Structure and Fertilizer Properties of Byproducts Formed in the Synthesis of EDDHA

ARTICLE *in* JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · JULY 2006

Impact Factor: 2.91 · DOI: 10.1021/jf0605749 · Source: PubMed

8 AUTHORS, INCLUDING:

Lourdes Hernández-Apaolaza
Universidad Autónoma de Madrid
31 PUBLICATIONS **503** CITATIONS
SEE PROFILE

Sonia García-Marco
Universidad Politécnica de Madrid
23 PUBLICATIONS **347** CITATIONS
SEE PROFILE

Miguel A Sierra
Complutense University of Madrid
249 PUBLICATIONS **3,715** CITATIONS
SEE PROFILE

Mar Gómez-Gallego
Complutense University of Madrid
106 PUBLICATIONS **1,592** CITATIONS
SEE PROFILE

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2006,** *54,* 4355−4363 **4355**

Structure and Fertilizer Properties of Byproducts Formed in the Synthesis of EDDHA

LOURDES HERNÁNDEZ-APAOLAZA, SONIA GARCÍA-MARCO, PALOMA NADAL, AND JUAN J. LUCENA*

Departamento de Química Agrícola, Facultad de Ciencias, CVII, Universidad Autónoma, 28049 Madrid, Spain

MIGUEL A. SIERRA, MAR GÓMEZ-GALLEGO,* PEDRO RAMÍREZ-LÓPEZ, AND ROSA ESCUDERO

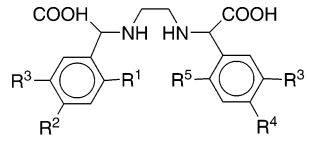
Departamento de Química Orgánica, Facultad de Química, Universidad Complutense, 28040 Madrid, Spain

The synthesis of commercial EDDHA produces *o,o*-EDDHA as the main reaction product, together with a mixture of regioisomers (*o,p*-EDDHA and *p,p*-EDDHA) and other unknown byproducts also able to complex Fe^{3+}. These compounds have been obtained by direct synthesis, and their structures have been determined by ESI-MS analysis as oligomeric EDDHA-like products, formed by polysubstitution in the phenolic rings. Short-term experiments show that the iron complexes of samples enriched in these oligomeric byproducts have adequate stability in solution, but a significant amount of them is lost after interaction with soils and soil materials. Mildly chlorotic cucumber plants are able to reduce iron better from *o,p*-EDDHA/Fe^{3+} than from the iron complexes of the oligomeric byproducts. In hydroponics, the chlorotic soybean susceptible plants have a lower potential for Fe absorption from these byproducts than from *o,o*-EDDHA/Fe^{3+} and from *o,p*-EDDHA/Fe^{3+}. In the studied conditions, the iron chelates of EDDHA byproducts do not have the long-lasting effect shown by *o,o*-EDDHA/Fe^{3+} and present a less efficient fast-action effect than the *o,p*-EDDHA/Fe^{3+}.

KEYWORDS: Iron chelates; fertilizers; *o,o*-EDDHA; *o,p*-EDDHA

INTRODUCTION

While polyaminocarboxylic acids bearing phenolate groups are massively used as micronutrient chelating fertilizers (*1*), very little is known about the structure, chelation properties, and agronomic behavior of all the compounds present in a commercial formulation. This is a matter of great importance from both agronomic and environmental points of view. In particular, one of the most efficient iron chelating agents employed to relieve iron chlorosis in plants, namely, ethylenediamine *N,N′*-bis(2-hydroxyphenylacetic acid (*o,o*-EDDHA) **1** (**Figure 1**) (*2*, *3*), and its analogues EDDH4MA **2** and EDDHCA **3** are industrially prepared by a Mannich-like reaction between phenol (or a substituted phenol), ethylenediamine, and glyoxylic acid (or glyoxylate) (**Scheme 1**) (*4*). In the case of EDDHA, this method is known to produce *o,o*-EDDHA **1** as the main component, together with a mixture of regioisomers that have been identified as *o,p*-EDDHA **5** and *p,p*-EDDHA **6** (**Figure 1**) accompanied by other unknown byproducts, formed in variable amounts depending on the reaction conditions (*5*, *6*). The commercial Fe^{3+} chelates are subsequently obtained by the addition of an iron salt to the reaction crude product, immediately after synthesis.



1 $R^1 = R^5 = OH$; $R^2 = R^3 = R^4 = H$ *o,o*-EDDHA
2 $R^1 = R^5 = OH$; $R^2 = R^4 = Me$; $R^3 = H$ EDDH4MA
3 $R^1 = R^5 = OH$; $R^2 = R^4 = H$; $R^3 = COOH$ EDDHCA
5 $R^1 = R^4 = OH$; $R^2 = R^3 = R^5 = H$ *o,p*-EDDHA
6 $R^2 = R^4 = OH$; $R^1 = R^3 = R^5 = H$ *p,p*-EDDHA

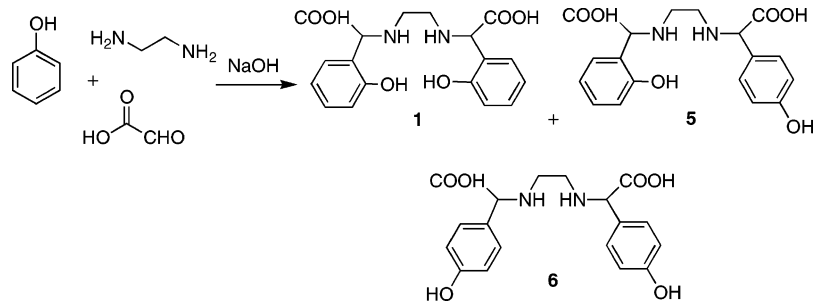
Figure 1.

Although in Europe more than 60 million dollars is spent every year in this class of agrochemicals (*7*), the interest about the structure and properties of the byproducts present in commercial fertilizers has just recently arisen. In this regard, we have reported a study about the structure and effectiveness of *o,p*-EDDHA **5** as an iron supplier (the main byproduct present

* Corresponding authors. (J.J.L.) E-mail: juanjose.lucena@uam.es; fax: +34 91 497 38 25. (M.G.-G.) E-mail: margg@quim.ucm.es; fax: + 34 91 3944310.

10.1021/jf0605749 CCC: $33.50
Published on Web 05/16/2006

Scheme 1



in EDDHA commercial iron chelates) (*8*). In fact, due to its good agronomical behavior, the European Regulation on fertilizers (EC Regulation No. 2003/2003) has now included *o,p*-EDDHA as an authorized chelating agent. On the other hand, the presence of two *p*-hydroxyphenyl groups makes the isomer *p,p*-EDDHA **6** unable to bind iron (*8*). Together with *o,o*-EDDHA/Fe^{3+} and *o,p*-EDDHA/Fe^{3+}, we have determined that most of the commercial EDDHA/Fe^{3+} fertilizers contain variable extra amounts of soluble iron (as much as 25% (*9*)) that did not correspond to any of the chelates declared (*10*). Additionally, studies of some commercially available samples of EDDHA have suggested that the byproducts present in commercial fertilizers could form 2:1 [Fe^{3+}/ligand] complexes (*11*). The presence of polycondensated byproducts able to complex iron has been also proposed in very recent studies of EDDHSA/Fe^{3+} commercial samples (*12*). Although these data suggest that the commercial formulations of EDDHA contain byproducts able to complex iron, their nature and their ability to transfer iron to plants remains unknown. Here, we report a study about the structure of these compounds using directed synthesis and ESI-mass spectrometry. The study will be carried out on samples prepared to be enriched in byproducts that are formed in the synthesis of *o,o*-EDDHA. We will also determine their role as substrates of the enzyme ferric chelate reductase (FCR) in cucumber plants (Fe-efficient strategy I plant) and their efficiency to provide iron to soybean plants (Fe-inefficient plant). To complete this study, the behavior in soils of these compounds will be addressed.

MATERIALS AND METHODS

All the ESI-MS experiments were carried out using an ESQUIRE-LC (Bruker Daltonic, Bremen, Germany) ion trap spectrometer in negative mode of detection. A syringe pump (model 74900, Cole-Palmer, Vernon Hills, IL) was used with MeOH solutions (1.5×10^{-5} mol L^{-1}) of the corresponding sample through a short length of 254 mm i.d. PEEK tubing (Upchurch Scientific, Oak Harbor, WA) with a flow rate of 3 mL min^{-1}. The stainless steel capillary was held at a potential of 5.0 kV. Nitrogen was used as nebulizer gas at a flow rate of 3.98 L min^{-1} (nebulizer pressure 11 psi) at 150 °C. The spectra reported are the averages of 15 scans using 450 ms as the accumulation time.

Preparation of the Samples. Na_2EDTA was purchased from Merck. Pure samples of *o,o*-EDDHA (*12*) and *o,p*-EDDHA (*5*) were synthesized following our reported procedures.

Preparation of Sample A. In a two-neck round-bottom flask equipped with reflux condenser and magnetic stirring bar, 100.0. g of phenol (1.063 mol) was melted at 40−45 °C. Then, dry ethylenediamine (2.46 g, 40.9 mmol) was added, and the mixture was stirred for 10 min. To the stirred mixture was added slowly, dropwise, 33% NaOH (4.97 g, 40.9 mmol) and 50% glyoxylic acid (12.12 g, 81.8 mmol). The resulting mixture was heated at 70−75 °C for 3 h. After reaching room temperature, water (120 mL) and CH_2Cl_2 (240 mL) were added, and the resulting mixture was vigorously stirred for 10 min. The organic layer was separated, the aqueous phase was washed with CH_2Cl_2 (3×50 mL), and the pH was adjusted to 6.9 with 5% HCl. After 72 h at room temperature, the precipitate obtained was filtered; washed successively with *i*-butyl acetate, water, and acetone; and dried, yielding 7.23 g (49%) of a pale yellow solid that was identified by ^1H NMR as *o,o*-EDDHA (1:1, meso/racemic mixture). The filtrate was washed with CH_2Cl_2 (3×50 mL), and the pH was adjusted to 6.63 with 5% HCl acid to obtain a new precipitate that after washing and drying yielded 0.24 g (2%) of a pale yellow solid that was identified by ^1H NMR as a 1:2 mixture of *o,o*-EDDHA and *o,p*-EDDHA (both as a mixture of isomers). The filtrate was concentrated under reduced pressure, the solid residue was dissolved in 50 mL of water, and the pH was adjusted to 5.7 with 5% HCl. The precipitate obtained after 24 h at room temperature was washed and dried to yield 0.19 g of a pale yellow solid that by ^1H NMR was formed by a 1:1:1 mixture of *o,o*-EDDHA, *p,p*-EDDHA, and *o,p*-EDDHA. The same sequence of operations was carried out with the filtrate, which only led to 20 mg of a mixture of EDDHA regioisomers by precipitation at pH 4.5. Finally, the filtrate was dried by evaporation under reduced pressure to give an orange solid (5.48 g, sample A**)**. The NMR analysis of this sample revealed the presence of *o,o*-EDDHA, *p,p*-EDDHA, and *o,p*-EDDHA together with signals of other unknown products.

Preparation of Sample B. Following the experimental procedure described previously, sample B was obtained from 7.7 g (81.8 mmol) of phenol, 2.46 g (40.9 mmol) of ethylenediamine, 12 g (81.8 mmol) of 50% glyoxylic acid, and 7.58 g (40.9 mmol) of 33% NaOH. After 4 h at 70−75 °C, the reaction mixture was allowed to reach room temperature. Then, water (30 mL) and CH_2Cl_2 (60 mL) were added, and the mixture was vigorously stirred for 10 min. The organic layer was separated, and the aqueous phase (pH 9.1) was washed with CH_2Cl_2 (3×50 mL) and evaporated to dryness under reduced pressure to give an orange solid that was washed thoroughly with acetone and dried (sample B). The analysis of the product (15.32 g) by NMR showed the presence of *o,o*-EDDHA, *o,p*-EDDHA, and *p,p*-EDDHA among other unknown products.

Preparation of Fe^{3+} Complexes. The solutions of the iron chelates employed in this work were prepared with the corresponding ligand and with NaOH (ligand/NaOH, 1:3 molar ratio) and $FeCl_3 \cdot 6H_2O$ (Merck) (5% Fe in excess of the molar amount of the ligand) following the previously reported procedure (*13*).

Stability in Solution versus pH. Iron chelate solutions (0.01 M in Fe) of EDTA/Fe^{3+}, *o,o*-EDDHA/Fe^{3+}, *o,p*-EDDHA/Fe^{3+}, sample A/Fe^{3+}, and sample B/Fe^{3+} were prepared. One milliliter of each solution, 4 mL of $CaCl_2$, 0.125 M and 4 mL of a biological buffer (MES for pHs between 5 and 6; HEPES for pHs between 7 and 8; AMPSO for pH 9; and CAPS for pHs 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, 11.0, 12.0, and 13.0, either with HCl or NaOH solutions as it 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 and total soluble iron were assessed by AAs.

Fe Solubilization from Soils and Fe Oxides. The Fe solubilization from soils and iron oxides by the samples was evaluated by following the procedure previously described (*10*). Briefly, three synthetic iron oxides and two soils were allow to react with the chelating agents, EDTA, *o,o*-EDDHA, *o,p*-EDDHA, sample A, and sample B from 1 h to 64 days. The formed chelates were determined by HPLC (*9*, *14*)

(see the Supporting Information for complete detailed experimental procedure). A modified Langmuir equation (amount of Fe in solution vs time) was applied to the experimental data

$$[M] = \frac{[M_{max}]t}{t_{1/2} + t}$$

where [M] is the amount of soluble metal per mass unit (μmol g^{-1}), $t_{1/2}$ (halftime) is the time used to dissolve half of the maximum concentration of the metal, and M_{max} is the maximum amount of metal dissolved.

Chelate Adsorption in Soils and Soil Materials. The sample adsorption rate in soils and soil materials was determined by a known procedure (*15*). The soil used was a clay loam with pH (water extract) of 7.75, 15 g kg^{-1} organic matter, 430 and 140 g kg^{-1} total and active lime, respectively, and 12.1 mg kg^{-1} DTPA (Soltanpour and Schawb method) extractable Fe (Soltanpour and Schawb method) (*10*). The soil materials used were (a) a standard of calcareous soil (*16*); (b) ferrihydrite ($5Fe_2O_3 \cdot H_2O$) (*17*, *18*); (c) acidic mountain *Sphagnum* peat, provided by Tolsa S.A. (Buyos, Lugo, Spain) [its chemical characteristics were pH (saturated paste), 4.0; dichromate oxidizable OM (%), 85.4; total OM (%) (determined by loss of weight by ashing), 99.5; C in humic acid (%), 30.2; C in fulvic acid (%), 18.3; Nkj (%), 1.4%; C/N, 35.4; CEC ($cmol_c$ kg^{-1}), 150; and DTPA-extractable Fe and Mn, 295 and 8.2 mg kg^{-1}, respectively]; (d) calcium-montmorillonite (STX-1, Gonzalez County, TX), obtained from the Clay Minerals Society Source (Clay Minerals Repository, Department of Geology, University of Missouri, Columbia, MO) [this reference material has been well-characterized elsewhere (*19*), and so despite its differences, it has been considered as an acceptable model for soil smectites]; and (e) $CaCO_3$ analytical grade (Panreac).

Studies with FCR in Green Stressed Cucumber Plants. In this study, cucumber plants were used since they are efficient and induce the Fe chelate reductase when iron is limited. Cucumber seeds (*Cucumis sativus* L. cv. Ashley) were germinated on standard seed germination papers moistened with a macronutrient solution in diffuse light in a growth chamber for 7 days. Uniform seedlings were selected, and stems of two individual plants were wrapped together with polyurethane foam and placed in a 12 L polypropylene bucket (12 pairs of plants per bucket) containing a continuously aerated EDTA buffered nutrient solution with the following composition: macronutrients (mM) 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, and 0.1 KH_2PO_4; cationic micronutrients (μM) 5.0 EDTA/Fe^{3+}, 2.5 $MnSO_4$, 1.0 $CuSO_4$, 10 $ZnSO_4$, 1.0 $CoSO_4$, 1.0 $NiCl_2$, and 115.5 $EDTANa_2$; anionic micronutrients (μM) 35 NaCl, 10 H_3BO_3, and 0.05 Na_2MoO_4; 0.1 mM HEPES; and 1 g L^{-1} $CaCO_3$ to buffer pH at 7.5 to simulate conditions in a calcareous soil. Plants were grown for 14 days in this nutrient solution in a Dycometal type CCK growth chamber provided with fluorescent and sodium vapor lamps with a 16 h/30 °C and 50% humidity day and 8 h/25 °C and 70% humidity night regime. Water was added every 2 days, and the nutrient solution was renewed every 7 days. The amount of iron added (5 μM) was found as the most adequate to produce green cucumber plants but with a high FCR activity (stressed plants) in an assay with similar experimental conditions (*20*).

For the measurement of FCR activity, 300 mL beakers, wrapped up with tin foil to avoid light exposure, were placed in the growth chamber. Each beaker contained 200 mL of reduction assay solution consisting of macronutrient solution as in the growth period, 100 μM Fe chelate (*o*,*o*-EDDHA/Fe^{3+}, *o*,*p*-EDDHA/Fe^{3+}, sample A/Fe^{3+}, sample B/Fe^{3+}, and EDTA/Fe^{3+}), 2 mM MES to buffer the pH at 6, and 300 μM Na_2-BPDS as the Fe^{2+} trapping and colorimetric reagent. Each solution was continuously aerated. The roots of 21 day old plants were washed three times in a macronutrient solution containing 37.5 μM Na_2BPDS before each individual pair of plants was transferred to one beaker. Aliquots of 3 mL were withdrawn at 0, 10, 20, and 60 min after transfer for absorbance measurements. Five replicates were prepared for each treatment, and also five replicate blanks per chelate, consisting of solutions without plants, were used.

The $Fe^{2+}(BPDS)_3$ concentration was calculated (*20*) after the determination of the absorbencies at 535 nm (maximum absorbance of the $Fe^{2+}(BPDS)_3$) and at 480 nm (near the maximum absorbance *o*,*o*-EDDHA/Fe^{3+} and *o*,*p*-EDDHA/Fe^{3+}). Since EDTA/Fe^{3+} does not present a significant absorbance at 535 or 480 nm, the [$Fe^{2+}(BPDS)_3$] concentration was directly calculated from the absorbency at 535 nm.

The slope of the plots of Fe^{2+} (μmol g^{-1} dry root) produced versus time (h) was used as the Fe^{2+} reduction rate for each pair of plants. Data were expressed as the mean reduction rate and the standard error, corresponding to five plant replications for each treatment.

Efficiency to Provide Fe to Soybean Plants. Soybean plants were used in this experiment since they are considered susceptible to chlorosis and are considered as a model for crops normally treated with chelates. Soybean seeds (*Glycine max* L. cv. Oshumi) were germinated at 28 °C on paper moistened with 1 mM $CaSO_4$ in the dark for 3 days. Afterward, the seedlings were placed in 10 L containers (27 seedlings per container) filled with a 1/5 diluted EDTA buffered nutrient solution of the same composition as in the cucumber experiment and grown for 7 days. On the eighth day, to induce iron chlorosis, seedlings were transferred to 12 L polypropylene buckets containing an aerated full-strength EDTA buffered nutrient solution but without an Fe source. Plants were grown under these conditions until severe symptoms of Fe deficiency were observed in the upper leaves (6 days), and then plants were placed in 2 L pots (2 plants per pot) covered with black plastic to avoid light exposure, and treatments (5 μM *o*,*o*-EDDHA/Fe^{3+}, *o*,*p*-EDDHA/Fe^{3+}, sample A/Fe^{3+}, or sample B/Fe^{3+}) were applied. The nutrient solution contained macronutrients and anionic micronutrients as for the cucumber experiment, and cationic micronutrients were added at (μM) 1.0 $MnSO_4$, 0.5 $CuSO_4$, 0.5 $ZnSO_4$, 0.1 $NiCl_2$, and 0.1 $CoSO_4$. Water was added every 2 days, and the solution was renewed weekly. The treatments were repeated four times in a completely randomized design. Plants were harvested after 14 days. The growth chamber conditions were the same as those used in the cucumber experiment.

During the experiment, SPAD readings with a chlorophyll meter (Minolta SPAD-502) were taken for all the leaf stages (average of three readings per leaf) at several times, although only values measured for the second leaf stage (the youngest fully open leaf at the start of the treatment period, $t = 0$) have been presented in the results since they were the most representative of the whole plant. Changes in the SPAD index (ΔSPAD) between the readings at the end of the experiment and at the beginning of the treatments ($t = 0$) have been used instead of the actual SPAD readings due to the initial variability of developing leaves. ΔSPAD gives a quantitative measurement of the recovery of the plants from chlorosis and the relative effectiveness of Fe fertilization treatment (*21*, *22*). Negative values of ΔSPAD imply a lack of recovery from the chlorosis, while positive values for a treatment mean that the treatment is effective.

Root and shoot were separated and washed following the reported procedure (*23*). Fresh and dry weights were determined, and after their digestion, Fe, Mn, Cu, and Zn were assessed by atomic absorption spectrometry (Perkin-Elmer Analyst 800).

Data were processed using the Statistical Package Social Science PC 12.0. Duncan's multiple range test ($\alpha = 0.05$) was used to test for differences among means.

RESULTS AND DISCUSSION

Synthesis and Structure. Samples A and B were prepared from phenol, ethylenediamine, glyoxylic acid, and NaOH, following the known Mannich-like industrial procedure (*4*). Sample A is representative of the mixture of compounds present in a commercial EDDHA. In the preparation of sample A, a great excess of *o*,*o*-EDDHA (the main product in the crude reaction) was removed by successive precipitations at pH 6.87 and 6.63, respectively. Small amounts of *o*,*p*-EDDHA and *p*,*p*-EDDHA were also removed by further precipitation at pH 5.70. By following this procedure, sample A is enriched in the rest of byproducts formed during the reaction. On the other hand, sample B has been directly obtained by a modification of the industrial method, carrying out the reaction in stoichiometric amounts of phenol, ethylenediamine, and glyoxylic acid. Under

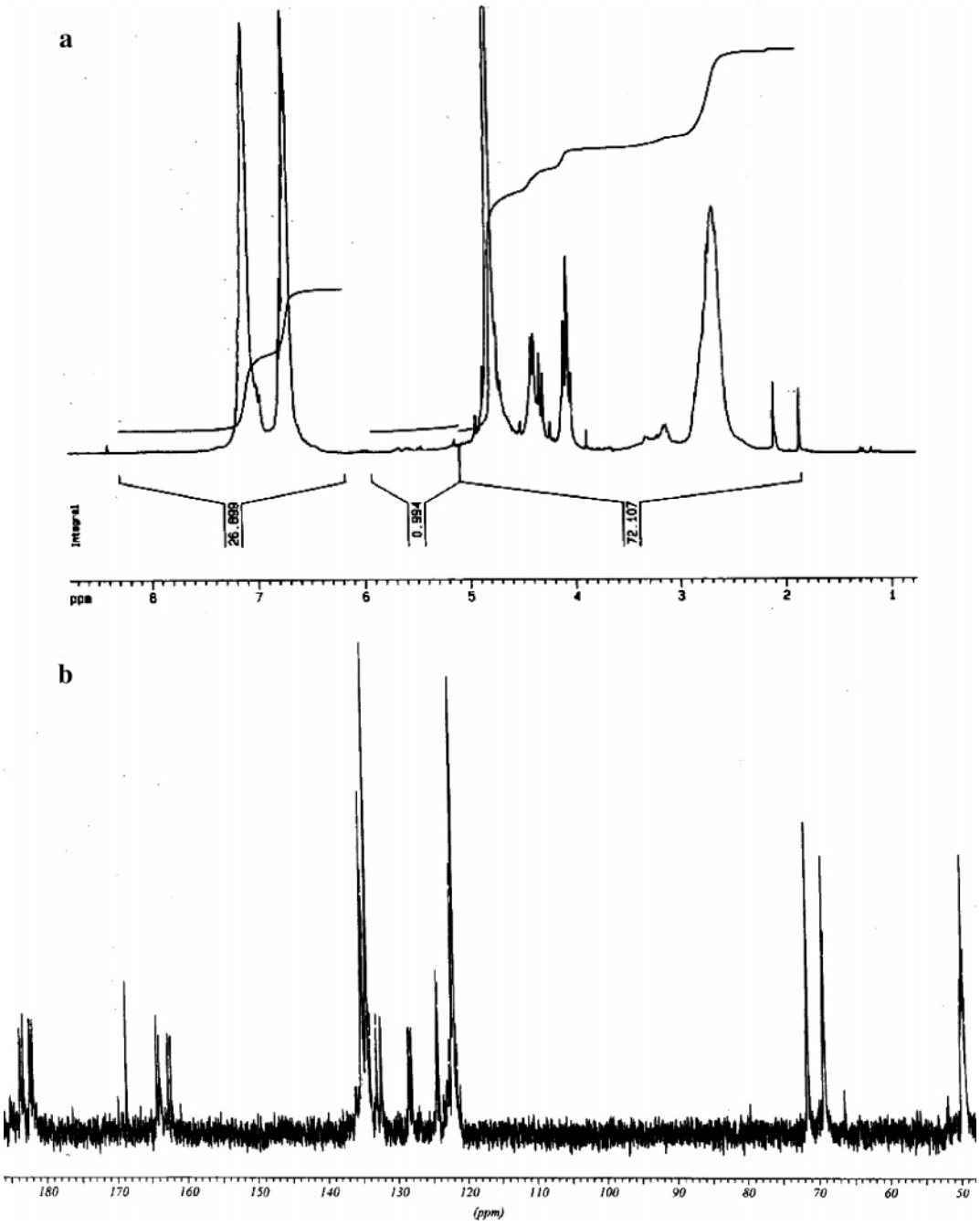


Figure 2. (**a**) ^{1}H NMR (D_2O/Na_2CO_3) of sample B; (**b**) ^{13}C NMR (D_2O/Na_2CO_3) of sample B.

these conditions, *o*,*o*-EDDHA is not formed in excess to the rest of the reaction byproducts.

The compositions of both samples have been analyzed by HPLC following the method developed by Lucena et al. (*24*, *25*) and by photometric titrations with the Fe^{3+} solution at 480 nm. The amount of *p*,*p*-EDDHA in the samples prepared cannot be determined by this method, as this isomer is not able to form iron chelates. The results in **Table 1** reveal that both samples A and B contain *o*,*o*-EDDHA and *o*,*p*-EDDHA together with significant amounts of other byproducts also able to complex iron. In both samples, the major component able to complex iron is the byproduct (58% and 77% in samples A and B, respectively). In sample A, a large contribution of the *o*,*p*-EDDHA isomer (30%) was also observed. This analysis makes sample B very adequate for the study and characterization of the unknown byproducts present in EDDHA commercial chelates.

Table 1. Amount of Fe That Can Be Complexed by Samples A and B Determined by Photometric Titration at 480 nm and Its Distribution among the Known Chelating Agents and Byproducts As Determined by HPLC

product	% Fe (g of Fe/100 g of product)	% of Fe-chelated (g of Fe/100 g of product)	
sample A	7.42	*o*,*p*-EDDHA	2.22
		o,*o*-EDDHA	0.89
		byproducts	4.31
sample B	10.19	*o*,*p*-EDDHA	1.48
		o,*o*-EDDHA	0.81
		byproducts	7.90

The determination of the structure and number of byproducts present in samples A and B was not possible by standard NMR techniques. Their ^{1}H NMR spectra ($D_2O-Na_2CO_3$) revealed

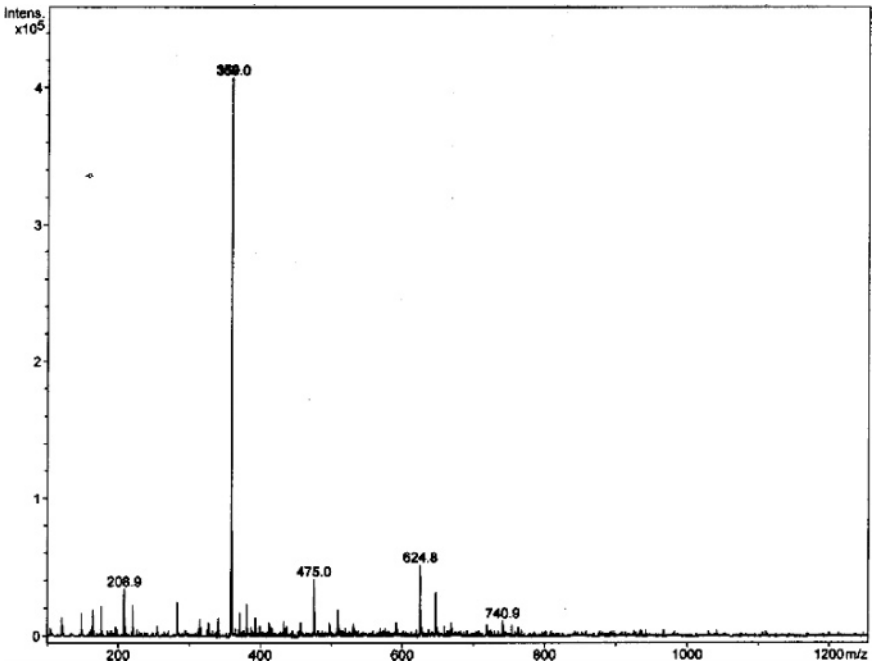


Figure 3. ESI-MS spectrum of sample B.

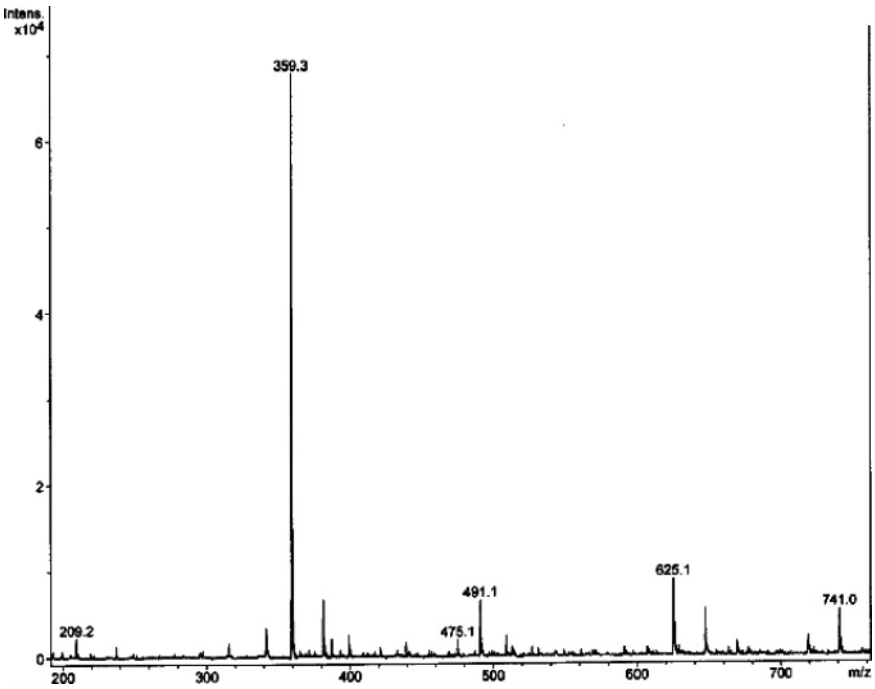


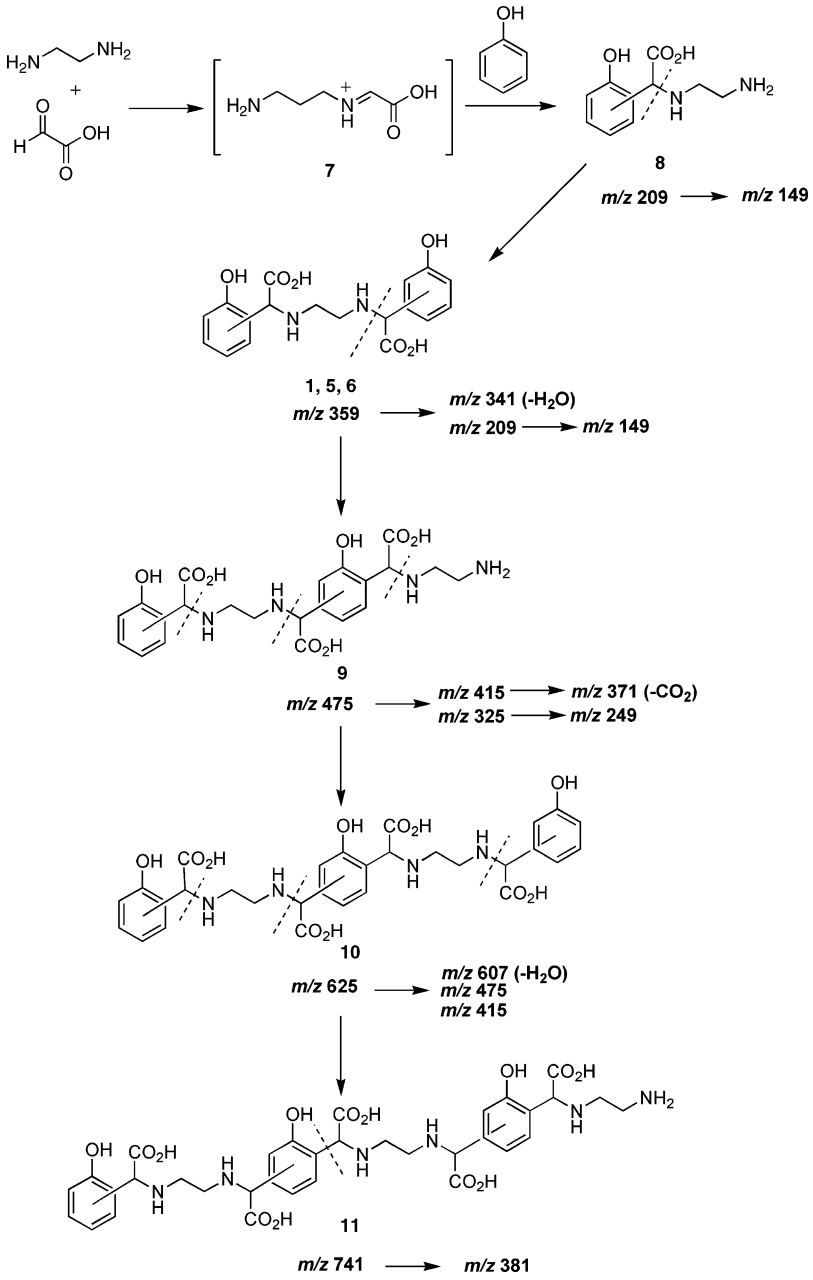
Figure 4. ESI-MS spectrum of sample A.

(together with the signals of the aromatic protons) groups of signals between 4.4 and 4.3 ppm and between 4.1 and 4.0 ppm assignable to CH protons (66.7, 65.4, 65.1, 64.5, and 64.3 ppm in ^{13}CNMR) together with a broad area between 2.71 and 2.62 ppm assignable to CH_2 groups (47.2−44.5 ppm in ^{13}C NMR) (**Figure 2**). No other types of signals were shown in NMR, which could be interpreted in the sense that the byproducts formed in the reaction are somewhat structurally related to EDDHA. Although the samples prepared contain *o*,*o*-EDDHA and *o*,*p*-EDDHA (see **Table 1**), their signals cannot be clearly identified in the NMR spectra.

Electrospray ionization mass spectrometry (ESI-MS) is a technique that has become one of the preferred soft methods to generate mass spectra from solutions of organic, inorganic, and bioorganic analytes, and it has revealed itself as particularly useful in the case of highly polar compounds (*26*). The ESI-MS analysis (negative mode) of sample B (**Figure 3**) reveals an intense peak at *m*/*z* 359 together with other intense peaks at *m*/*z* 209, 475, 625, and 741, respectively, which could correspond to the pseudomolecular $[M - H]^-$ ions of the different types of compounds present in the sample. Clearly, the most intense signal at *m*/*z* 359 should belong to the regioisomers *o*,*o*-EDDHA, *o*,*p*-EDDHA, and *p*,*p*-EDDHA already present in the mixture. The remaining signals must then be assigned to the rest of the byproducts formed in the reaction (**Figure 3**).

The peaks observed in the ESI-MS spectrum of sample B were independently analyzed to establish their structure and to determine whether they belonged to $[M - H]^-$ ions of different compounds or if they were only formed by a fragmentation process. The MS/MS study revealed different fragmentation

Scheme 2



patterns for each peak. Thus, the analysis of the pseudomolecular $[M - H]^-$ ion at *m*/*z* 741 indicates the loss of EDDHA ($[M - H] - 360$, *m*/*z* 381) as the main fragmentation process. On the other hand, the pseudomolecular $[M - H]^-$ ion at *m*/*z* 625 loses a water molecule, to form the most intense peak at *m*/*z* 607. Other fragmentation peaks at *m*/*z* 475 $[M - H] - 150]^-$ and their fragments at *m*/*z* 415 (loss of ethylenediamine) and 371 (loss of CO_2) are also clearly visible in the spectrum. These latter peaks are also revealed in the MS/MS study of the pseudomolecular $[M - H]^-$ ion at *m*/*z* 475, together with the $[M - H] - 150]^-$ ion at *m*/*z* 325 and its fragmentation peak at *m*/*z* 249. Finally, the EDDHA's pseudomolecular $[M - H]^-$ ion at *m*/*z* 359 mainly loses a water molecule (*m*/*z* 341), but also fragments leading to peaks at *m*/*z* 209 and 149, respectively. Considering the results of the MS/MS analysis, it is not clear if the peaks shown at *m*/*z* 475 and 209 in **Figure 3** correspond to single compounds or if they are just formed as a result of the fragmentation of other peaks (or both). All the MS/MS spectra are collected in the Supporting Information.

The ESI-MS spectrum of sample A reveals a distribution of peaks at *m*/*z* 209, 359, 491, 475, 625, and 741 similar to that discussed for sample B (**Figure 4**). Interestingly, now the peak at *m*/*z* 475 is not as intense as in **Figure 3**, and a new *m*/*z* 491 signal is clearly visible. The MS/MS analysis of this peak (see Supporting Information) reveals the same fragmentation pattern just described for the *m*/*z* 475 peak, leading, among others, to peaks at *m*/*z* 447 (loss of CO_2) and the $[M - H] - 150]^-$ ion at *m*/*z* 341 as the main fragment. The ESI-MS spectra of samples A and B confirm that, although in different amounts, both contain a similar mixture of byproducts.

The structure and formation of all these byproducts can be understood by considering the reaction conditions in which they have been formed and studying the different fragmentation patterns observed in the ESI-MS spectra (**Scheme 2**). The reaction between ethylenediamine and glyoxylic acid is known to form a reactive iminium intermediate **7**, a good electrophile that in the presence of phenol yields the different products observed. Initially, **8** could be formed, and depending on the

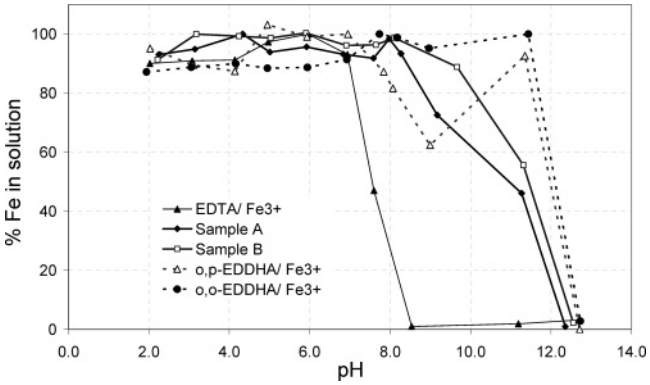


Figure 5. Percent of soluble Fe recovered at different pHs in 10 mM Ca^{2+} solution.

orientation of the electrophilic attack in the reaction with a new phenol ring, the different EDDHA isomers **1**, **5**, and **6** can be obtained. The activated rings of the EDDHA could also react with the iminium electrophile present in the reaction medium, leading to structures **9**−**11**. The different possibilities of orientation in the successive electhophilic aromatic substitutions in the phenolic rings, and the presence of several stereogenic centers on the compounds formed, increase the number of possible stereoisomers present in the reaction mixture. These facts could justify the broad areas of signals corresponding to CH and CH_2 groups in the NMR spectra. The ESI-MS analysis of samples A and B confirms that the byproducts formed in the synthesis of EDDHA are polysubstitution EDDHA-like compounds. Their structures are well-compatible with the chelation of iron, which justifies the origin of the unknown amounts of complexed iron experimentally analyzed in EDDHA commercial samples.

Stability and Reactivity of the Chelates. To establish the fertilizer properties of the polysubstitution byproducts present in EDDHA commercial formulations, solutions of iron chelates of samples A and B were studied, and their properties were compared with those of the known chelates *o,o*-EDDHA/Fe^{3+}, *o,p*-EDDHA/Fe^{3+}, and EDTA/Fe^{3+}.

The stability versus pH was studied first (**Figure 5**). Samples A and B lead to iron complexes more stable than EDTA/Fe^{3} but not as stable as those formed from *o,o*-EDDHA. In both cases, almost the total amount of soluble iron was recovered between pH 2.0 and 8.0, but above this point, the stability of their iron complexes was considerably reduced.

The ability of the byproducts in samples A and B to solubilize iron from an insoluble form was addressed by the determination of the iron concentration in solution after the interaction between the chelating agent and the different iron oxides (goethite (α-FeOOH), maghemite (γ-Fe_2O_3), and amorphous Fe oxide (Fe-$(OH)_3$)) (*10*) and soils (standard calcareous soil and two agricultural soils). Compared to *o,p*-EDDHA/Fe^{3+}, *o,o*-EDDHA/Fe^{3+}, and EDTA/Fe^{3+}, samples A and B solubilized almost negligible amounts of iron from all the solid phases. (See the Supporting Information for a more detailed presentation of the results.)

The effectiveness of an iron chelate also depends on its ability to maintain Fe in the soil solution despite its reaction with different soil components. The average value of the chelates recovered after the interaction with the soils and soil materials was 91% for *o,o*-EDDHA/Fe^{3+}, 65% for EDTA/Fe^{3+}, and 62% for the Fe chelated by the polysubstituted byproducts. Particularly, the standard of calcareous soil presented a 93, 30, and 26% recovery of chelated Fe for *o,o*-EDDHA/Fe^{3+}, EDTA/Fe^{3+}, and Fe chelated by the polysubstituted byproducts, respectively. The most reactive material was the ferrihydrite that allowed a low recovery of the chelates (76, 5, and 0% for *o,o*-EDDHA/Fe^{3+}, EDTA/Fe^{3+}, and the Fe chelated by the polysubstituted byproducts, respectively). In general, the iron complexes of the byproducts present in samples A and B were only slightly more retained by the soil materials and soils tested than the *o,o*-EDDHA/$Fe^{3+}$and *o,p*-EDDHA/Fe^{3+} complexes, except for ferrihydrite, in which a high retention was obtained. This behavior could be due to their chemical structures, which have more charged functional groups prone to interact with the soil surfaces.

Table 2. Rate of Fe^{3+} Reduction (μmol of Fe^{2+} h^{-1} g^{-1}) in Root Dry Weight (DW) and Fresh Weight (FW) Basis from Iron Chelates Used for Cucumber Plants[a]

	μmol of Fe^{2+} h^{-1} g^{-1} root	
treatments	DW	FW
o,o-EDDHA/Fe^{3+}	13 c	0.45 b
o,p-EDDHA/Fe^{3+}	25 a	0.85 a
sample A/Fe^{3+}	20 ab	0.63 ab
sample B/Fe^{3+}	14 bc	0.46 b
EDTA/Fe^{3+}	24 a	0.83 a

[a] Different letters in the same column denote significant differences according to the Duncan test ($\alpha = 0.05$)'.

Iron Complexes of EDDHA Byproducts as Substrates of the FCR in Green Stressed Cucumber Plants. Cucumber plants were grown as described by Lucena and Chaney (*20*) in a way to produce plants that were Fe stressed but still green. The FCR activity with respect to the different iron chelates used as the enzyme substrates is presented in **Table 2**.

It is clear that cucumber root FCR activity is dependent on the chelate used, significantly reducing more Fe^{3+} when *o,p*-EDDHA/Fe^{3+} and EDTA/Fe^{3+} were the iron sources. The iron chelates of samples A and B present an intermediate behavior between *o,p*-EDDHA and *o,o*-EDDHA, with this latter product being the worst substrate for FCR. These results are in agreement with our recent results that show how the ability of EDDHA/Fe^{3+} complexes toward reduction increases when one of the coordination sites in the close octahedral environment around the iron is replaced by a water molecule (*27*). Sample A was noticeably better than sample B as a substrate for the enzyme, which could be due to the amount of *o,p*-EDDHA already present in this sample (see **Table 1**). Since the byproducts are the main component of sample B (their concentration is 5 times higher than in sample A), it seems clear that these polysubstituted EDDHA-like structures do not play an important role in the FCR behavior.

Efficiency of the Iron Complexes of EDDHA Byproducts To Provide Fe to Soybean Plants. The efficacy of iron treatments to correct iron chlorosis in soybean plants was estimated by the SPAD index variation (**Figure 6**).

Plants treated with 5 μM sample B/Fe^{3+} complexes showed severe chlorosis symptoms during the experiment (14 days after treatment application), whereas plants treated with 5 μM *o,o*-EDDHA/Fe^{3+} and sample A/Fe^{3+} complexes presented an increase in the SPAD index only during the first week (*o,o*-EDDHA/Fe^{3+}) or just at the end of the experiment. However, 5 μM *o,p*-EDDHA/Fe^{3+} is enough to obtain a good recovery from chlorosis during all the experimental periods.

Plant dry weights at the end of the experiment did not present significant differences. Plants grown with *o,p*-EDDHA/Fe^{3+} presented higher iron concentrations in leaves than plants treated

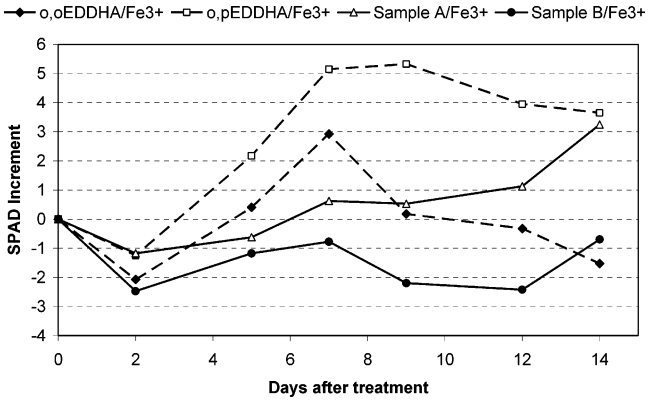


Figure 6. Variation of the SPAD index (ΔSPAD) for the first leaf stage (youngest fully open leaf) with time.

Table 3. Growth and Mineral Content of Plants Treated with Low Doses of Different Chelates[a]

treatment	biomass (g of plant^{-1} DW)	leaves (mg kg^{-1} DW) Fe	Mn	Cu	Zn	Fe/Mn
5 μM *o,o*-EDDHA/Fe^{3+}	3.12 ns	42 b	78 a	9.3 a	33 b	0.54 b
5 μM *o,p*-EDDHA/Fe^{3+}	3.81	47 a	63 b	7.1 b	37 b	0.75 a
5 μM sample A/Fe^{3+}	3.38	38 c	64 b	7.6 ab	31 b	0.59 b
5 μM sample B/Fe^{3+}	3.62	34 d	64 b	9.1 a	43 a	0.53 b

[a] Different letters in the same column denote significant differences among treatments according to Duncan's multiple range test ($\alpha = 0.05$).

with *o,o*-EDDHA/Fe^{3+} (**Table 3**). These results are in good agreement with previous findings (*10*). Sample B/Fe^{3+} yielded the lowest iron concentrations, followed by sample A/Fe^{3+}, indicating that the byproducts are not good sources of iron to the plants in this short-term experiment. This trend is related to that obtained with the SPAD index increment. Our results in **Table 3** also show that the iron source plays an important role in copper concentration in leaves. Plants treated with *o,o*-EDDHA/Fe^{3+} and sample B/Fe^{3+} presented higher Cu concentrations in leaves than plants treated with *o,p*-EDDHA/Fe^{3+} and sample A/Fe^{3+}. Since *o,p*-EDDHA forms quite strong complexes with Cu^{2+} (*8*), it may impede Cu to enter into the plant. No differences in Zn concentration in leaves were found between the different chelates tested, except for plants treated with the sample enriched in byproducts (sample B), which showed the highest value.

Fe concentration in leaves is not sometimes an adequate iron nutritional index (*22*). The Fe/Mn ratio has been considered as a better index for several crops, mainly those grown in hydroponics (*28*). An increase of the Fe/Mn ratio implies a recovery from iron chlorosis (**Table 3**). According to the Fe/Mn ratio, plants treated with *o,p*-EDDHA/Fe^{3+} presented a better recovery from chlorosis than those treated with *o,o*-EDDHA/Fe^{3+} and with the iron chelates of samples A and B. The Fe/Mn index is well-correlated with the data of FCR activity for all the chelates tested.

Discussion of Fertilizer Properties. Fe-EDTA/Fe^{3+} presents a low stability in high pH soil, so it is only recommended for supplying Fe to efficient crops and in systems where the pH can be controlled below 6.5 such as in drip irrigation or hydroponic-like systems (*29*). *o,o*-EDDHA/Fe^{3+} is very well-known to be a good Fe fertilizer even in high lime content soil since it maintains an adequate stability in the soil and has a long-lasting effect, due to its low reactivity with soil mineral phase materials (*29*). Recently, *o,p*-EDDHA/Fe^{3+} has been revealed as a fast-action iron fertilizer since the kinetics of native iron solubilization and release to the plants is quite fast (*10*). As also presented in this paper, it can alleviate chlorotic plants very quickly. However, its reactivity with soil materials limits its presence to a few days after application. The synthesized EDDHA byproducts here studied are stable enough in calcium solutions (**Figure 5**) but present a higher reactivity in soils than *o,p*-EDDHA/Fe^{3+}. Moreover, solubilization of Fe by the byproduct chelating agents is low, and the formed chelate is stable only for a few hours. While the kinetics of the Fe release to the plants seems to be quite fast, chlorotic susceptible plants have a low potential for Fe absorption from these byproducts (**Table 3**). These data indicate that in the studied conditions, the iron chelates of the EDDHA byproducts synthesized by us do not have the long-lasting effect presented by *o,o*-EDDHA/Fe^{3+} and present a less efficient fast-action effect than the *o,p*-EDDHA/Fe^{3+}.

In conclusion, the synthesis of commercial EDDHA yields a mixture of *o,o*-EDDHA, *o,p*-EDDHA, *p,p*-EDDHA, and other byproducts also able to chelate iron. The structure of these compounds has been determined by direct synthesis and ESI-MS analysis. These products are formed during the Mannich-like reaction between ethylenediamine and glyoxylic acid, by polysubstitution of the activated phenolic rings. Our short OLINIT-term assays suggest that the iron complexes of these oligomeric EDDHA-like compounds have a limited value as iron fertilizers. They neither have the long-lasting effect of *o,o*-EDDHA/Fe^{3+} nor the fast action of *o,p*-EDDHA/Fe^{3+}. Further research is needed to establish their efficacy in field conditions.

Supporting Information Available: Fe solubilization from soils and iron oxides and NMR and MS/MS spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Mengel, K.; Kirkby, E. A.; Kosegarten, H.; Appel, T. Iron. In *Principles of Plant Nutrition*; Kluwer Academic: Dordrecht, The Netherlands, 2001; pp 553−571.

(2) Norvell, W. A. Reactions of metal chelates in soils and nutrient solutions. In *Micronutrients in Agriculture*; Mortvedt, C., Shuman, W., Eds.; SSSA Book series 4; Soil Science Society of America: Madison, WI, 1991; pp 187−227.

(3) Yunta, F.; García-Marco, S.; Lucena, J. J.; Gómez-Gallego, M.; Alcázar, R.; Sierra, M. A. Chelating agents related to ethylenediamine bis(2-hydroxyphenyl)acetic acid (EDDHA): Synthesis, characterization, and equilibrium studies of the free ligands and their Mg^{2+}, Ca^{2+}, Cu^{2+}, and Fe^{3+} chelates. *Inorg. Chem.* **2003**, *42*, 5412−5421.

(4) Petree, H. E.; Myatt, L.; Jelenevsky, A. M. Preparation of phenolic ethylenediaminepolycarboxylic acids. U.S. Patent 4,130,582, 1978.

(5) Gómez-Gallego, M.; Sierra, M. A.; Alcázar, R.; Ramírez, P.; Piñar, C.; Mancheño, M. J.; García-Marco, S.; Yunta, F.; Lucena, J. J. Synthesis of *o,p*-EDDHA and its detection as the main impurity in *o,o*-EDDHA iron chelates. *J. Agric. Food Chem.* **2002**, *50*, 6395−6399.

(6) Álvarez-Fernández, A.; Cremonini, M. A.; Sierra, M. A.; Placucci, G.; Lucena, J. J. Nature of Impurities in Fertilizers containing EDDHMA/Fe^{3+}, EDDHSA/Fe^{3+}, and EDDCHA/Fe^{3+} Chelates. *J. Agric. Food Chem.* **2002**, *50*, 284−290.

(7) Álvarez-Fernández, A.; García-Marco, S.; Lucena, J. J. Evaluation of synthetic iron (III)-chelates (EDDHA/Fe^{3+}, EDDHMA/Fe^{3+}, and the novel EDDHSA/Fe^{3+}) to correct iron chlorosis. *Eur. J. Agron.* **2005**, *22*, 119−130.

(8) Yunta, F.; García-Marco; S.; Lucena, J. J. Theoretical speciation of ethylenediamie-*N*(*o*-hydroxyhenylacetic)-*N*′(*p*-hydroxyhenylacetic) acid (*o,p*-EDDHA) in agronomic conditions. *J. Agric. Food Chem.* **2003**, *51*, 5391−5399.

(9) García-Marco, S.; Torreblanca, A.; Lucena, J. J. Chromatographic Determination of Fe Chelated by Ethylenediamine-*N*(*o*-hydroxyphenylacetic)-*N*′(*p*-hydroxyphenylacetic) acid in Commercial EDDHA/Fe^{3+} Fertilizers. *J. Agric. Food Chem.* **2006**, *54*, 1380−1386.

(10) García-Marco, S.; Martínez, N.; Yunta, F.; Hernández-Apaolaza, L.; Lucena, J. J. Effectiveness of ethylenediamine-*N*(*o*-hydroxyphenylacetic)- *N*′(*p*-hydroxyphenylacetic) acid (*o*,*p*-EDDHA) to supply iron to plants. *Plant Soil* **2006**, *279*, 31−40.

(11) Cremonini, M. A.; Álvarez-Fernández, A.; Lucena, J. J.; Rombola, A.; Marangoni, B.; Placucci, G. Nuclear magnetic resonance analysis of iron ligand EDDHA employed in fertilizers. *J. Agric. Food Chem.* **2001**, *49*, 3527−3532.

(12) Sierra, M. A.; Gómez-Gallego, M.; Alcázar, R.; Lucena, J. J.; Álvarez, A.; Yunta, F. New Procedure for the Preparation of Bis-hydroxyaryl acetic acids using Cyanide Transfer Reagents. EP WO02/00604, European Patent, 2002.

(13) Bailey, N. A.; Cummins, D.; McKenzie, E. D.; Worthington, J. M. Iron(III) Compounds of Phenolic Ligands. The Crystal and Molecular Structure of Iron(III) Compounds of the Sexadentate Ligand *N*,*N*′-ethylene-bis-(*o*-hydroxyphenylglycine). *Inorg. Chim. Acta* **1981**, *50*, 111−120.

(14) García-Marco, S.; Cremonini, M. A.; Esteban, P.; Yunta, F.; Hernández-Apaolaza, L.; Placucci, G.; Lucena, J. J. Gradient ion-pair chromatographic method for the determination of iron *N*,*N*′-ethylenediamine-di-(2-hydroxy-5-sulfophenylacetate) by HPLC/APIES-MS. *J. Chromatogr. A* **2005**, *1064*, 67−74.

(15) Álvarez-Fernández, A.; Gárate, A.; Lucena, J. J. Interaction of Fe chelates with several soil materials and with a soil standard. *J. Plant Nutr.* **1997**, *20*, 559−572.

(16) Álvarez-Fernández, A.; Sierra, M. A.; Lucena, J. J. Reactivity of synthetic iron chelates with soils and soil components. *Plant Soil* **2002**, *241*, 129−137.

(17) Pérez-Sanz, A.; Lucena, J. J. Synthetic iron oxides as sources of Fe in a hydroponic culture of sunflower. In *Iron nutrition in soils and plants*; Abadia, A., Ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 1995; pp 241−246.

(18) Sims, J.; Bingham, F. Retention of boron by layer silicates, sesquioxides, and soil materials. II. Sesquioxides. *Soil Sci. Soc. Am. Proc.* **1968**, *32*, 364−369.

(19) van Olphen, H. *Data handbook for clay minerals another nonmetallic minerals*; Pergamon Press: London, 1979.

(20) Lucena, J. J.; Chaney, R. Synthetic iron chelates as substrates of root ferric chelate reductase (FCR) in green stressed cucumber plants. *J. Plant Nutr.* **2006**, *29*, 423−439.

(21) Peryea, F. J.; Kammereck, R. Use of Minolta SPAD-502 chlorophyll meter to quantify the effectiveness of mid-summer trunk injection of iron on chlorotic pear trees. *J. Plant Nutr.* **1997**, *20*, 1457−1463.

(22) Pestana, M.; de Varennes, A.; Araújo Faria, E. Diagnosis and correction of iron chlorosis in fruit trees: a review. *Food, Agric., Environ.* **2003**, *1*, 46−51.

(23) Álvarez-Fernández, A.; Pérez-Sanz, A.; Lucena, J. J. Evaluation of effect of washing procedures on mineral analysis of orange and peach leaves sprayed with seaweed extracts enriched with iron. *Commun. Soil Sci. Plant Anal.* **2001**, *32*, 157−170.

(24) Lucena, J. J.; Barak, P.; Hernández-Apaolaza, L. Isocratic iron pair high liquid chromatrographic method for the determination of various iron(III) chelates. *J. Chromatogr. A* **1996**, *727*, 349−356.

(25) Hernández-Apaolaza, L.; Barak, P.; Lucena, J. J. Chromatographic determination of commercial Fe(III) chelates of ethylenediamine tetraacetic acid, ethylenediamine di(*o*-hydroxyphenylacetic) acid, and ethylenediamine di(*o*-hydroxy-*p*-methylphenylacetic) acid. *J. Chromatogr. A* **1997**, *789*, 453−460.

(26) Gaskell, S. J. Electrospray Mass Spectrometry, Principles and Practice. *J. Mass Spectrom.* **1997**, *32*, 677−688.

(27) Gómez-Gallego, M.; Pellico, D.; Ramírez-López, P.; Mancheño, M. J.; Romano, S.; de la Torre, M. C.; Sierra, M. A. Understanding of the Mode of Action of Fe(III)-EDDHA as an Iron Chlorosis Corrector based on its Photochemical and Redox Behavior. *Chem. Eur. J.* **2005**, *11*, 5997−6005.

(28) Lucena, J. J.; Gárate, A.; Ramón, A. M.; Manzanares, M. Iron nutrition of a hydroponic strawberry culture (*Fragaria vesca* L.) supplied with different Fe chelates. *Plant Soil* **1990**, *123*, 9−15.

(29) Lucena, J. J. Iron Fertilizers in Correcting Iron Deficiencies in Plants. In *Iron Nutrition in Plants and Rhizospheric Microorganism*; Barton, L. L., Abadía, J., Eds.; Kluwer Academic: Dordrecht, The Netherlands, 2006; Ch. 5, pp 103−127.

Received for review February 28, 2006. Revised manuscript received April 5, 2006. Accepted April 6, 2006. Financial support by the Spanish Ministerio de Ciencia y Tecnología (Grants CTQ2004-06250, UCM group, and AGL2004-07849-C02-01/AGR, UAM group).

JF0605749