NMR Analysis of the Iron Ligand Ethylenediaminedi(*o*-hydroxyphenyl)acetic Acid (EDDHA) Employed in Fertilizers

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The exceptional efficiency of the iron chelate of ethylenediaminedi(*o*-hydroxyphenyl)acetic acid (*o*,*o*-EDDHA) in correcting iron chlorosis in plants and the medical applications of various metallic chelates of this compound have long been recognized. As commercial preparations of *o*,*o*-EDDHA usually contain impurities, a method for their detection is proposed. By using one- and two-dimensional nuclear magnetic resonance two impurities were identified. The structure of one of these compounds was assigned to an isomer of EDDHA containing at least one *p*-hydroxyphenyl moiety. The structure of the other impurity was tentatively assigned to a byproduct of the EDDHA synthesis: 2,6-di[CH(COOH)NHCH$_2$CH$_2$NHCH(COOH)Ar]phenol (Ar = hydroxyphenyl). Both compounds were also detected in the EDDHA extracted from a commercial iron fertilizer.

Keywords: *EDDHA; NMR; fertilizers; iron chelates*

INTRODUCTION

Ethylenediaminedi(*o*-hydroxyphenyl)acetic acid (*o*,*o*-EDDHA, **1**, Figure 1) is one of the most important Fe^{3+} chelating agents used to increase iron availability and to control iron chlorosis in calcareous and alkaline soils (*1*). It forms a 1:1 complex with Fe^{3+} that is readily available to plant roots because it is soluble in water and remarkably stable over a wide range of pH values (*2*). *o*,*o*-EDDHA is one of the six possible −OH positional isomers on the benzene ring of EDDHA, and it is the only one that has these properties. Other EDDHA/Fe^{3+} complexes are much less stable than *o*,*o*-EDDHA. Industrial preparation of the EDDHA/Fe^{3+} chelate is commonly carried out by addition of iron salts to unpurified EDDHA immediately after EDDHA synthesis (*3*, *4*). Therefore, it is reasonable to assume that positional isomers other than *o*,*o* are often present in commercial preparations containing EDDHA. This raises the problem of their identification and quantification because the agronomic effectiveness of these isomers is unknown. Furthermore, only chelates formed by the *o*,*o* isomer are allowed as fertilizer in Europe (Directive 98/3/CE).

Quite recently, Hernández-Apaolaza et al. (*5*) put forward the proposal that the EDDHA used to prepare some commercial EDDHA/Fe^{3+} chelates might contain measurable amounts of *o*,*p* or *p*,*p* isomers. This hypothesis was necessary to explain the presence of two unexpected minor peaks during a high-pressure liquid chromatography (HPLC) analysis of some commercial EDDHA/Fe^{3+} samples. However, it could not be fully confirmed by comparison with authentic samples because of their unavailability as HPLC standards. This prompted us to pursue the study of Hernández-Apaolaza et al. (*5*) by employing a different technique that could yield structural information about the unknown compounds without the need for comparison with an authentic specimen.

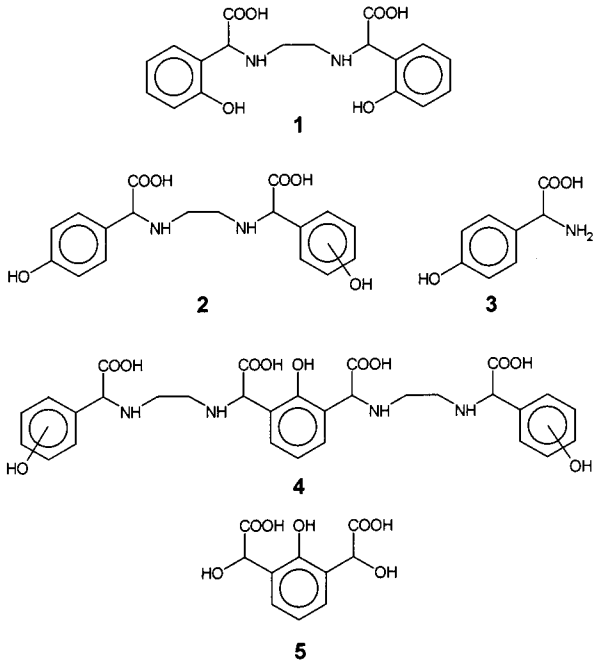

Figure 1. Structures of **1**−**5**.

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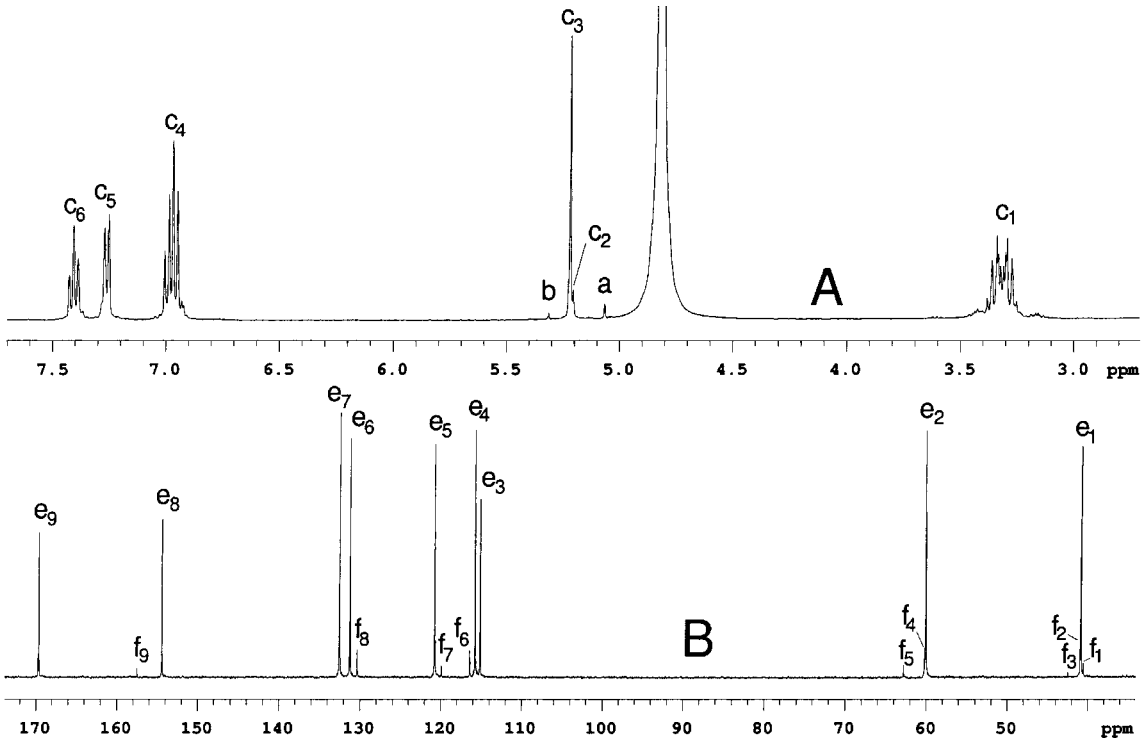

Figure 2. NMR spectra of a commercial sample of EDDHA in D_2O at 308 K (400 MHz): (A) ^1H spectrum; (B) ^{13}C spectrum.

In this paper we show that mono- and bidimensional nuclear magnetic resonance spectroscopy (1D- and 2D-NMR) can be successfully used to identify EDDHA impurities in commercially available *o,o*-EDDHA. In an effort to extend our study also to EDDHA/Fe^{3+} chelates, the EDDHA contained in a commercial fertilizer was extracted with a simple chemical pretreatment and the purity was qualitatively determined by 1D-NMR.

MATERIALS AND METHODS

o,o-EDDHA was purchased from Sigma (lot 26H5012). The present preparation was analyzed by 1D-NMR in alkaline conditions according to the method of Patch et al. (*6*) and found to be a mixture of the two expected diastereomers (meso and racemic) in an 80:20 ratio instead of the expected 50:50. It has been noted in the past that commercial *o,o*-EDDHA is an "unspecified mixture" of meso and racemic isomers (*6*, *7*). A sample of commercial fertilizer containing the EDDHA/Fe^{3+} complex was also obtained. All reagents used were of analytical grade.

1D- and 2D-NMR spectra were recorded at 200 and 400 MHz with Bruker AC 200, using simultaneous (SIM) mode, and Varian Mercury VX 400 instruments, and all were processed with the program VNMR-SGI provided by Varian. Bruker free induction decays (FIDs) were converted to the VNMR format with the macro "convertbru" and subsequently processed by backward linear prediction of the first three points before complex Fourier transformation, so as to obtain very flat baselines. Library NMR sequences were always used except for nuclear Overhauser enhancement (NOE) experiments that were implemented according to the method of Banci et al. (*8*). Samples for NMR were prepared by dissolving EDDHA in 650 μL of D_2O at a pH of ~0. This permitted dissolution of ~20−30 mg of the compound. Despite the lesser solubility of EDDHA at the above pH, acidic conditions were preferred over basic conditions because of the better spectral resolution achieved at acidic pH. All of the chemical shifts are referenced to the methyl signal of acetic acid (^1H = 2.04 ppm; ^{13}C = 20.0 ppm) added in small amounts as internal standard into the NMR tubes. Chemical shift calculations were carried out with the program ACD/HNMR predictor by Advanced Chemistry Development Inc., Toronto, Canada.

EDDHA was extracted from the commercial fertilizer containing the EDDHA/Fe^{3+} complex with the following procedure, similar to the one used by Bannochie and Martell (*2*): To ~4 g of fertilizer was added 25 mL of nitrogen-saturated 3 M KOH, and the resulting solution was allowed to stand for 15 min at room temperature in the dark to avoid photodegradation of EDDHA (*9*). The precipitated $Fe(OH)_3$ was eliminated by centrifugation at 75600*g* with a Beckmann J-25 centrifuge. The clean solution was acidified at pH 5.5 and allowed to stand overnight. The precipitated EDDHA was recovered by centrifugation at 75600*g*, washed twice with distilled water, and dried in an oven at 105 °C. The resulting brown powder was submitted to NMR analysis. Actually, preparation of an iron-free fraction of the commercial fertilizer is required to avoid the dramatic broadening of the NMR lines caused by iron paramagnetism (*10*). The extraction procedure was tested with a sample of EDDHA/Fe^{3+} chelate synthesized for this purpose from Sigma EDDHA and $FeCl_3$ according to the method of Petree et al. (*3*). Although ligand recovery was ~70%, the relative amounts of peaks *a*, *b*, and c_2+c_3 measured by NMR (vide infra) were comparable to those found in the starting EDDHA. It is worth noting that if the precipitation of EDDHA at pH 5.5 is stopped after 15−30 min, the small amount of solid recovered by centrifugation (~5−10% of the starting ligand) is noticeably enriched in compound **2**. This behavior was exploited to recognize the pair of signals belonging to the benzylic CHs of the meso and racemic diastereomers of compound **2** in Figure 6C.

RESULTS AND DISCUSSION

The ^1H NMR spectrum of a commercial sample of EDDHA in D_2O at 308 K is shown in Figure 2A. Besides the HDO residual peak at 4.83 ppm, five peaks appear at 3.33 (c_1), 5.21 (c_2), 5.23 (c_3), 6.98 (c_4), 7.27 (c_5), and 7.42 ppm (c_6). They can be assigned, respectively, to aliphatic CH_2's, benzylic methenes of the two diastereomers and four aromatic protons, two of which overlap (*11*). Unexpectedly, two more peaks are detected at 5.08 (*a*) and 5.32 ppm (*b*), the integrals of which equaled about 4.2 and 2.5% compared to the sum of the c_2+c_3 peaks. Their presence is indicative of two compounds,

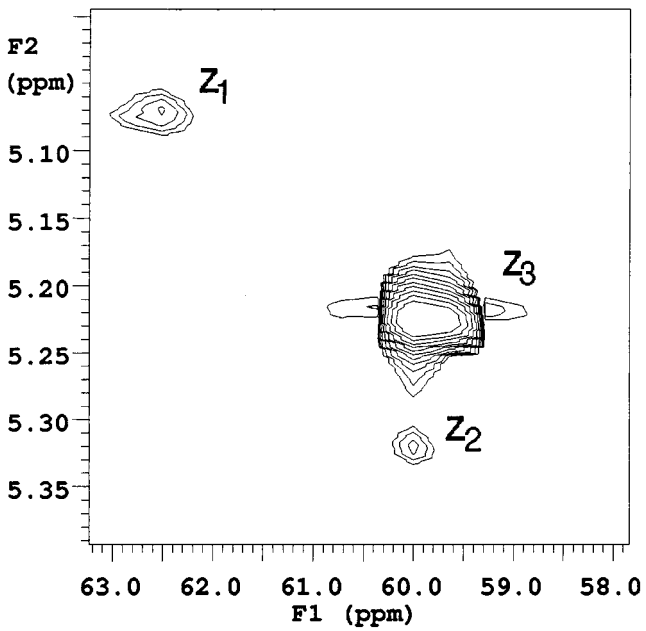

Figure 3. Expansion of a 400 MHz 2D gradient HSQC spectrum (298 K) of a commercial sample of EDDHA.

hereafter termed impurities A and B. Unassigned resonances were found also in the ^{13}C spectrum of *o,o*-EDDHA (Figure 2B), where minor peaks (indicated by the letter "*f*") flank most of the carbon signals, thus indicating a likely chemical resemblance between *o,o*-EDDHA and the two unknown compounds.

It seems reasonable to search for the structure of impurities A and B by starting from the hypothesis of Hernández-Apaolaza et al. (*5*) that these minor compounds are isomers of **1**. Accordingly, signals *a* and *b* might belong to benzylic CH groups of A and B, the other missing lines being buried under the much more intense signals of **1**.

Support for the above hypothesis comes from the inspection of a 2D gradient HSQC spectrum (*12*); its relevant CH part is shown in Figure 3. In fact, the hydrogens that give rise to peaks *a* and *b* are connected via cross-peaks (cp's) z_1 and z_2 to carbons having chemical shifts (respectively, 62.6 and 60.0 ppm) in the range expected for benzylic methenes; they are also very close to the EDDHA ^{13}C benzylic CH resonance (z_3, at 60.0 ppm). More importantly, the three cp's appear as F_2 doublets when ^{13}C decoupling is switched off during acquisition, thus strengthening the assignment.

Further connections are found by exploiting the long-range proton−carbon coupling constants in a gradient-HMBC 2D experiment (*13*). It is worth assigning first the major peaks due to compound **1** (Figure 4A) so as to understand the type of information gathered from an HMBC map. Following the line at $F_2 = 5.23$ ppm, corresponding to peak c_3 in Figure 2A, five cp's are encountered, corresponding to long-range connections between the CH benzylic proton of **1** and the carboxylic carbon (y_1 at 170.0 ppm), aromatic C_2, C_6, and C_1 (y_2, y_3, and y_4 at 154.4, 131.2, and 115.5 ppm, respectively), and aliphatic CH_2 (y_5 at 40.8 ppm). The assignment choice between C_6 and C_1 is straightforward; the HSQC lacks a cp between an aromatic proton and 115.5 ppm (data not shown), thus permitting the assignment of the carbon at that frequency as quaternary. Moving vertically at the C_6 frequency, H_4 can be recognized at $F_2 = 7.46$ ppm (y_6). Its assignment is justified by the well-known difference in magnitude between $^3J_{CH}$ (∼7 Hz) and $^2J_{CH}$ (∼1 Hz) in substituted aromatics (*14*). The same reasoning aids location of H_6 from C_2 at $F_2 = 7.30$ ppm (y_7) and C_4 at $F_1 = 132.2$ ppm (y_8) moving from H_6. Finally, H_3 and H_5 are assigned at 7.00 and 7.03 ppm, respectively, because of the presence of two small HMBC cp's (due to $^2J_{CH}$) at the frequencies of C_2 and C_6 (respectively, y_9 and y_{10}). C_5 and C_3 are detected at 120.5 and 116.0, respectively, once more via $^3J_{CH}$ (cp's y_{11} and y_{12}).

When the HMBC spectrum is plotted with a lesser threshold (Figure 4B), new peaks appear, owing to the long-range heteronuclear couplings stemming from the CH protons of species A and B. In particular, at $F_2 = 5.32$ ppm (corresponding to peak *b* in Figure 2A) five peaks are found at carbon frequencies almost equal to those previously reported for **1** at the proton benzylic CH frequency (cp's x_1-x_5, Figure 4B). At $F_2 = 5.08$ ppm (corresponding to peak *a*, Figure 2A) only four cp's are detected; their ^{13}C chemical shifts are not much different from those described above but for the fact that no long-range coupling to an aromatic C−OH is found (cp's w_1-w_4). The latter finding allows us to propose the assignment of impurity A to the *p*-EDDHA isomer proposed by Hernández-Apaolaza et al. (*5*). However, we prefer to indicate compound A as **2** (Figure 1) because there is no way to tell by NMR whether only one or both phenyls carry the −OH at position 4.

Structure **2** can be further confirmed by carrying out an NOE difference experiment in which peak *a* is saturated long enough to allow detection of both direct and relayed enhancements at the phenyl protons (*15*). In fact, the direct and relayed spatial connectivities can be recognized in the aromatic part of Figure 5C as positive and negative peaks centered at 7.30 and 6.97 ppm that correspond to the couples of equivalent protons $H_{2,6}$ and $H_{3,5}$ in **2**, respectively. The characteristic AA′BB′ shape of the multiplet at 7.30 ppm (Figure 6A) and the required distortion of its NOE relayed companion at 6.97 ppm (*16*) both confirm para −OH substitution. Furthermore, the two NOE difference generated signals are almost identical to those obtained by irradiating the benzylic CH of pure *p*-hydroxyphenylglycine **3** (Figure 1) but for a slight difference in the chemical shift (Figure 6B).

The chemical shifts of the aromatic protons of **2** permit the assignment of most of its ^{13}C chemical shifts that can eventually be compared to the minor peaks appearing in Figure 2B. Thus, at the frequency of the pair $H_{2,6}$, three HMBC cp's are detected at 157.5, 130.6, and 62.6 ppm (cp's w_5-w_7 in Figure 4B and, respectively, peaks f_9, f_8, and f_5 in Figure 2B). They correspond to three long-range connections (via $^3J_{CH}$) between H_2 (or H_6) and C_4, C_6 (or C_2), and the benzylic carbon. Both equivalent carbons $C_{6,2}$ are obviously coupled to the benzylic proton at $F_2 = 5.08$ ppm, yielding cp w_2, the remaining cp's at 170.0, 119.8, and 40.5 ppm (w_1, w_3, and w_4) being reasonably assigned to the ^{13}C chemical shifts of COOH, C_1, and methylenic carbons. The pair $C_{3,5}$ can be assigned to peak f_6 in Figure 2B on the basis of its intensity and chemical shift (116.4 ppm).

Whereas impurity A can be tentatively assigned structure **2**, it is difficult to determine the structure of B on the basis of NMR only because of the almost complete overlap of its peaks (and most of its 2D cp's) with those of **1**. Some information is, however, available. First, the pattern of HMBC cp's at the frequency of the

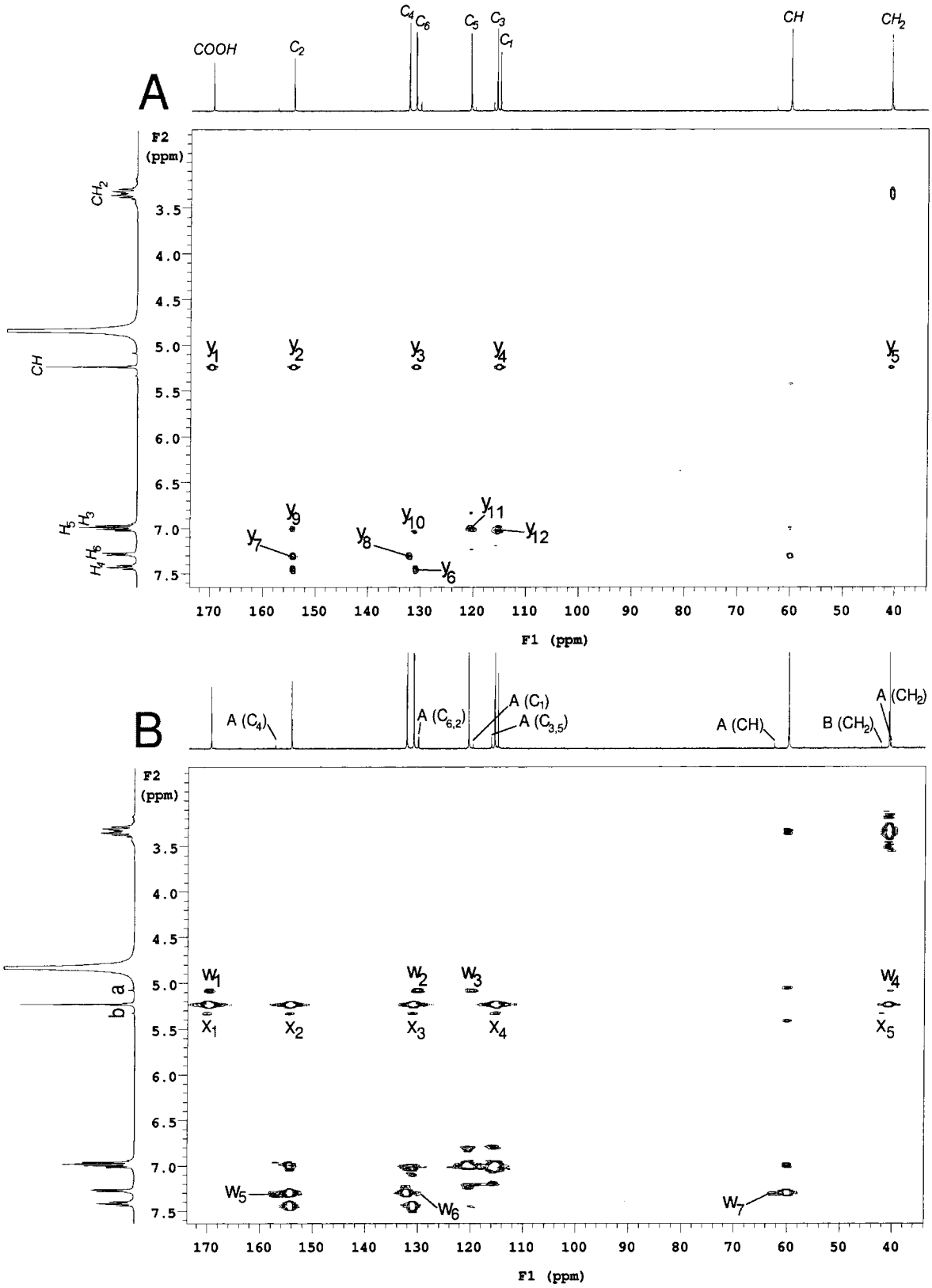

Figure 4. HMBC 2D spectra of a commercial sample of EDDHA recorded at 400 MHz and 298 K: (A) greater threshold; (B) lesser threshold.

benzylic proton (cp's x_1-x_5, Figure 4B) indicates that B should bear an −OH at one of the two ortho positions, the other one being unsubstituted. As this pattern is identical to that found in **1** (cp's y_1-y_5, Figure 4A), the moiety comprising the benzylic proton should resemble that in **1**. Second, from an NOE difference experiment, a spatial proximity is evidenced between the benzylic CH and a CH_2 group at 3.45 ppm (Figure 5D). Third, by the same NOE experiment, an enhancement is detected also at the ortho proton at 7.45 ppm, more deshielded than the corresponding H_6 in **1**. On the basis of these findings we tentatively propose the structure

Table 1. ^{1}H and ^{13}C NMR Signal Assignments for Compounds 1, 2, and 4 at Acidic pH[a]

atom		1		2[b]		4[c]	
$C\underline{H}_2$	($\underline{C}H_2$)	3.33	(40.8)	3.38[d]	(40.5)	3.45[d]	(42.3)
$C\underline{H}$	($\underline{C}H$)	5.23/5.21[e]	(60.0/nd)[e]	5.08/5.06[e,f]	(62.6/nd)[e,f]	5.32	(60.0)
	($\underline{C}OOH$)		(170.0)		(170.0)		(170.0)[g]
	($\underline{C}_1$)		(115.5)		(119.8)		(115.5)[g]
H_2	(C_2)		(154.4)	7.30[d]	(130.6)		(154.4)[g]
H_3	(C_3)	7.00	(116.0)	6.97[d]	(116.4)		(115.5)[g]
H_4	(C_4)	7.46	(132.2)		(157.5)	7.45[d]	(131.2)[g]
H_5	(C_5)	7.03	(120.5)	6.97[d]	(116.4)	nd	(nd)
H_6	(C_6)	7.30	(131.2)	7.30[d]	(130.6)	7.45[d]	(131.2)[g]

[a] In units of ppm at 9.4 T (^{1}H, 400 MHz; ^{13}C, 100.59 MHz) unless otherwise stated. [b] Moiety containing the *p*-hydroxyphenyl group. [c] Central disubstituted hydroxyphenyl moiety (−OH is bonded to C_2). [d] From NOE experiments at 200 MHz. [e] Meso/racemic. nd, not detected. [f] Assignment interchangeable. [g] By comparison with **1**.

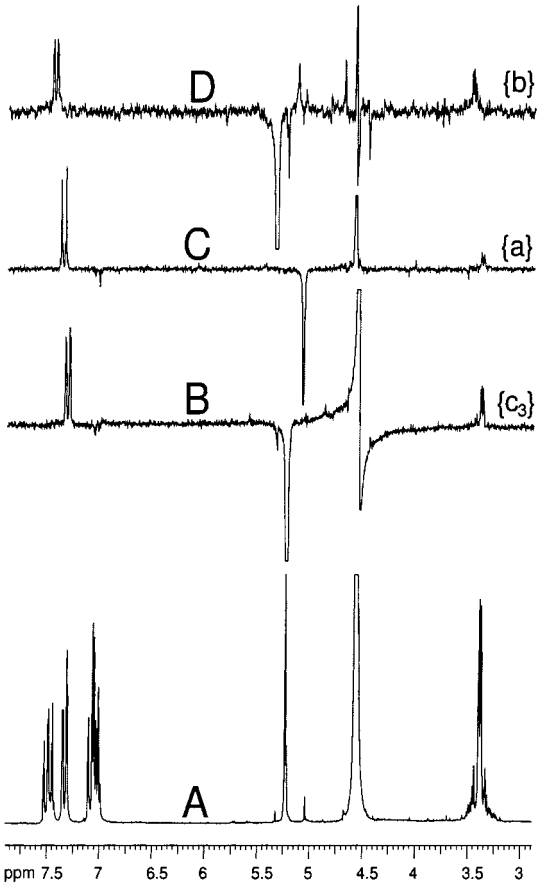

Figure 5. NOE difference (200 MHz, 308 K) experiments of a commercial sample of EDDHA: (A) reference ^{1}H spectrum; NOE difference spectra obtained by irradiating (B) the c_3 signal, (C) the *a* signal, and (D) the *b* signal.

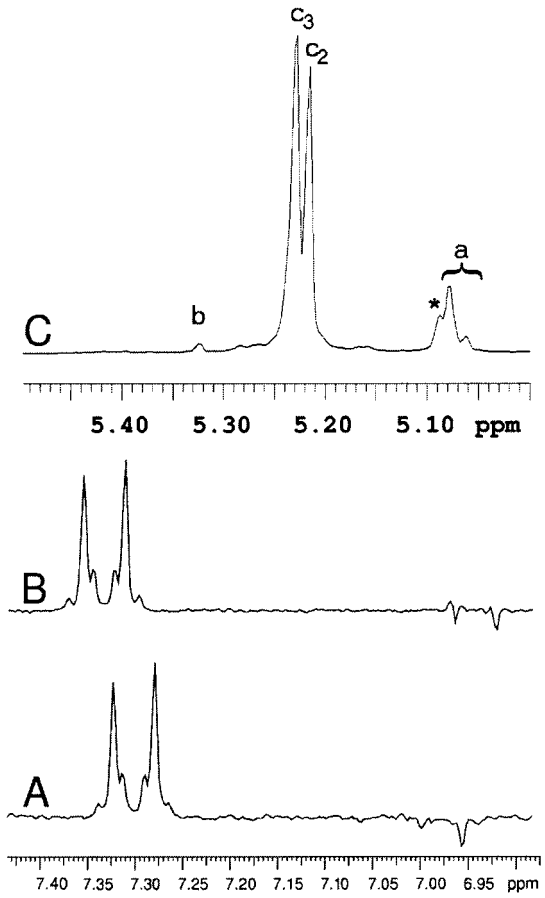

Figure 6. NOE difference signals (200 MHz, 308 K) generated at the phenyl protons by irradiating (A) the *a* ^{1}H signal of a commercial sample of EDDHA and (B) the benzylic CH of pure *p*-hydroxyphenylglycine. (C) Expansion of the CH region of the 200 MHz (308 K) ^{1}H spectrum of EDDHA extracted from a commercial fertilizer containing the EDDHA/Fe^{3+} chelate. The asterisk indicates an unidentified minor compound. The peak labels can be compared with those in Figure 2A.

of B as **4** (Figure 1). In **4** both benzylic protons bonded to the symmetrical central hydroxyphenyl moiety and both aromatic protons ortho to each of them are equivalent, in agreement with our detection of a single resonance for each type of proton. Moreover, the latter protons are indeed calculated to be more deshielded than H_6 in **1**, due to the presence of two $-CH(^+NH_2R)$-COOH electron-withdrawing substituents at the phenyl ring (at acidic pH) instead of only one as in **1**. Thus, cp's x_1-x_5 (Figure 4B) are due to long-range couplings between each of the benzylic methenes bonded to the central moiety of **4** and the connected COOH (x_1, 170.0 ppm), C_2 (x_2, 154.4 ppm), C_4 (or C_6, x_3, 131.2 ppm), C_1 (or C_3, x_4, 115.5 ppm) and the CH_2's (x_5, 42.3 ppm and peak f_3 in Figure 2B). The NMR assignments of compounds **1**, **2**, and **4** are summarized in Table 1. The presence of compound **4** can be explained by recalling that, to the best of our knowledge, the most common method of industrial synthesis of *o,o*-EDDHA proceeds through a Mannich-type reaction between phenol and a ethylenediamine/glyoxylic acid adduct at basic pH (*3*, *17*, *18*). In these conditions a molecule of phenol could undergo a double electrophilic attack by two molecules of the adduct, thus giving rise to the central, doubly substituted, hydroxyphenyl moiety of **4**. The reaction would then normally incorporate the other two phenol molecules. Interestingly, a related disubstituted compound, **5** (Figure 1), was detected as an impurity during

the synthesis of *o*-hydroxymandelic acid which proceeds in conditions similar to those above by direct electrophilic attack of glyoxylic acid on phenol under basic conditions (*19*).

The ^{1}H NMR spectrum of a sample of EDDHA extracted from a commercial fertilizer (see Material and Methods) is reported in Figure 6C. It is apparent that the same impurities as those found in commercial EDDHA are present, together with another unknown minor compound. Differently from Figure 2A the meso/racemic ratio of **1** is now close to 55:45, whereas that of **2** is close to 85:15 (in either order); however, no other stereoisomers of **4** are detected, possibly because of an accidental isocrony of the CH signals. Integration data of the ^{1}H signals c_2+c_3, *a*, and *b* are, respectively, 87, 12, and 1%. It is evident that the purity of *o*,*o*-EDDHA is well below 100%, which is not surprising because the industrial synthesis of EDDHA/Fe^{3+} chelates from *o*,*o*-EDDHA excludes a purification step (*3*, *4*).

In this work we have shown that NMR spectroscopy can be used as a tool to detect impurities in the organic part of the *o*,*o*-EDDHA/Fe^{3+} chelate contained in commercial fertilizers. Although we are aware that the reported example might not be representative of commercial products, we believe that the low purity of *o*,*o*-EDDHA is mainly due to the way EDDHA is produced and is thus unlikely to be much greater in other samples. Further work is in progress to test other samples based on EDDHA as well as on other ligands allowed by European law.

ABBREVIATIONS USED

HPLC, high-pressure liquid chromatography; EDDHA, ethylenediaminedi(hydroxyphenyl)acetic acid; NMR, nuclear magnetic resonance; FID, free induction decay; cp, cross-peak; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; NOE, nuclear Overhauser enhancement.

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