JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 5143–5147 5143
DOI:10.1021/jf900116z

Facile Deferration of Commercial Fertilizers Containing Iron Chelates for Their NMR Analysis

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Ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid (*o*,*o*-EDDHA) is widely used in commercial formulations as a Fe^{3+} chelating agent to remedy iron shortage in calcareous and alkaline soils. Commercially available *o*,*o*-EDDHA−Fe^{3+} formulations contain a mixture of EDDHA regioisomers (*o*,*p*-EDDHA and *p*,*p*-EDDHA), together with other, still uncharacterized, products. NMR spectroscopy can be applied to their study as long as iron is accurately removed prior to the observation. This paper shows that it is possible to obtain a deferrated solution of the organic ligands present in commercial fertilizers containing the EDDHA−Fe^{3+} chelate by treating the chelate with ferrocyanide, thus forming Prussian Blue that can be easily removed by centrifugation. This iron removal process does not cause significant losses of the *o*,*o*-EDDHA ligand or its minor structural isomers.

KEYWORDS: EDDHA; EDDHA regioisomers; NMR; fertilizers; iron chelates; iron removal; Prussian Blue

INTRODUCTION

Iron is one of the most abundant elements in Earth's crust. However, its availability to plants is hampered by its low solubility, in particular in calcareous soils (*1*, *2*). The consequence of a low supply of iron is a nutritional disorder named iron chlorosis that is manifested by the gradual disappearance of the green coloring of the plant (*3*). Remediation of iron chlorosis is efficiently achieved by the use of formulations based on iron chelates. These compounds are generally polyaminocarboxylic acids [the most common product is ethylenediamine-*N*,*N*′-bis-(*o*-hydroxyphenylacetic) acid, *o*,*o*-EDDHA, **Figure 1**] having Fe-stability constants in the range 10^{27}−10^{38} (*4*). The commercial formulations of this type of agrochemical must indicate on the label the amount and type of iron chelate present in the fertilizer (*5*). This requirement is particularly important in the formulations based on EDDHA because studies have demonstrated that regioisomers of *o*,*o*-EDDHA such as *o*,*p*-EDDHA are also efficient as chlorosis correctors (*6*), although these results have been recently challenged (*7*, *8*).

Quality control of fertilizers containing iron chelates is typically carried out by ion-pair reversed-phase HPLC (*9*, *10*), but other chromatographic methods exist (for a review of the most recent and some official analytical methods to assess the quality of commercial iron-chelate fertilizers see refs (*11*) and (*12*) and references cited therein). However, focusing on the EDDHA−Fe^{3+} commercial formulations, it is known that they contain other compounds apart from the agronomically useful *o*,*o*-EDDHA−Fe^{3+} and *o*,*p*-EDDHA−Fe^{3+} complexes (*10*), and to date the identification and quantification of these impurities by HPLC remain elusive. Moreover, it has been repeatedly reported that these impurities must play an important role in raising the amount of dissolved iron with respect to that allowed by considering the active ingredient only (*7*, *13*).

On the other hand, the use of NMR (*14*) for the quality control of fertilizers containing iron chelates is precluded by the strong paramagnetism of Fe^{3+}. Reduction of the transverse relaxation times of all protons to the range of milliseconds (*15*) renders NMR lines broad and practically useless. Removal of iron from EDDHA and homologues based commercial iron chelates without modifying the composition of the fertilizing mixture would be an efficient solution of this problem, because the Fe-free organic material may be analyzable and quantifiable by NMR. However, attempts made in this direction have been only partially successful to date. It has been reported (*16*, *17*) that a treatment of a commercial *o*,*o*-EDDHA−Fe^{3+} chelate with a 3 M nitrogen-saturated KOH solution removed completely iron in the form of a $Fe(OH)_3$ precipitate. The *o*,*o*-EDDHA and its isomers could be recovered from the supernatant by precipitation at the isoelectric point (pH ≈5.5) in 70% yield, which is inconvenient for analytical purposes. Therefore, more efficient and if possible milder procedures to remove the metal from EDDHA and homologues based commercial iron chelates are needed.

Prussian Blue [PB, $Fe_4[Fe(CN)_6]_3$] has been used since the 18th century as blue dye for paintings and inks and nowadays as a low-cost pigment for some polymeric materials (*18*). PB is a very insoluble compound [$K_{sol} = 10^{-84.5}$ (*19*)], and this high insolubility allows for its formation even from very stable ferric chelates

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 Published on Web 05/21/2009

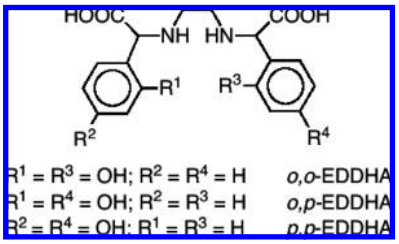

Figure 1. Structure of the EDDHA ligands.

such as EGTA−Fe^{3+} and HEDTA−Fe^{3+}, especially at low pH values (pH 0−3) (*19*). These properties make potassium ferrocyanide [$K_4Fe(CN)_6$] a very attractive reagent to displace the organic ligand from *o*,*o*-EDDHA−Fe^{3+} and its less stable isomer *o*,*p*-EDDHA−Fe^{3+}.

Here we report a procedure for deferrating commercial mixtures of EDDHA and homologue iron chelates, keeping the amount of fertilizer and solvent at the minimum necessary to obtain well-resolved NMR spectra of the organic ligands. The organic ligands remain dissolved in the reaction mixture throughout the procedure, which avoids losses of material. This iron removal process is applied to three commercial EDDHA−Fe^{3+} fertilizers.

MATERIALS AND METHODS

Chemicals and Reagents. *o*,*o*-EDDHA was purchased from Sigma (97%, lot 26H5012) and found to be an 80:20 mixture of the meso and racemic diastereomers (*17*). All commercially available compounds for the synthesis of *o*,*p*-EDDHA (*20*) and *p*,*p*-EDDHA (*21*) were used without further purification. Analytical grade $FeCl_3$ and $K_4[Fe(CN)_6]\cdot 3H_2O$ were obtained from Aldrich and Alfa Aesar. HPLC grade tetrabutylammonium hydroxide and acetonitrile were purchased from Scharlau-Ferosa.

Preparation of *o*,*p*- and *p*,*p*-EDDHA. Synthesis of *o*,*p*-EDDHA was carried out according to the method of ref (*20*); *p*,*p*-EDDHA (*21*) was prepared by following a modification of the reported procedure (sample A in ref (*22*)). In this case, the precipitate obtained at pH 5.7 was collected after 4 h as a white solid (10 mg) that turned out to be a mixture (about 6.4:1) of the two diastereomers of *p*,*p*-EDDHA (see mass spectrum and 1D- and 2D-NMR spectra in DCl/D_2O in the Supporting Information).

Commercial Samples. Three commercial fertilizers containing EDDHA−Fe^{3+} manufactured by three different companies were purchased in the Spanish market. The trade names of the fertilizers were Sequestrene (lot VPA3C19120159300, purchased from Syngenta), Kelom (lot 001032006, purchased from Anfico), and Greental (lot 04/011, purchased from JAER), hereafter referred to as samples A, B, and C, respectively. Samples for NMR were deferrated prior to analysis according to the procedure outlined below.

Deferration of EDDHA−Fe^{3+} Commercial Chelates. Approximately 10 mg of a fertilizer containing EDDHA−Fe^{3+} was dissolved in 500 μL of D_2O in an Eppendorf tube. According to European law (*5*), this should correspond to at least 2.6 mg of EDDHA, but a more realistic estimate of the total iron chelate concentration can be obtained from visible light absorbance at 480 nm using the average molar extinction coefficient of meso and racemic *o*,*o*-EDDHA−Fe^{3+} [4765 ± 45 M^{-1} cm^{-1} (*21*)]. About 300 μL of a 40 mM solution of potassium ferrocyanide [$K_4[Fe(CN)_6]\cdot 3H_2O$] in D_2O was then added to reach a final estimated ferrocyanide/iron chelate ratio in excess with respect to the stoichiometric ratio for the formation of PB. The addition of $K_4[Fe(CN)_6]$ led to the almost immediate formation of a PB blue suspension. The pH of the solution was then lowered to about 0.7 with HCl (g) to allow the complete dissolution of the organic ligands (*17*). PB was removed by centrifugation at 18000*g* for 5 min in a Beckman Coulter Eppendorf centrifuge model Microfuge 18. Although residual low-spin ferrocyanide ion $[Fe(CN)6]^{4-}$ is diamagnetic and would not alter the spectrum, it is readily oxidized by the dissolved oxygen to paramagnetic ferricyanide $[Fe(CN)6]^{3-}$, the presence of which would broaden the NMR spectral lines. Therefore, an aliquot of 700 μL of the centrifugate was taken, and the excess ferrocyanide was removed by addition of a D_2O solution of $ZnSO_4$ until precipitation of $K_2Zn_3[Fe(CN)_6]_2$ was complete [$K_{sol} = 10^{-95}$ (*23*)] (typically 5−10 μL of a 1.15 M solution of $ZnSO_4$ was sufficient). Finally, $K_2Zn_3[Fe(CN)_6]_2$ was removed by centrifugation at 18000*g* for 5 min, leaving a clear pale yellow solution that was analyzed by NMR.

In Situ Synthesis and Deferration of EDDHA−Fe^{3+} for Testing Ligand Recovery. The iron chelate of EDDHA was synthesized according to the method of ref (*10*): 10.12 mg of 97% commercial EDDHA from Aldrich (corresponding to 9.82 mg of ligand) was placed in an Eppendorf tube and dissolved in 800 μL of D_2O containing a 3-fold molar amount of NaOH with respect to EDDHA. Then 5.34 mg of $FeCl_3$ was added; the pH was adjusted to 7.0, and the solution was left to stand overnight for allowing precipitation of the excess of iron as hydroxide. The solution was then centrifuged (as above) at 18000*g* for 5 min. To check the amount of the chelate present in solution, a small aliquot of the supernatant (45 μL) was made up to 1 mL and used for measuring visible absorbance at 480 nm. Quantification was done using a molar extinction coefficient of 4650 ± 50 M^{-1} cm^{-1}, which is the weighted average (80:20) of the molar extinction coefficients of the meso and racemic diastereoisomers of *o*,*o*-EDDHA−Fe^{3+} [respectively, 4574 ± 53 and 4955 ± 37 M^{-1} cm^{-1} (*21*)]. Then 600 μL of the iron chelate solution was deferrated following the procedure above, using 150 μL of a 112 mM solution of potassium ferrocyanide in D_2O; after centrifugation, 20 μL of $ZnSO_4$ 1.15 M in D_2O was added, and the solution was centrifuged again. Finally, 550 μL of the supernatant was placed in an NMR tube together with 50 μL of acetic acid (440 mM) as internal standard.

Note that ferrocyanide ion is relatively nontoxic; a World Health Organization review about the toxicology of the calcium, potassium, and sodium salts of ferrocyanide reported that the sodium ferrocyanide intake causing no toxicological effects in rats is 25 mg kg^{-1} and its LD_{50} in rats is 1600−3200 mg kg^{-1} (*24*). This low toxicity stems from the high kinetic stability of the ferrocyanide ion in solution that prevents it from releasing free cyanide even when used in acidic pH conditions similar to ours (*25*). Care, however, should be taken to store the ferrocyanide solution in the dark; otherwise, it may decompose at a rate up to 8% per hour (*25*).

HPLC Analysis. HPLC quantification of *o*,*o*-EDDHA−Fe^{3+} was done according to the ion-pair chromatography method described by Lucena et al. (*9*, *10*) using an LC-20 A chromatographic system (Shimadzu, Japan) equipped with a Lichrospher 100RP-18 (5 μm) column (Agilent Technologies, Madrid, Spain) thermostated at 25 °C, an LC-7A pump, an SIL-10A autosampler, an NSPD-M6A photodiode array detector, and chromatographic software CLASS-LC10 V.1.6. The flow rate was 1.5 mL/min, and the injection volume was 50 μL.

NMR Analysis. The NMR spectra were acquired at 400 MHz with a Mercury-Plus spectrometer (Varian, Palo Alto, CA, USA) using the PRESAT sequence, which saturates the residual H_2O signal, using a 90° pulse width of 6.3 μs, an SW of 6400 Hz, 16K total acquired complex points, and a recycle delay of 10 s. Care must be taken to avoid using too intense a B_1 field for presaturation. We found that in our conditions a B_1 field of 70 Hz applied at the frequency of the residual water signal for 1.5 s was sufficient to saturate the water line without affecting the nearby benzylic CH signals, thus permitting the accurate quantification of the organic ligands from their integral (vide infra). A known amount of pure acetic acid (50 μL of a 115.4 mM solution in D_2O) was added as internal reference for concentration and chemical shift (methyl ^1H signal, 2.04 ppm; ^{13}C signal, 20.0 ppm). A temperature of 35 °C was chosen to minimize the overlap between the residual water peak and the nearby EDDHA peaks.

The spectra were processed by means of VNMRJ 1.1D software from Varian. All 1D spectra were submitted to reference deconvolution (*26*) with the routine "fiddle" of VNMRJ 1.1D, using the methyl line of the internal standard (acetic acid) as line shape template. Reference deconvolution helps narrowing the spectral lines by converting an arbitrary line shape into lines of known shape, usually Gaussian. In our case reference deconvolution was essential for achieving better resolution in the benzylic region of *o*,*o*-, *o*,*p*-, and *p*,*p*-EDDHA so as to be able to perform line fitting and quantification through the routine "fitspec" of VNMRJ 1.1D.

RESULTS AND DISCUSSION

The effectiveness of the deferration procedure we propose is better studied from two complementary points of view: the first one is iron removal itself, and the second one is the recovery of the

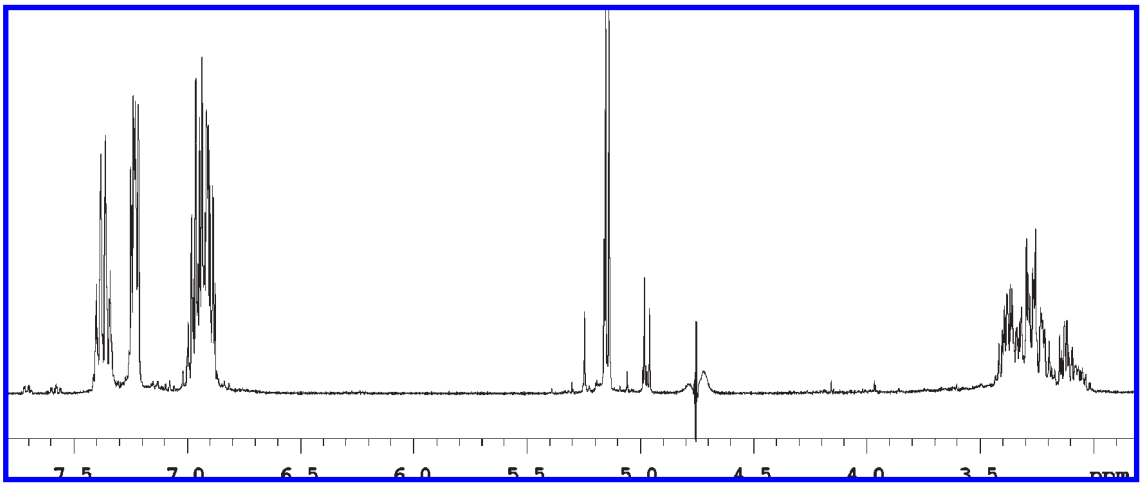

Figure 2. ^{1}H NMR spectrum of a deferrated solution of sample A in D_2O at 35 °C.

organic fraction *o*,*o*- and *o*,*p*-EDDHA and other byproducts of synthesis.

Iron Removal. Iron removal is easily checked by NMR and UV−vis spectroscopy. **Figure 2** shows the ^{1}H NMR spectrum of commercial sample A after deferration. The spectrum is well resolved, the average line width being 1.7 Hz, a value comparable to the average value of 1.3 Hz obtained for pure *o*,*o*-EDDHA in the same conditions of pH, concentration, and shimming (spectrum not shown). As line width is directly proportional to the concentration of iron in solution (*15*), this is evidence that only a very small amount of iron is present in the solution. The absence of most of the chelated iron is demonstrated by recording the UV−vis spectrum of a solution of a commercial chelate before and after deferration (**Figure 3**). The broad band centered at 480 nm [which is due to absorption of Fe^{3+}−phenolate bonds (*20*)] mostly disappears after the deferrating treatment.

NMR Analysis of the Organic Fraction Obtained from EDDHA-Containing Iron Fertilizers. The NMR spectra of solutions obtained by deferration of commercial fertilizer samples containing EDDHA show the expected signals for *o*,*o*-EDDHA and its regioisomers (*17*). In particular, the peaks between 6.8 and 7.5 ppm belong to aromatic protons, the peaks between 4.9 and 5.3 ppm belong to CH protons, and the complex multiplets between 2.9 and 3.5 ppm belong to the CH_2 protons. The CH spectral region has been studied in detail in ref (*17*) and found to comprise peaks ascribed to the two major species, that is, the meso and racemic diastereoisomers of *o*,*o*-EDDHA (respectively, at 5.15 and 5.13 ppm), and to minor species bearing *p*-EDDHA or *o*-EDDHA moieties (respectively, upfield and downfield of the CH *o*,*o*-EDDHA signals). It has been suggested that the *p*-EDDHA moieties mainly belong to *o*,*p*-EDDHA and *p*,*p*-EDDHA, whereas the *o*-EDDHA moiety at 5.24 ppm might belong to a synthesis byproduct (*17*). The two CH signals of the *o*-EDDHA moiety of *o*,*p*-EDDHA (each corresponding to a different diastereomeric couple of *o*,*p*-EDDHA enantiomers) were not detected in our previous study and hypothesized to be buried under the much more intense CH signals of *o*,*o*-EDDHA. The same was observed in the ^{1}H NMR spectra of one of our samples (sample C) for which the amount of *o*,*p*-EDDHA was very low, as revealed by the low intensity of the signals around 4.9 ppm. On the contrary, when a commercial fertilizer declaring a high amount of *o*,*p*-EDDHA on the label (sample A) was submitted to our deferration procedure, a new signal was detected downfield of the meso *o*,*o*-EDDHA signal (5.16 ppm, **Figure 4**), the other line still being missing. Assignment of the benzylic NMR signals of *o*,*p*-EDDHA was unambiguously achieved by

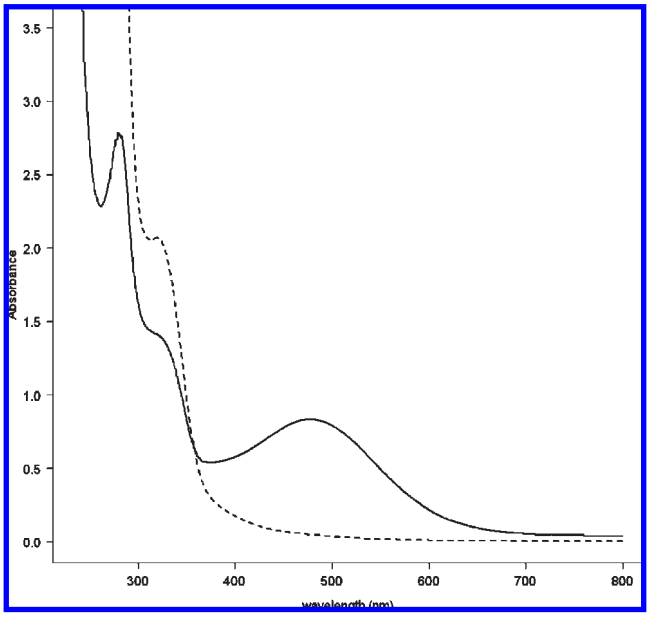

Figure 3. UV−vis spectrum of a water solution of sample A before (solid) and after (dashed) deferration.

spiking the solution with a few grains of a pure *o*,*p*-EDDHA powder. From **Figure 4** it is apparent that the peaks at 5.16, 4.98, and 4.96 ppm increase upon *o*,*p*-EDDHA addition. The peak at 5.13 ppm, corresponding to racemic *o*,*o*-EDDHA also increases, thus indicating superimposition between that line and the missing *o*,*p*-EDDHA NMR line. Therefore, the lines at 5.13/5.16 and 4.96/4.98 ppm should be assigned, respectively, to the benzylic protons of the ortho and para moieties of *o*,*p*-EDDHA.

The small lines at 4.99 and 4.97 ppm in the CH region of the ^{1}H NMR spectrum could be assigned, by exclusion, to the two diastereomers of *p*,*p*-EDDHA, although a more convincing proof of the assignment is desirable. Fortunately, while synthesizing *o*,*o*-EDDHA following the method of ref (*22*) as indicated under Material and Methods, we serendipitously obtained at pH 5.7 a very small amount of a white solid, which turned out to be a mixture of the two diastereomers of *p*,*p*-EDDHA. The CH region of the NMR spectrum obtained after the deferrated solution of sample A had been spiked with *p*,*p*-EDDHA is shown in **Figure 5**. The increase of lines at 4.99 and 4.97 ppm confirms that these lines must indeed be assigned to the CH protons of the *p*,*p*-EDDHA diastereoisomers.

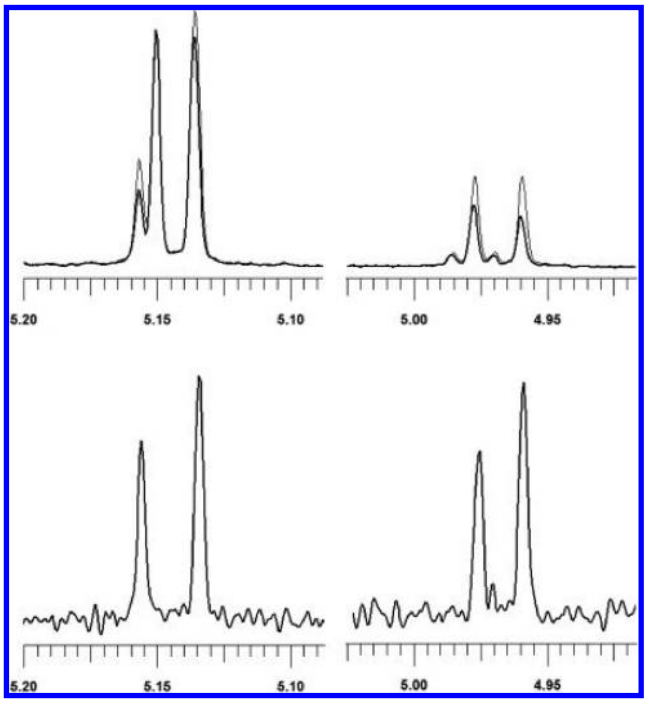

Figure 4. (Bottom) ^{1}H NMR spectrum of the benzylic region of *o,p*-EDDHA. (Top) Benzylic region of the same spectrum shown in **Figure 2** before (black) and after (gray) spiking with a few grains of solid *o,p*-EDDHA.

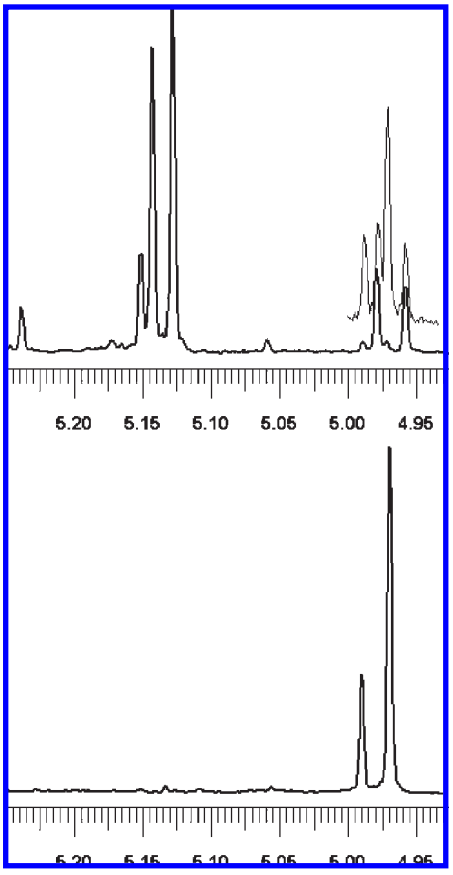

Figure 5. (Bottom) ^{1}H NMR spectrum of the benzylic region of *p,p*-EDDHA. (Top) Benzylic region of the same spectrum shown in **Figure 2** before (black) and after (gray) spiking with a few grains of solid *p,p*-EDDHA.

Overall, the treatment of commercial samples declaring *o,o*-EDDHA−Fe^{3+} in their composition can be efficiently deferrated using $K_4[Fe(CN)_6]$. The method proceeds in situ with a minimum manipulation of the sample, and the resulting solution can be used for quantifying the dissolved *o,o*-EDDHA and *o,p*-EDDHA using the NMR signal of the benzylic protons. Evidently, the complete recovery of the organic ligand should be unambiguously achieved.

Table 1. ^{1}H NMR and HPLC Quantification of EDDHA Isomers Contained in Three Commercial Fertilizers Analyzed in Triplicate (Units, % w/w ± Standard Deviation)

compound	sample A	sample B	sample C
		NMR	
o,o-EDDHA	21.1 ± 1.0	24.6 ± 0.9	36.4 ± 0.8
o,p-EDDHA	10.1 ± 0.2	7.2 ± 0.1	nd[a]
p,p-EDDHA	1.3 ± 0.4	1.5 ± 0.1	nd
		HPLC	
o,o-EDDHA	21.3 ± 0.6	25.2 ± 0.8	36.1 ± 1.3

[a] nd, not detected.

Recovery of Ligands. Recovery of *o,o*-EDDHA from the iron chelate was first tested with a solution of *o,o*-EDDHA−Fe^{3+} freshly prepared from 9.82 mg of pure *o,o*-EDDHA (see Materials and Methods). After complete formation of the iron chelate was achieved (the absorbance at 480 nm being 0.7023, which corresponded to 9.68 ± 0.10 mg of ligand in the parent solution), the solution was deferrated in situ according to the procedure reported under Materials and Methods; the ligand concentration measured by NMR with respect to the internal standard (acetic acid) was 23.70 ± 0.14 mM, which corresponded to 33.67 ± 0.20 mM iron chelate in the parent solution or 9.71 ± 0.06 mg of ligand, or nearly complete ligand recovery. Note that here the given errors are the standard deviations (0.68%) from three different measures of the integrals in the same EDDHA NMR spectrum having a S/N ratio of 440; our relative standard deviation compares well with the one expected in similar S/N conditions (*27*).

Recovery of *o,o*-EDDHA from the iron chelate was assessed by comparison with HPLC experiments (**Table 1**). In the three samples analyzed, the agreement between the two techniques is remarkable, the results being not significantly different ($p > 0.95$) at the Wilcoxon test (*28*). The complete recovery of *o,o*-EDDHA in the centrifugate prompts us to postulate that its regioisomers *o,p*- and *p,p*-EDDHA are also quantitatively recovered in our experimental conditions. This hypothesis is corroborated by the finding that the relative percentages between *o,o*- and *o,p*-EDDHA measured by NMR in sample A (67.7 and 32.3%, respectively) agree well with the average values declared on the label by the manufacturer (60.7 and 39.3%, respectively). Of course, as no proof of complete recovery of *p,p*-EDDHA can be given, our *p,p*-EDDHA quantification must be taken as tentative, although it is reasonable to think that this very minor compound is also quantitatively recovered because of its structural resemblance to the other EDDHA isomers.

In conclusion, a facile method for removing iron from a water solution of EDDHA−Fe^{3+} chelates used to relieve iron chlorosis has been developed. The NMR spectra of the resulting solutions are well resolved and therefore suitable to be used for the detection and quantification of minor structural isomers. Further applications of our procedure can be envisaged for studying the structure of the impurities and/or byproducts in all cases when the amount of dissolved iron is higher than expected as, for example, in fertilizers based on the iron chelates of EDDHMA (*29*) and EDDHSA (*30*).

ABBREVIATIONS USED

o,*o*-EDDHA, ethylenediamine-*N*,*N*′-bis-(*o*-hydroxyphenylacetic)acid; *o*,*p*-EDDHA, ethylenediamine-*N*-(*o*-hydroxyphenylacetic)-*N*′-(*p*-hydroxyphenylacetic) acid; *p*,*p*-EDDHA ethylenediamine-*N*,*N*′-bis-(*p*-hydroxyphenylacetic)acid; HPLC, high-performance liquid chromatograhy; NMR, nuclear magnetic resonance.

Supporting Information Available: Chromatograms of samples A−C, ^{1}H NMR spectra of samples B and C after deferration; ^{1}H NMR spectra (1D, 2D gHSQC and gHMBC) of *p*,*p*-EDDHA, and mass spectrum of *p*,*p*-EDDHA; photograph showing how the color of the solution changes along the deferration procedure. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received January 12, 2009. Revised manuscript received May 1, 2009.