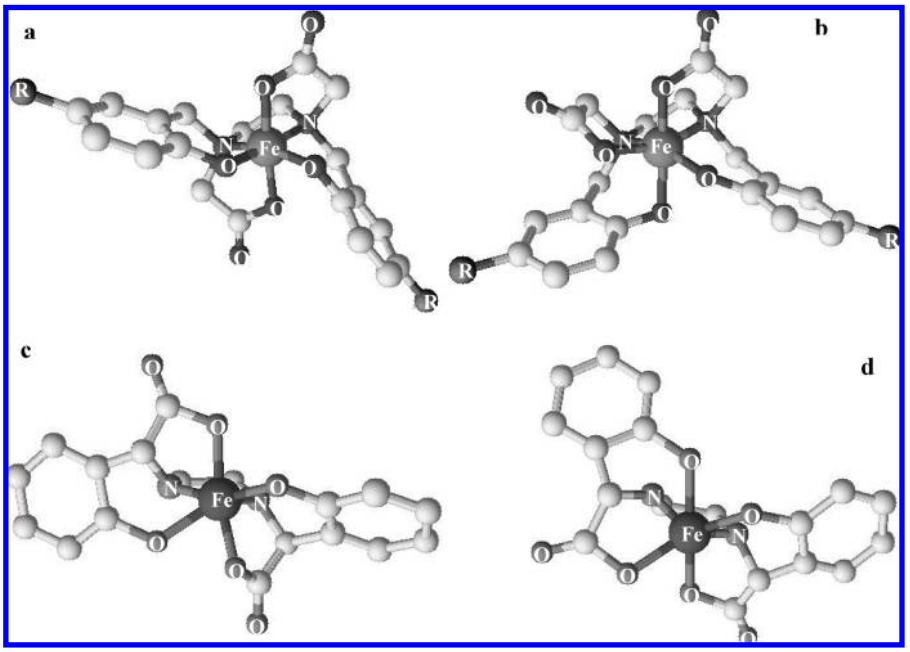


Figure 1. Spatial structures of Fe(III) chelates of (**a**) HJB (R = CH_3) and HBED (R = H) with (6,5,6) arrangement, (**b**) HJB (R = CH_3) and HBED (R = H) with (6,5,5) arrangement, (**c**) *rac-o,o*-EDDHA, and (**d**) *meso-o,o*-EDDHA.

being displaced by other cations or being retained by soil surfaces, (b) its capacity to deliver Fe to the plant, and (c) its facility to chelate native Fe from the soil, having delivered a Fe ion to the plant (shuttle effect) (*9*). This work focus on items a and c, which mainly depend on chemical characteristics of the chelates. Halvorson and Lindsay (*10*) developed computer programs to calculate the equilibrium levels of chelated metals in nutrient solutions and in soil systems, considering solid phases and the presence of oxides. This type of modelization has been already applied to study the stability of several Fe chelates (*3*, *11*), showing a good correlation between the modelization results and their behavior in agronomic conditions. In addition, different soil tests have been proposed (*7*, *12*−*16*), in order to evaluate and compare the losses of available Fe from chelates by their retention on solid surfaces and/or Fe replacement by different ions, mainly H^+, Ca^{2+}, or Cu^{2+}. According to Álvarez-Fernández et al. (*13*), ferrihydrite and acid peat are the most reactive soil components with respect to Fe-EDDHA. The *meso* isomer is mainly retained by organic matter, and the *rac* isomer by iron oxides (*12*, *16*).

The quantification of the Fe chelates is a prerequisite for the reactivity studies and a useful tool to determine the purity in commercial products. Previously, an ion-pair chromatography method was developed to determine Fe chelates of EDTA, DTPA, CDTA, EDDHA, EDDHMA, HBED, and HBEP, with the TBAOH as the ion-pair reagent (*17*). This method has been accepted as European Norm (*18*) but its application to HJB has not been yet demonstrated.

The aim of this work is to know the potential use of Fe-HJB and Fe-HBED as Fe fertilizers by studying their speciation in soils. Theoretical studies, using calculated stability constants and reactivity in soils conditions are evaluated. Also the usefulness of the ion-pair chromatographic method currently used for other chelates is evaluated for Fe-HJB and Fe-HBED.

MATERIAL AND METHODS

The procedures employed to determine the purity of the chelating agents *o,o*-EDDHA, HBED and HJB, and the stability constants of HJB have been previously described in detail (*3*, *11*). A brief description is given below.

Chelating Agents and Fe Chelates. Standard chelating agents *o,o*-H_4-EDDHA and HBED·HCl were purchased from Promochem and Strem Chemicals, respectively. Samples of HJB·HCl and HBED·HCl obtained as in ref *8* were kindly provided by PCC ADOB.

The ^{1}H NMR and ^{13}C NMR spectra of HJB were kindly provided by PCC ADOB, and HBED was characterized on a Bruker 200-AC spectrometer (200.13 MHz for ^{1}H and 50.03 MHz for ^{13}C).

The titrimetric purity of the chelating agents was determined by using a photometric method (*3*). In brief, ligand solutions of about $1\cdot10^{-4}$ M were titrated with a $4.48\cdot10^{-4}$ M Fe(III) standard solution until the absorbance at 480 nm presented no changes. Titrations were carried out at 25.0 ± 0.5 °C in a sealed, water-jacketed glass vessel and under purified N_2 atmosphere. Ionic strength was maintained at 0.1 M with NaCl, and pH was fixed at 6.0 with 2 mM MES. The HJB/Fe^{3+} and HBED/Fe^{3+} molar absorptivities at 480 nm were also obtained.

For preparing each Fe chelate solution, ligand was dissolved in NaOH (1:3 molar ratio or 1:4 in the case of HBED·HCl and HJB·HCl). Then $Fe(NO_3)_3$ solution (5% Fe in excess of molar amount) was added and pH adjusted to 7.0. The solution was left to stand overnight, filtered, and made up to volume.

Protonation Constants of HJB. The protonation constants have been determined by using both photometric and potentiometric methods. The first and second protonations occur at high pH, where potentiometric measurements become inaccurate. Then their protonation constants were determined spectrophotometrically (*19*) since the combination of protons with the phenolic groups is accompanied by extensive changes in the absorption spectra (at 301.6 and 238.6 nm). Twelve $1.0\cdot10^{-4}$ M solutions were prepared with the ionic strength adjusted to 0.100 M with NaCl and pH adjusted from 10.00 to 13.30 at 0.3−0.5 pH intervals. Spectra (200−400 nm) were obtained using a Shimazdu UV−vis spectrophotometer. The wavelength on the maximum absorbance and molar absorptivities of L^{4-} and LH_2^{2-} species were initially estimated at pH 13.5 and 9.8, respectively, and used as seeds for the following calculations. The calculations themselves involved a least-squares minimization of calculated versus observed absorbances, varying the first and second protonation constants and the molar absorptivities (*3*).

The lowest protonation constants were determined by potentiometric titration (*20*). HJB chelating agent solution ($1.0\cdot10^{-3}$ M; μ = 0.1 M (NaCl)), previously dissolved in four equivalents of NaOH per equivalent of chelating agent, was back-titrated with 0.0483 M HCl solution at 25.0 ± 0.5 °C under N_2 atmosphere. The third and fourth ligand protonation constants, corresponding to the two amino groups, were calculated using the program Hyperquad 2006 (*21*). The protonation constants

Table 1. Selected Chemical Characteristics of the Two Agricultural Soils Used

		pH					$CaCO_3$ ($g \cdot kg^{-1}$)					
	texture	H_2O	KCl	EC ($dS \cdot m^{-1}$)	OM ($g \cdot kg^{-1}$)	N_{kj} ($g \cdot kg^{-1}$)	total	active	Fe[a] ($mg \cdot kg^{-1}$)	Mn ($mg \cdot kg^{-1}$)	Cu ($mg \cdot kg^{-1}$)	Zn ($mg \cdot kg^{-1}$)
Sudanell soil	sandy clay loam	7.82	7.23	0.188	24.0	1.40	179	52	26.7	5.4	47.1	27.4
Picassent soil	sandy loam	7.70	7.10	0.270	9.2	0.30	380	89	2.3	1.9	0.7	2.5

[a] Micronutrients determined as in ref *28*.

corresponding to the carboxylate groups (K_5^H and K_6^H) could not be determined because of their low values, below the titration's pH.

Ca and Mg Stability Constants of HJB. Potentiometric titrations were also used to determine the Ca(II) and Mg(II) stability constants. HJB/Ca^{2+} and HJB/Mg^{2+} chelates in a 1:1 rate were previously formed and then were back-titrated with 0.0483 M HCl solution at 25 °C under N_2 atmosphere.

Total Ca(II) and Mg(II) concentrations were measured by AAS (Perkin-Elmer Analyst 800). The Ca(II) and Mg(II) stability constants were calculated using the program Hyperquad 2006 (*21*).

Fe(III) and Cu(II) Stability Constants of HJB. Stability constants for the Fe(III) and Cu(II) chelates were calculated from spectrophotometric data obtained after titration (*3*). Four equivalents of standard base (0.200 M) were added to the chelating agent, and then the ionic strength was maintained at 0.100 M with NaCl. Solutions of Fe(III) and Cu(II) chelates (1:1 metal/ligand ratio) were prepared under N_2 at 25.0 ± 0.5 °C, by slow addition of Fe(III) and Cu(II) standard solutions. For the Fe(III) chelate, the pH was raised to 12 by the addition of NaOH. The $1 \cdot 10^{-4}$ M experimental solution was titrated with aqueous 2.0 M HCl titrant to pH 0.5. The $1 \cdot 10^{-3}$ M Cu(II) chelate solution was adjusted to pH 1.0 and titrated with aqueous 0.200 M NaOH titrant to pH 12.0. The absorbance of the solutions was measured at 480 nm for the Fe(III) chelate and 650 nm for the Cu(II) chelate.

Total Fe(III) and Cu(II) concentrations were measured by AAS. The stability constants (K_{FeL}, K_{FeHL}, K_{CuL}, K_{CuHL}, and K_{CuH2L}) were calculated from the data by an in-house program using Microsoft Excel Solver utilizing mass balance and known equilibrium constant constraints while minimizing the least-squares absorbance fit to the observed spectral curves (*3*).

Theorical Chelate Stability. The behavior of the Fe(III) chelates was studied in a nutrient solution system and in soil conditions by modelization. Percentage of chelated Fe was obtained using the equilibrium speciation model VMINTEQ program (*22*). The component database was modified in order to include the HJB and HBED chelating agents as new components. The thermodynamic database was also modified including every protonation, Ca(II), Mg(II), Cu(II), and Fe(III) stability constants for each species formed. The percentage of Fe chelated by HJB, HBED, *rac o,o*-EDDHA, *meso o,o*-EDDHA, and EDTA was calculated at pH range 4−13 in two theoretical models considering their agronomic use.

The first model was used to determine the behavior of the chelates in hydroponic conditions. Hoagland nutrient solution was used and $Fe(OH)_3$(amp) was introduced in the system as a solubility controller.

The second model was used to predict the behavior of the chelates in soil conditions. All soil components that could have some effect on HJB/Fe^{3+} and HBED/Fe^{3+} stability were considered. Two soil types with unlimited and limited Cu(II) availability were proposed in order to predict the stability of HJB/Fe^{3+} and HBED/Fe^{3+} with high and low Cu(II) levels in soil, respectively. (See Supporting Information for details of the model conditions.)

Chromatographic Method for the Determination of HBED/Fe^{3+} and HJB/Fe^{3+}. The ion-pair HPLC method proposed by Lucena et al. (*17*), which was adopted as European Standard by CEN (*18*) for the determination of Fe chelated by *o,o*-EDDHA and *o,o*-EDDHMA, was tested for the determination of Fe chelated by HJB and HBED. A Waters 2695 Separation Module, a Waters 996 Photodiode Array Detector, and a Symmetry C-18 (150 × 3.9 mm and d_p = 5 μm) column were used. The mobile phase consisted of 30% acetonitrile in 0.03 M TBAOH aqueous solution at pH 6.0 at a flow rate of 1.5 $mL \cdot min^{-1}$. Injection volume was 20 μL. Spectra were recorded between 200 and 600 nm.

In order to identify interferences, iron chelates of EDTA, *o,p*-EDDHA, *o,o*-EDDHA, EDD4HMA and EDDHSA were also injected and their retention times compared to those of HJB/Fe^{3+} and HBED/Fe^{3+}. Chelates were prepared from standard chelating agents with high purity as described above. For HJB/Fe^{3+} and HBED/Fe^{3+}, solutions adjusted to pH 5 and 9, were prepared as well.

Stability in Solution versus pH. One milliliter of each Fe chelate solution (0.01 M), 4 mL of 0.125 M $CaCl_2$ and 4 mL of a biological buffer (MES for pH values between 5 and 6; HEPES for pH values between 7 and 8; AMPSO for pH 9; and CAPS for pH values 10−13) were added to a 50 mL volumetric flask. Then, 30 mL of water was added, and the pH was adjusted to 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 10.5, 11.0, 11.5, 12.0, 12.5, and 13.0, either with HCl or NaOH solutions as was needed. Samples were transferred to plastic vessels and were shaken at 25 °C for 3 days. At the end of this period, pH values by potentiometric and total soluble iron by AAS were assessed (*7*).

Chelate Retention on Soils and Soil Materials. Twenty-five milliliters of chelate solutions containing $4.48 \cdot 10^{-4}$ M Fe, 0.01 M $CaCl_2$, and 0.01 M HEPES buffer (pH 7.5) were added to soil materials: (1) 0.50 g of Ca-montmorillonite, (2) 0.50 g of acid pet, (3) 0.25 g of ferrihydrite, (4) 2.00 g of $CaCO_3$, (5) 5.0 g of a standard calcareous soil (SCS) (*13*), (6) 5.0 g of a calcareous soil from Picassent (Valencia, Spain), and (7) 5.0 g of a calcareous soil from Sudanell (Lleida, Spain). Details about soil materials and SCS are described elsewhere ((*13*); see Supporting Information). The main characteristics of Picassent soil (PS) and Sudanell soil (SS) are given in **Table 1**.

After 1 h of agitation, samples were allowed to interact for 3 days at 25 °C. Then solutions were filtered, and pH and soluble Fe by AAS were measured. Chelated Fe was also determined as described above.

Fe Solubilization from Fe Oxides. Three Fe oxides (ferrihydrite, maghemite, and goethite) were used for the evaluation of Fe solubilization by the chelating agents, as described by García-Marco et al., (*15*). Briefly, 0.1 g of each Fe oxide were mixed with 10 mL of a solution containing $5.0 \cdot 10^{-5}$ M of chelating agent (HJB, HBED, and *o,o*-EDDHA), 0.01 M $CaCl_2$, and 0.01 M HEPES buffer (pH 7.5) and allowed to react for 1, 3, 7, and 14 h, and 1, 3, 7, 14, 28, and 56 days. After shaking, solutions were filtered and acidified, and Fe concentrations were measured by AAS.

A modified Langmuir equation was applied to the experimental data:

$$[Fe] = \frac{[Fe_{max}] \cdot t}{T_{1/2} + t}$$

where [*Fe*] is the amount of soluble metal per mass unit (μmol g^{-1}), *t* is the time of interaction, $T_{1/2}$ (half-time) is the time used to dissolve half of the maximum concentration of the metal, and Fe_{max} is the maximum amount of metal dissolved, expressed as μmol $Fe \cdot g^{-1}$ oxide. Fe_{max} values were also expressed as a percentage of the theoretical chelating capacity of each ligand. (5 μmol $Fe \cdot g^{-1}$). Kinetic parameters $T_{1/2}$ and Fe_{max} in the Langmuir equation were calculated from the experimental data of soluble Fe ([Fe]) for each chelating agent, using the Microsoft Excel Solver tool.

RESULTS

Purity of the Chelating Agent. NMR spectra of the HJB and HBED samples show that they present a high degree of purity (see Supporting Information). NMR has been frequently used to determine the purity of the free chelating agents. However, despite its accuracy, inorganic impurities and water content cannot be evaluated by this technique (*3*).

The titrimetric purities of the HBED standard (Strem Chem.) and samples of HJB and HBED (Adob) determined by

Table 2. Log Protonation and Log Stability Constants[a] for the Chelating Agents

quotient	HJB	*o,o*-EDDHA *3*	*rac*-EDDHA *3*	*meso*-EDDHA *3*	HBED *2*, *19*
$[HL^{3-}]/[H^+][L^{4-}]$	12.35	11.94	11.88	11.90	12.64 *2*
$[H_2L^{2-}]/[H^+][HL^{3-}]$	9.86 ± 0.10	10.73	10.80	10.89	11.03 *2*
$[H_3L^-]/[H^+][H_2L^{2-}]$	7.94 ± 0.26	8.66 ± 0.04	8.67 ± 0.01	8.58 ± 0.04	8.34 *2*
$[H_4L^\circ]/[H^+][H_3L^-]$	4.61 ± 0.40	6.18 ± 0.06	6.28 ± 0.11	6.16 ± 0.02	4.40 *2*
$[CaL^{2-}]/[Ca^{2+}]\,[L^{4-}]$	7.62 ± 0.16	7.29 ± 0.30	7.99 ± 0.42	7.56 ± 0.49	9.29 *19*
$[CaHL^-]/[Ca^{2+}]\,[H^+]\,[L^{4-}]$	15.76 ± 0.07	16.77 ± 0.33	17.42 ± 0.39	17.10 ± 0.65	17.98 *19*
$[CaH_2L^\circ]/[Ca^{2+}]\,[H^+]^2\,[L^{4-}]$	22.70 ± 0.06	25.95 ± 0.50	26.87 ± 0.37	26.41 ± 0.64	25.48 *19*
$[MgL^{2-}]/[Mg^{2+}]\,[L^{4-}]$	8.72 ± 0.17	9.76 ± 0.05	10.13 ± 0.03	9.44 ± 0.08	10.51 *19*
$[MgHL^-]/[Mg^{2+}]\,[H^+]\,[L^{4-}]$	17.45 ± 0.03	18.18 ± 0.15		17.51 ± 0.25	18.66 *19*
$[MgH_2L^\circ]/[Mg^{2+}]\,[H^+]^2\,[L^{4-}]$	25.11 ± 0.33	25.36 ± 0.24		26.56 ± 0.35	25.67 *19*
$[FeL^-]/[Fe^{3+}]\,[L^{4-}]$	33.86 ± 0.04	35.09 ± 0.28	35.86	34.15	39.0 *2*
$[FeHL^\circ]/[Fe^{3+}]\,[H^+]\,[L^{4-}]$	34.68 ± 0.06	36.89 ± 0.21	35.08	36.56	40.52 *2*
$[FeOHL^{2-}]/[Fe^{3+}]\,[H^+]^{-1}[L^{4-}]$		23.66 ± 0.27	23.12	22.81	
$[CuL^{2-}]/[Cu^{2+}]\,[L^{4-}]$	21.51 ± 0.22	23.90	24.94 ± 0.05	23.68 ± 0.02	22.95 *2*
$[CuHL^-]/[Cu^{2+}]\,[H^+]\,[L^{4-}]$	30.32 ± 0.22	31.94	32.87 ± 0.04	32.30 ± 0.00	31.73 *2*
$[CuH_2L^\circ]/[Cu^{2+}]\,[H^+]^2\,[L^{4-}]$	35.58 ± 0.16	36.92	37.33 ± 0.07	37.25 ± 0.01	36.85 *2*

[a] m = 0.1 M (NaCl); t = 25 °C.

photometry (see Supporting Information) are 89.72 ± 0.43%, 82.01 ± 0.37%, and 93.72 ± 0.24%, respectively, which is in good agreement with their elemental composition, including all of the ligands, one HCl molecule, and one (for standard HBED) or three (for sample HJB) water molecules. Because of their high purity, the three ligands may be used as standards for modeling their chemical stability and for the analysis and quantification of commercial chelates. The Strem Chem. standard of HBED and sample HJB were selected for the study. The molar absorptivities (ε) at 480 nm obtained for HJB/Fe^{3+} (4199 ± 93) and HBED/Fe^{3+} (4061 ± 59) are lower than the ε value for *o,o*-EDDHA/Fe^{3+} (4721 ± 16) (*6*) because the absorption maximum for the Fe-phenolate bands of HJB and HBED are λ_{max} = 510.0 and 490.0 nm, respectively.

Protonation Constants. The first four protonation constants corresponding with the protonation of the two phenolate groups (K_1^H and K_2^H) and the two amino groups (K_3^H and K_4^H) calculated for HJB ligand are shown in **Table 2**. These values are compared with those of *o,o*-EDDHA, *rac*-*o,o*-EDDHA, *meso*-*o,o*-EDDHA obtained in an earlier work using the same methodology (*3*) and with those of HBED previously reported by Ma et al. (*2*).

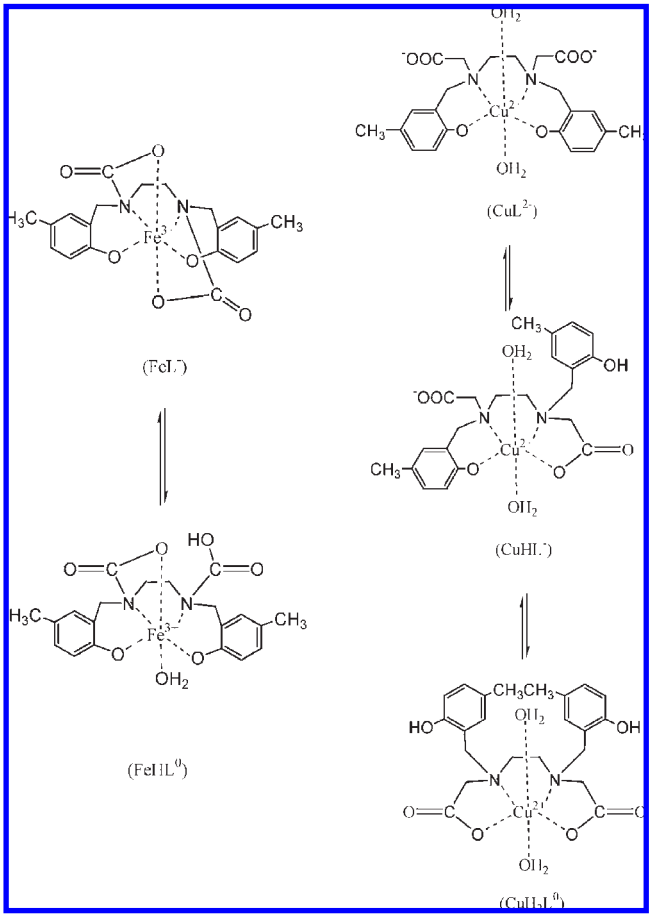
HJB presents a K_1^H value higher than those of *o,o*-EDDHA, *rac*-*o,o*-EDDHA and *meso*-*o,o*-EDDHA but similar to that of its analogue HBED. However, the second protonation constant of HJB is notably the lowest, surely due to the influence of methyl substituent in HJB. The rest of the protonation constants are similar to those of HBED but lower than those of *o,o*-EDDHA. The protonation of the tertiary amines (HJB and HBED) is more difficult than in the secondary amines (*o,o*-EDDHA).

Ca and Mg Stability Constants. HJB/Ca^{2+} and HJB/Mg^{2+} stability constants are in general lower than those of the HBED complexes (**Table 2**), probably due to the influence of the methyl groups in the benzene rings of HJB. The lower rigidity of HBED complexes with respect to those of the *o,o*-EDDHA complexes is the cause of its higher constants.

The three species are important at the pH range of calcareous soils (7.5−9) and they involve the coordination of the metal with the two carboxylic and the two amino groups.

Fe and Cu Stability Constants. Table 2 shows the stability constants of Fe(III) and Cu(II) chelates. The HJB/Fe^{3+} species are represented in **Scheme 1**. The species FeL involves the coordination with the amino nitrogen atoms, the carboxylate oxygens, and the phenolate groups. Between pH 0 and 1, a protonation occurs in one of the phenolates, and a water molecule occupies the vacant position forming the species FeHL. Fe(III) forms chelates with HJB of similar log K° compared to that of *o,o*-EDDHA but less stable than HBED.

Scheme 1. HJB/Fe^{3+} and HJB/Cu^{2+} Species Formed



The different HJB/Cu^{2+} species are also represented in **Scheme 1**. At low pH values, a blue Cu(II) complex is observed, which no doubt involves only coordination to the ethylenediamine nitrogens, the carboxylate oxygens, and two water molecules (CuH_2L). A new green complex (CuHL) is formed as the pH rises, involving the two amino groups, one carboxylate group,

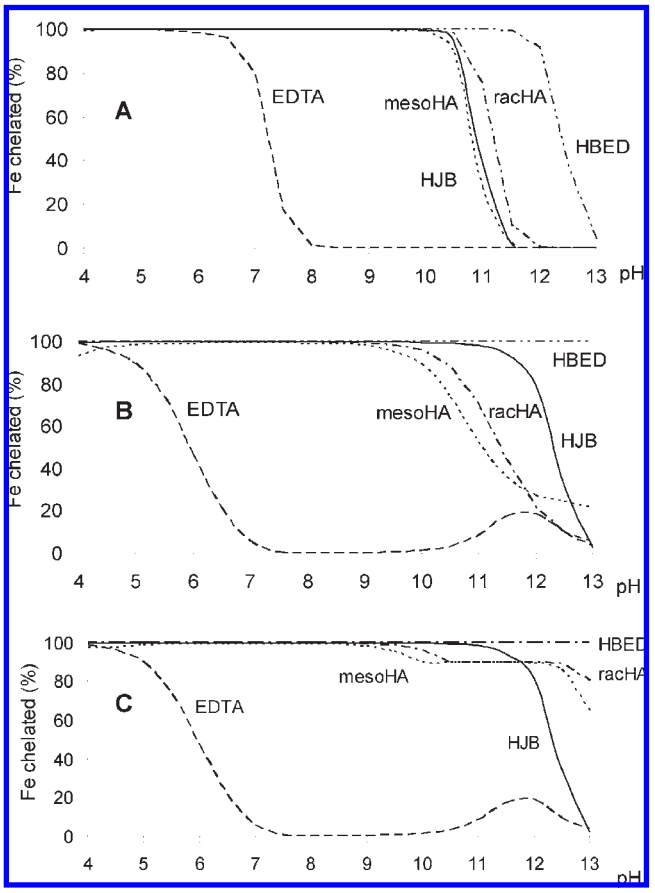


Figure 2. Percentage of chelated Fe(III), in the presence of HJB, HBED, *meso o,o*-EDDHA, *rac o,o*-EDDHA, and EDTA chelating agents in (**A**) nutrient solution and soil conditions with (**B**) unlimited Cu^{2+} and (**C**) limited (normal) Cu^{2+}.

and one phenolate group. Finally, CuL appears at high pH values and involves the two amino groups and the two phenolate groups. Carboxylate groups in the axial position may help to stabilize the CuL chelate.

Fe and Cu chelates of *o,o*-EDDHA and analogous ligands with six Fe-donor bonds form the same species with an additional hidroxylated FeL species at high pH (*3*).

Fe(III) and Cu(II) stability constants for HJB are lower than those for HBED, *o,o*-EDDHA, *rac-o,o*-EDDHA, and *meso-o,o*-EDDHA ligands (**Table 2**). However, the lower stability of the HJB/Cu^{2+}chelate may be favorable for the HJB/Fe^{3+} chelate formation because of the low competence of the Cu(II) ion.

Fe(III) Chelate Stability. Comparisons among HJB/Fe^{3+}, HBED/Fe^{3+}, *rac-o,o*-EDDHA/Fe^{3+}, *meso-o,o*-EDDHA/Fe^{3+}, and EDTA/Fe^{3+} in nutrient solution and soil conditions are shown in **Figure 2**. The most stable chelate is HBED/Fe^{3+} in the three models and EDTA/Fe^{3+} the least. In the nutritive solution system (see **Figure 2A**), Fe(III) is chelated by HJB in a percentage similar to those of *rac-o,o*-EDDHA and *meso-o,o*-EDDHA. In soils with limited Cu(II) levels (normal soils, see **Figure 2C**), HJB/Fe^{3+} chelate shows the same stability compared to that in the model with unlimited Cu(II) (**Figure 2B**). It is also clear that for *rac-o,o*-EDDHA and *meso-o,o*-EDDHA the main competitor is Cu(II) since in the limited Cu(II) model, the stability is considerably higher. While *o,o*-EDDHA and HJB may present similar stability in soil conditions, when high Cu(II) concentrations are present HJB presents as an advantage that Cu(II) competition is not as important as for the *o,o*-EDDHA.

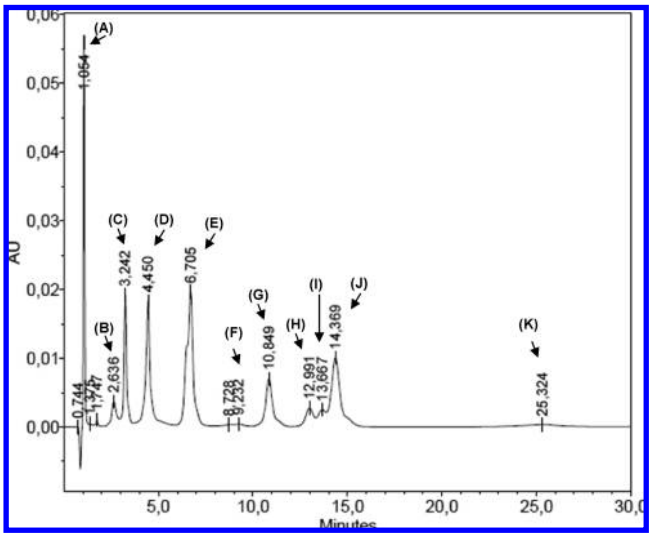


Figure 3. Chromatogram of a mixture of Fe chelates. Column, Waters Symmetry C18; eluent, 0.03 M TBAOH, 30% acetonitrile (pH 6.0); flow rate, 1.5 mL/min; injection volume, 20 μL; detection wavelength, 280 nm. (A) Fe-EDTA, (B) Fe-*o,p*-EDDHA, (C) Fe-*rac-o,o*-EDDHA, (D) Fe-*meso-o,o*-EDDHA, (E) Fe-HBED (peak 1) and Fe-*meso*-EDDHMA (unresolved peaks), (F) Fe-HBED (peak 2), (G) Fe-*rac*-EDDHMA, (H) and (I) Fe-EDDHSA, (J) Fe-HJB (peak 1), and (K) Fe-HJB (peak 2).

Chromatographic Method for the Determination of HBED/Fe^{3+} and HJB/Fe^{3+}. Figure 3 shows the chromatogram of a mixture of the Fe chelates of EDTA, *o,p*-EDDHA, *o,o*-EDDHA, HBED, EDDH4MA, EDDHSA, and HJB. The EN 13368−2:2007 method (*18*) allows the separation of HJB/Fe^{3+} and HBED/Fe^{3+} from EDTA and both *o,o*- and *o,p*-EDDHA Fe chelates. However, the second peak of EDDHSA/Fe^{3+} may interfere with HJB/Fe^{3+}, and the peaks corresponding to HBED/Fe^{3+} and *meso*-EDDH4MA/Fe^{3+} are not properly resolved. Single runs of EDDHSA/Fe^{3+}, HJB/Fe^{3+}, EDDH4MA/Fe^{3+}, and HBED/Fe^{3+} are shown in **Figure 4**, together with the spectra of overlapping peaks. Since chromatograms and spectra of EDDHSA/Fe^{3+} and EDDH4MA/Fe^{3+} are quite different from those of HJB/Fe^{3+} and HBED/Fe^{3+}, respectively, identification of chelates with this method is achievable.

Two different peaks are observed for freshly prepared HJB/Fe^{3+} and HBED/Fe^{3+} standards. Consecutive runs of the same chelate sample at different times give as a result two main peaks whose contribution to total area of the chromatogram rises for the first peak and decreases for the second one (**Figure 5A** and **B**). In addition, a slight increase of total areal with time is observed. Then, HJB/Fe^{3+} and HBED/Fe^{3+} chelates prepared at different pH values (5, 7, and 9) were compared, in order to minimize the contribution of the second peak. We conclude that pH should be adjusted to 5 instead to 7 after $Fe(NO_3)_3$ addition. **Figure 5C** and **D** shows the chromatogram of both HJB/Fe^{3+} and HBED/Fe^{3+} at pH 5.

Stability in Solution versus pH. The variation of soluble Fe with the solution pH, calculated as the percentage of soluble Fe recovered from each chelate, is presented in **Figure 6**. In all cases, the total amount of soluble Fe is recovered between pH 2.0 and pH 10.0 despite the competition with Ca(II). Although HJB/Fe^{3+} is not as stable as *o,o*-EDDHA/Fe^{3+}, around 85% of the Fe chelated by HJB remains in solution at pH 11. HBED/Fe^{3+} forms extremely stable complexes even at pH values above 12. These results are in good agreement with those from the theoretical modelization (**Figure 2A**).

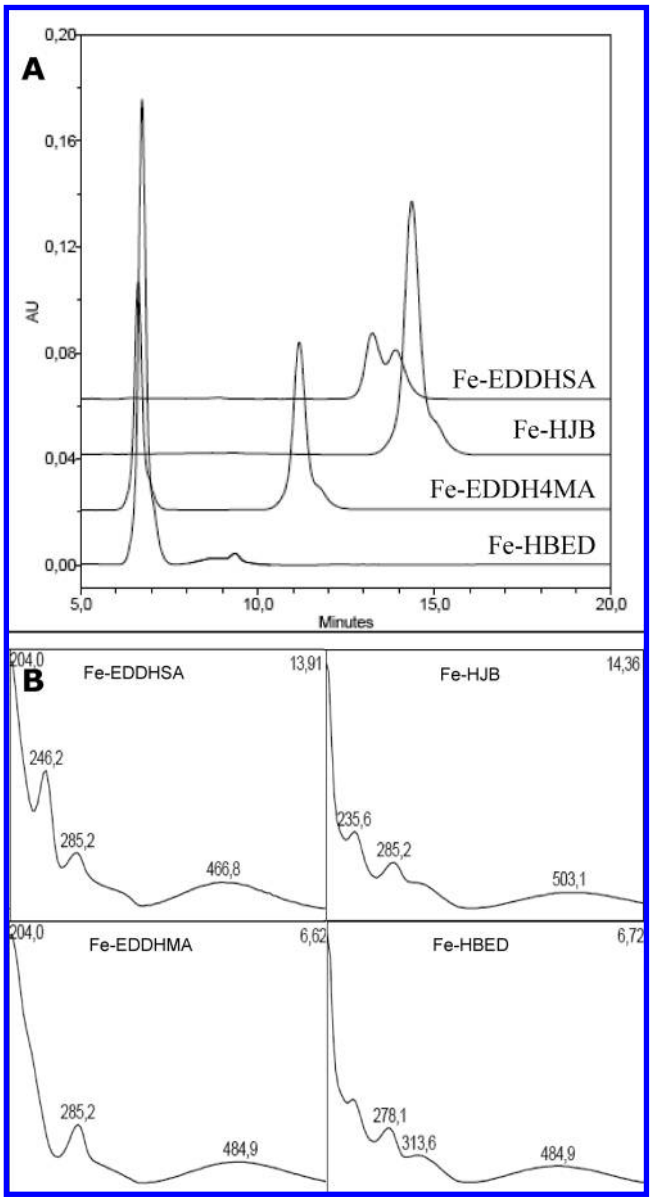


Figure 4. Chromatograms of single runs (**A**) and spectra (**B**) of Fe-EDDHSA, Fe-HJB, Fe-EDDHMA, and Fe-HBED. Conditions are as described in **Figure 3**.

Chelate Adsorption in Soils and Soil Materials. Figure 7 presents the percentage of chelated Fe remaining in solution with respect to the initial amount of chelate, after the interaction with soil materials and soils. The results are similar to those of total Fe obtained by AAS, indicating that all of the Fe in solution came from the chelates. HJB/Fe^{3+} is more retained in peat and Ca-montmorillonite, while the recovery is not affected when interacting with ferrihydrite and $CaCO_3$. HJB/Fe^{3+} is able to maintain nearly 100% of added Fe in the two agricultural soils, while the SCS, which contains an appreciable amount of montmorillonite, presents a relatively high reactivity with HJB/Fe^{3+}. Fe displacement by Cu was also assessed by determining Cu concentration in solutions from interaction from soils (data not shown), but it did not account for the Fe loss from HJB/Fe^{3+} when reacting with SCS. The less adsorbed Fe chelates are HBED/Fe^{3+} and *racemic* *o*,*o*-EDDHA/Fe^{3+}. The amount of chelated Fe was higher than 90% for HBED/Fe^{3+} for all of the soils and soil materials, only slightly affected by acid peat. As expected, peat and ferrihydrite react more extensively with *meso*-*o*,*o*-EDDHA/Fe^{3+}.

Table 3. Fe Dissolution from Oxides in the Presence of *o*,*o*-EDDHA, HBED, and HJB[a]

Fe source	chelating agent	$T_{1/2}$ (h)	Fe_{max} μmol Fe g^{-1}	Fe_{max} %	r^2	model validity
ferrihydrite	HJB	1.3	4.41	88.2	0.98	56 days
	HBED	1.0	3.83	76.5	0.99	56 days
	o,*o*-EDDHA	1.4	2.58	51.7	0.98	1 day
maghemite	HJB	3.1	4.61	92.2	1.00	56 days
	HBED	2.7	4.04	80.8	0.95	56 days
	o,*o*-EDDHA	4.2	3.27	65.4	0.98	1 day
goethite	HJB	3.8	4.84	96.8	0.94	56 days
	HBED	3.9	4.49	89.8	0.94	56 days
	o,*o*-EDDHA	2.4	4.62	92.3	0.94	56 days

[a] $T_{1/2}$ is the time required to dissolve half of the maximum concentration of Fe, and Fe_{max} is the maximum amount of Fe dissolved and expressed as μmol Fe g^{-1} oxide and as percentage of the theoretical chelating capacity of the ligand (5 μmol·g^{-1}).

Fe Solubilization from Fe Oxides. The ability of HJB, HBED, and *o*,*o*-EDDHA to dissolve Fe from oxides was studied by the determination of Fe concentration in solution after the interaction between each chelating agent and different Fe oxides. **Table 3** shows the kinetic parameters $T_{1/2}$ (hours) and Fe_{max} (expressed as μmol Fe g^{-1} oxide and as the percentage of theoretical chelating capacity) for the different chelating agents (HJB, HBED, and *o*,*o*-EDDHA) and oxides (ferrihydrite, maghemite, and goethite). For *o*,*o*-EDDHA, a slight chelate decrease is appreciated in the long term; therefore, model fitting was only applied to data corresponding to chelate formation. HJB and HBED did not experience a significant decrease, and Langmuir kinetics can be assumed during the entire experiment (56 days) and for all of the substrates tested.

HJB dissolves more Fe than the other chelating agents, (i.e., higher Fe_{max}), for all of the substrates. Since the lower the $T_{1/2}$ the faster the Fe solubilization, it can be inferred that HBED is the fastest in chelating Fe from ferrihydrite and maghemite. All of the chelating agents solubilize more Fe from goethite, which is higher for HJB and lower for HBED. Both HJB and HBED need more time than *o*,*o*-EDDHA to solubilize Fe from goethite.

Although the solubilization rate depends on the solid phase, HJB is the chelating agent that dissolves the highest amount of Fe from all of the substrates, while HBED solubilizes a higher or similar amount of Fe than *o*,*o*-EDDHA. Besides, they are able to maintain the soluble Fe in solution during the entire experiment.

DISCUSSION

In this work, the new chelating agent HJB is presented and chemically characterized, in order to predict its efficacy as an Fe fertilizer. HJB has been compared to two well-known ligands: *o*,*o*-EDDHA, generally employed to control iron chlorosis, and HBED, a strong chelator mainly used in biomedical applications that has not been chemically tested before as an Fe fertilizer. All of them present the same donor groups, but the existence of methyl groups in the benzene ring seems to have an effect on the chemical behavior of HJB. Methylation of the aromatic rings results in an increase of the negative charge density and thus leads to an increase of the basicity of phenol (phenol, pK_a = 9.89; 4-methylphenol, pK_a = 10.17). However, this effect does not correspond with the low value of log K_2^H (corresponding to the protonation of the second phenolate) of HJB compared to that of HBED. The difference between log K_1^H and log K_2^H for HJB (2.49) is considerably higher than that observed for *o*,*o*-EDDHA (1.21) and HBED (1.61), surely due to a stronger interaction between the two phenolate groups in HJB caused by steric effects

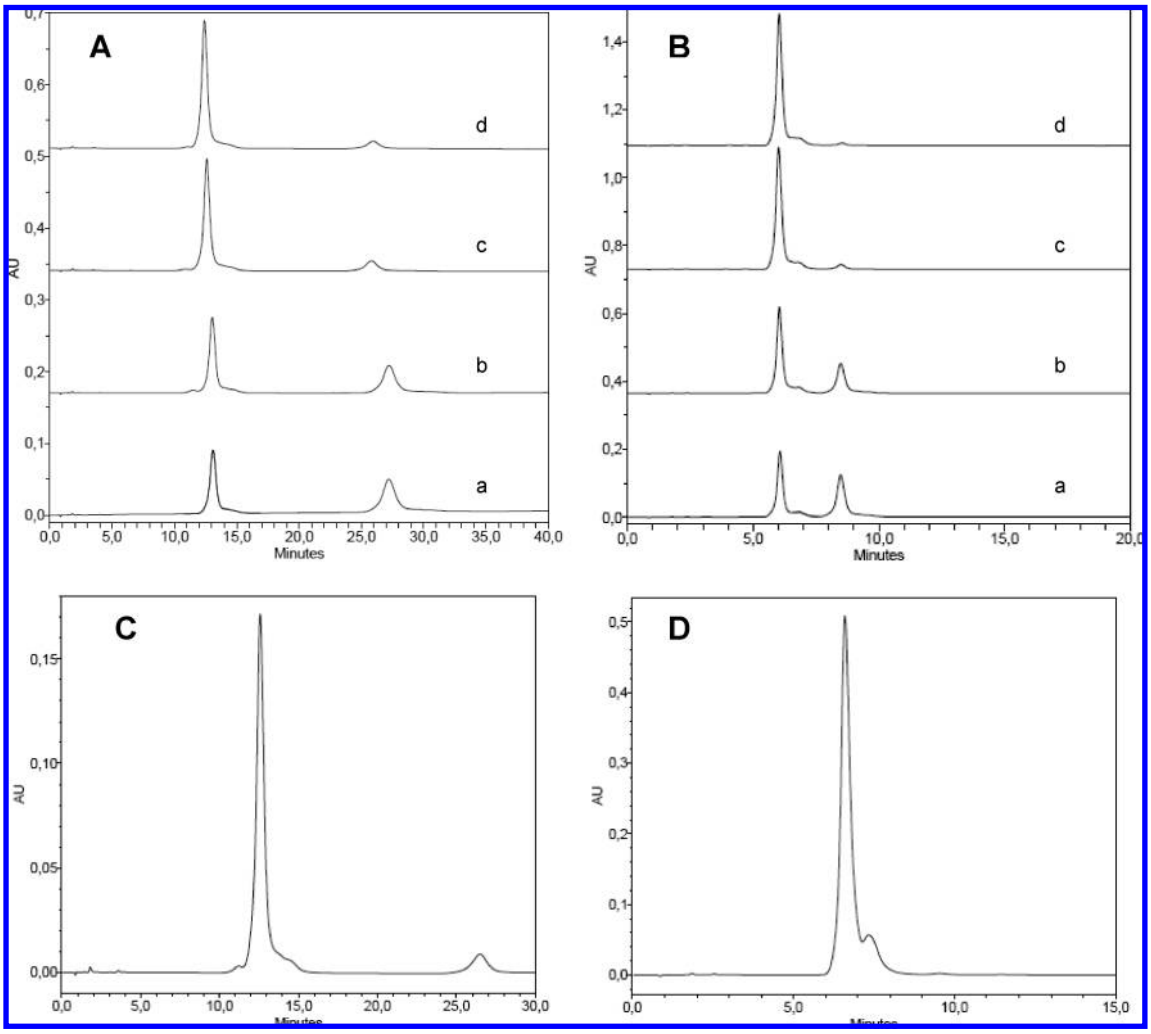


Figure 5. Chromatograms of Fe-HJB (**A**,**C**) and Fe-HBED (**B**,**D**) at different times after chelate preparation at pH 7 (**A**,**B**): (a) 30 min after sample preparation, (b) 24 h, (c) 7 days, (d) 10 days, and at pH 5 (**C**,**D**). Conditions are as described in **Figure 3**.

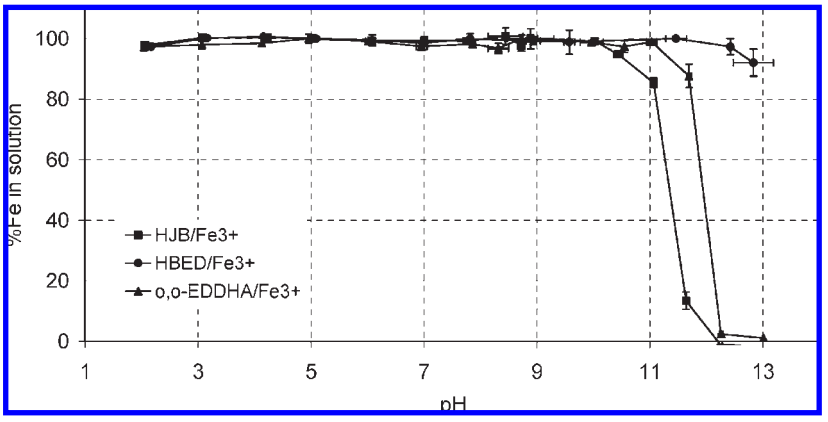


Figure 6. Percentage of soluble Fe recovered at different pH values in 10 mM Ca(II) solution. Error bars represent the standard error ($n = 3$).

of the methyl groups. The higher log protonation constants log K_3^H and log K_4^H, corresponding to the protonation of the two amino groups, for *o*,*o*-EDDHA than for both HBED and HJB are related to the fact that the basicity of secondary amines (*o*,*o*-EDDHA) is higher than the basicity of tertiary amines (HBED and HJB). The protonation constants corresponding to the carboxylate groups could not be determined because the chelating agents precipitate after the addition of the fourth equivalent of acid.

As discussed elsewhere (*23*), the higher Fe(III) affinity of HBED (log $K = 39.01$) relative to that of *o*,*o*-EDDHA (log $K = 35.09$) is due to a more favorable steric orientation of donor groups. Methylation of aromatic rings in HJB exerts a significant effect on Fe(III) stability constants since the magnitude of HJB stability constant for Fe(III) is 5.15 log units lower than that of HBED. Yunta et al. (*3*) found that both EDDH4MA and EDDH5MA were weaker ligands for binding Fe(III) than *o*,*o*-EDDHA, this result being partially explained by a geometric

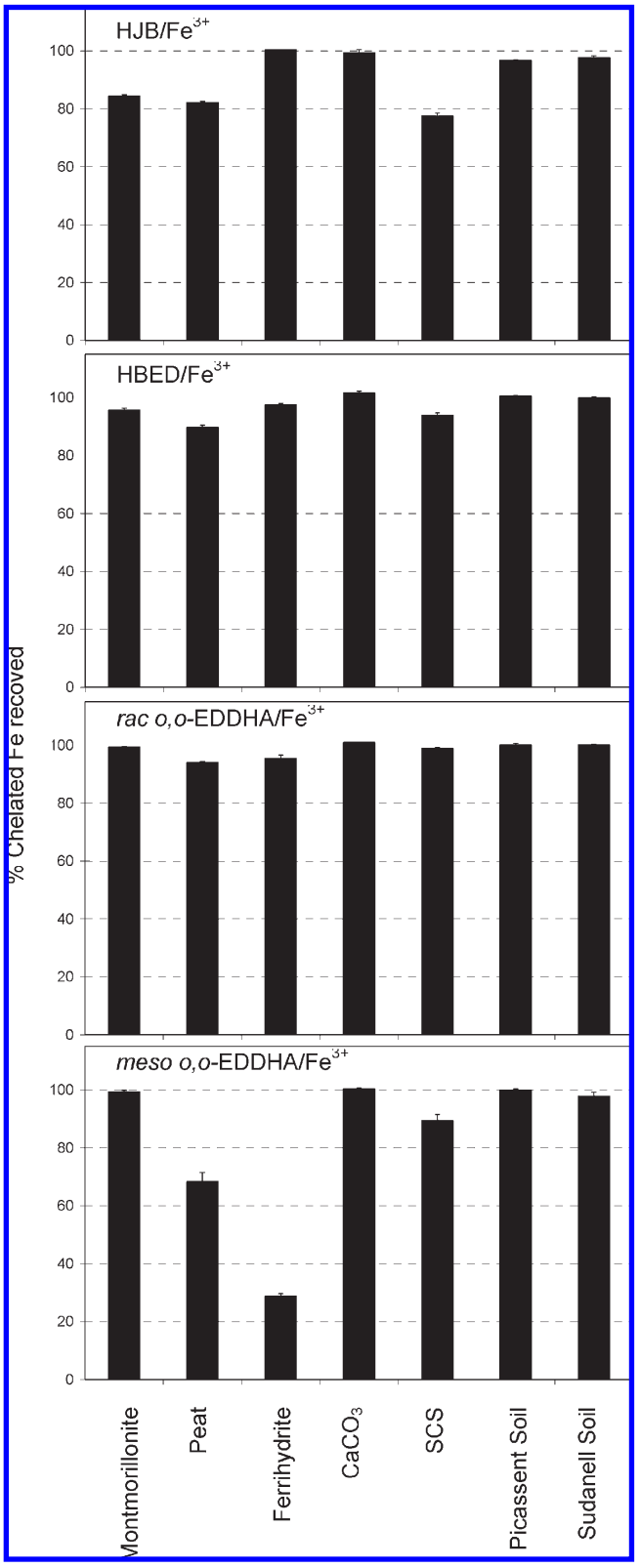


Figure 7. Percentage of chelated Fe that remained in solution after the interaction of Fe chelates with solid phases. Error bars represent the standard error ($n = 3$).

effect. It should be noted that, despite the lower stability constant of HJB for Fe(III), modelization of the relative chelating ability under agronomic conditions denotes a good performance of HJB, even better than *o*,*o*-EDDHA when Cu(II) availability is high.

Chromatographic determinations provide additional information on the spatial arrangement of donor groups in HBED and HJB. These ligands do not have chiral C atoms, although N atoms work as asymmetric centers. Three spatial arrangements of the donor groups have been suggested when HBED forms a complex with Fe(III) (*17*). These spatial arrangements can be denoted as (5,5,5), (6,5,5), and (6,5,6) depending on the number of atoms in the three equatorial chelate rings (*24*). Lucena et al. (*17*), with the same chromatographic methodology, found two peaks on a HBED/Fe^{3+} chromatogram, the second peak more than 10-fold smaller than the first one. In the present work, we have obtained two peaks for HBED/Fe^{3+} and for HJB/Fe^{3+}, and the relative area of the peaks is time-dependent. Krokhin et al. (*24*) found the same phenomena for Fe(III) and Co(III) HBED chelates in ion electrokinetic chromatography, and according to them, the first largest peak corresponds to the most stable (6,5,6) arrangement, with the carboxylate in axial positions, (**Figure 1a**) (*25*), and the second is likely the (6,5,5) structure, less favored, but initially formed at 50%. Since we have reported a slight increase of total area with time for both HJB and HBED, we suggest that the conditions of the Fe(III) chelate formation from the chelating agents should be carefully controlled, particularly the final pH. As the pH is lowered, the Fe chelate stability also lowers, and then an internal conversion of N atom becomes more probable, which is necessary to turn the (6,5,5) arrangement into the (6,5,6) one. The slight increase of the sum of areas is probably due to differences in absorptivities of the conformers at 280 nm.

The second set of experiments investigates the reactivity of chelates and chelating agents and gives complementary information about the efficacy of Fe chelates. In this work, the pH-Ca^{2+} effect on soluble Fe, interaction of Fe chelates with soil constituents and soils, and Fe solubilization by chelating agents have been considered. Similar chelate stability in solution when varying pH values has been obtained in the theoretical modeling and in the interaction experiment, revealing that all of them are highly stable Fe chelates in a wide pH range, despite competition with Ca. Only with pH values above 11 is the percentage of HJB/Fe^{3+} that remains in solution lower than those of *o*,*o*-EDDHA/Fe^{3+} and HBED/Fe^{3+}. Nevertheless, HJB/Fe^{3+} performance in these conditions is similar to that of other Fe chelates currently in the market, such as EDDHSA/Fe^{3+} (*13*). In the interaction experiment with soil and soil components, the three polyphenolic chelates present low reactivity with soils and soil materials and high recovery of chelated Fe. In this type of study, a decrease of the Fe concentration in solution can be due to chelate sorption, Fe displacement from the chelate and precipitation, or chelate degradation. The novel HJB/Fe^{3+} is most retained by Ca-montomorillonite. Several adsorption mechanisms have been suggested (namely, H-bonding, salt bridges, and ligand exchange (*26*)), and all of them are more likely to occur with the chelating agent than with the Fe chelate (*26*). Probably, the Fe chelate, negatively charged, is bound to the clay surface by a bridging mechanism involving Ca (*12*). The amount of chelated Fe remaining in solution is reduced by peat for all of the chelates; probably, the lower final pH (all of the solutions from peat interactions had pH values between 3.5 and 3.7) may play a role. The behavior of *o*,*o*-EDDHA/Fe^{3+} diasteroisomers have been related to their stability constants, which suggests that the association of the ligand with the surface requires prior rupture of some bonds between Fe and the ligand (*12*, *13*). In our results, *meso*-*o*,*o*-EDDHA/Fe^{3+} reacts more extensively than HJB/Fe^{3+} with peat, the latter having a lower stability constant with Fe; thus, other factors may be affecting the retention processes of Fe chelates. The same binding mechanism has been suggested for

chelate retention on Fe oxides; however, only *meso*-*o*,*o*-EDDHA/Fe^{3+} is significantly affected. Finally, the ability of HJB and HBED to dissolve Fe from iron oxides is in general similar to that of *o*,*o*-EDDHA, the formed chelates being stable for at least 8 weeks. Our results show that HBED is the fastest ligand in chelating Fe from ferrihydrite and maghemite, which does not correspond with the finding in Lucena and Chaney (*27*), where slow kinetics of the displacement reactions in solution involving HBED were observed. The decrease of formed *o*,*o*-EDDHA/Fe^{3+} with time may be explained by the surface sorption of the *meso* form, as pointed out by the retention experiment, rather than chelate degradation.

In summary, the novel HJB/Fe^{3+} has a promising potential as an Fe fertilizer. Its stability constants are comparable to those of *o*,*o*-EDDHA, and HJB can be used as an iron chelate in both hydroponics and soil conditions, even when Cu^{2+} is highly available in soil. As expected, theoretical modelization of HBED/Fe^{3+} behavior predicts high ability of HBED to bind Fe under agronomic conditions. The results from interaction experiments indicate that HJB/Fe^{3+} and HBED/Fe^{3+} are, at least, as effective as *o*,*o*-EDDHA/Fe^{3+} in maintaining Fe in soil solution. Given that EDDHA/Fe^{3+} commercial formulations consist of a mixture of *o*,*o*-EDDHA, *o*,*p*-EDDHA, *p*,*p*-EDDHA, and other byproducts with different chelating ability and reactivity (*3*, *7*), and that HJB and HBED synthesis yields a purer product (*8*) as we observed by potetentiometric titration and NMR analysis, the new chelating agent HJB is a promising alternative to EDDHA in Fe fertilizers. Besides, HBED has been proved to maintain available Fe in solution under different agronomic conditions. Further plant experiments are needed to demonstrate their efficacy to provide Fe to plants.

ABBREVIATIONS USED

HJB, *N*,*N*′-bis(2-hydroxy-5-methylbenzyl)ethylenediamine-*N*,*N*′-diacetic acid; HBED, *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-diacetic acid; *o*,*o*-EDDHA, ethylenediamine-*N*,*N*′-bis(2-hydroxyphenylacetic) acid; *o*,*p*-EDDHA, ethylenediamine-*N*-(*o*-hydroxyphenylacetic acid)-*N*′-(*p*-hydroxyphenylacetic acid); *p*,*p*-EDDHA, ethylenediamine-*N*-(*p*-hydroxyphenylacetic acid)-*N*′-(*p*-hydroxyphenylacetic acid); EDDH4MA, ethylenediaminedi-(2-hydroxy-4-methylphenylacetic) acid; EDDH5MA, ethylenediaminedi-(2-hydroxy-5-methylphenylacetic) acid; EDTA, ethylene diamine tetraacetic acid; DTPA, diethylenetriminepentaacetic acid; CDTA, *trans*-l,2-cyclohexanediaminetetraacetic acid; EDDHSA, *N*,*N*′-ethylenediamine-di(*o*-hydroxy-*p*-sulfoxy-phenylacetic) acid; HBEP, *N*,*N*′-bis(2-hydroxybenzyl)ethylenediamine-*N*,*N*′-dipropionic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); MES, 2-(*N*-morpholino)ethanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonicacid; AMPSO, *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; TBAOH, tetrabutylammonium hydroxide; NMR, nuclear magnetic resonance; HPLC, high-performance liquid chromatography; AAS, atomic absorption spectroscopy.

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Supporting Information Available: Soils and soil material characteristics of the chelate retention experiment, ^{1}H NMR and ^{13}C NMR spectra of HJB and HBED chelating agents, and spectrophotometric measurements for protonation constants and Fe^{3+} and Cu^{2+} stability constants. This material is available free of charge via the Internet at http://pubs.acs.org.

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