JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Chromatographic Determination of Fe Chelated by Ethylenediamine-*N*-(*o*-hydroxyphenylacetic)-*N*′-(*p*-hydroxyphenylacetic) Acid in Commercial EDDHA/Fe^{3+} Fertilizers

SONIA GARCÍA-MARCO, ANA TORREBLANCA, AND JUAN J. LUCENA*

Sección de Química Agrícola, Universidad Autónoma de Madrid, 28049 Madrid, Spain

EDDHA/Fe^{3+} chelates are the most common fertilizers used to solve Fe chlorosis in established crops. Commercial products contain two regioisomers, ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid (*o*,*o*-EDDHA)/Fe^{3+} and ethylenediamine-*N*-(*o*-hydroxyphenylacetic)-*N*′-(*p*-hydroxyphenylacetic) acid (*o*,*p*-EDDHA)/Fe^{3+}. Although several chromatographic methods exist for the determination of Fe^{3+} chelated by the *o*,*o*-EDDHA isomer, no method has been described for the quantification of Fe^{3+} chelated by *o*,*p*-EDDHA. In this work, factors that affect the behavior of *o*,*p*-EDDHA/Fe^{3+} in ion pair chromatography are reviewed: pH, ion pair reagent, and organic modifier. The best chromatographic performance was obtained with an aqueous mobile phase at pH 6.0 containing 35% acetonitrile and 5 mM tetrabutylammonium hydroxide under isocratic elution conditions. This method was applied to the quantification of commercial samples.

KEYWORDS: EDDHA/Fe^{3+}; Fe^{3+} chelates; fertilizers; HPLC; *o*,*o*-EDDHA/Fe^{3+}; *o*,*p*-EDDHA/Fe^{3+}

INTRODUCTION

Iron (Fe) deficiency chlorosis is a widespread agricultural problem that affects the development and decreases the yield of many susceptible crops growing on calcareous and alkaline soils (*1*, *2*). Nowadays, fertilization with synthetic Fe^{3+} chelates is the most common agricultural practice (*3*), and ethylenediamine-*N*,*N*′-bis(*o*-hydroxyphenylacetic) acid (*o*,*o*-EDDHA) is the best known member of these products used to relieve this problem. This compound (**Figure 1**, structure **3**) has two phenol groups on a diaminocarboxylic acid backbone and is able to form soluble Fe^{3+} complexes of relatively low reactivity in soils and high stability in either neutral or alkaline solutions (*4*) that can transport Fe to the plant rhizosphere.

Industrial synthesis of EDDHA/Fe^{3+} commercial products is carried out by addition of inorganic Fe salts to unpurified EDDHA immediately after its synthesis (*5*). This synthesis produces a mixture of three regioisomeric compounds, *o*,*o*-EDDHA, *o*,*p*-EDDHA, and *p*,*p*-EDDHA, and polycondensation products (*6*−*8*) in variable amounts (**Figure 1**). After addition of Fe salts to the chelating agent, the *o*,*o*-EDDHA/Fe^{3+} and *o*,*p*-EDDHA/Fe^{3+} chelates are formed, but *p*,*p*-EDDHA, since the two *p*-hydroxyphenyl groups are sterically impeded to bind Fe^{3+}, does not form the Fe^{3+} complex (*4*, *9*).

Ethylenediamine-*N*-(*o*-hydroxyphenylacetic)-*N*′-(*p*-hydroxyphenylacetic) acid (*o*,*p*-EDDHA), together with EDDHSA, has been included as an authorized chelating agent in the present European regulation on fertilizers (EC Regulation No. 2003/2003 and its modification, No. 2076/2004), due to the good agronomic behavior of commercial products containing both *o*,*o*-EDDHA and *o*,*p*-EDDHA. The effectiveness of its Fe^{3+} complex as an Fe source in plant nutrition has recently been studied, and results suggest that the combination of the long-lasting effect of *o*,*o*-EDDHA/Fe^{3+} and the fast action of *o*,*p*-EDDHA/Fe^{3+} is adequate to solve Fe chlorosis and to maintain a correct Fe nutrition in plants (*10*).

According to the current EC regulation, synthetic commercial

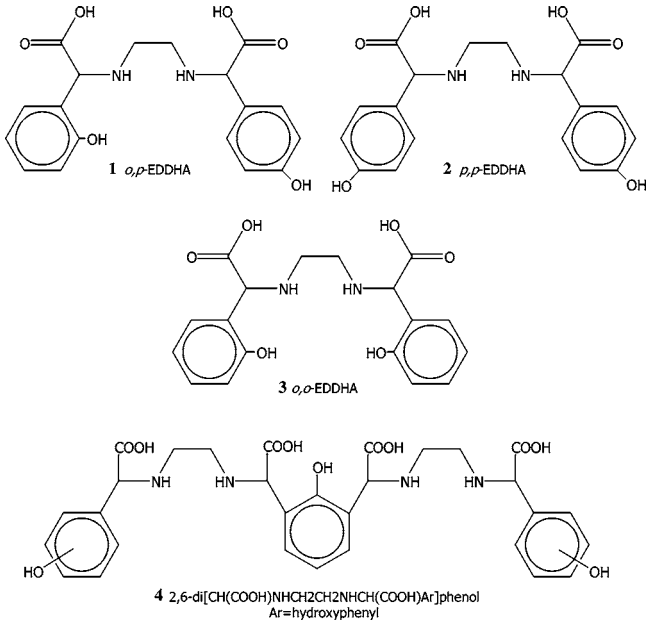

Figure 1. Structures of the chelating agents described in the text.

* To whom correspondence should be addressed. Phone: +34-91-4973968. Fax: +34-91-4973825. E-mail: juanjose.lucena@uam.es.

10.1021/jf051745x CCC: $33.50
Published on Web 01/20/2006

Table 1. ^{1}H and ^{13}C Signal Assignments (ppm) for the *o,p*-EDDHA 2 Standard in D_2O/Na_2CO_3 (pH > 10)[a]

proton	δH	carbon	δC
CH (aromatics)	7.10–6.98 (m, 4H), 6.55–6.69 (m, 4H)	CH (aromatics)	128.05, 127.98, 127.40, 127.31, 116.45, 116.07
		ipso C (aromatics)	160.85, 157.53, 124.83, 121.98
CHNH	4.33 (s, 1H), 4.38 (s, 1H), 3.91 (s, 1H), 3.94 (s, 1H)	COOH	178.11, 175.86, 175.75
$NHCH_2CH_2NH$	2.59–2.66 (m, 4H)	CHNH	62.52, 65.12
		$NHCH_2CH_2NH$	43.29, 43.62

[a] m = multiplet, and s = singlet.

Fe^{3+} chelates should be products obtained by chemical combination of the Fe^{3+} ion with one or several permitted chelating agents and must contain a minimum of 5% (w/w) of water-soluble iron, of which the chelated fraction is at least 80%. Information about the water-soluble Fe content, the Fe chelated fraction, and the concentration of Fe chelated by the declared chelating agents, when higher than 2%, must be included in the label.

There is no analytical method for the quantification of Fe chelated by *o,p*-EDDHA in fertilizers containing EDDHA/Fe^{3+}; thereby, the EC has requested to develop it to be able to control their compliance with the present fertilizer regulation (EC Standardization Mandate 335, 2003). Many chromatographic techniques have been applied for the analysis of this type of fertilizer (*11*−*16*), but HPLC has proven to be the most useful technique in this field (*17*).

An ion pair chromatographic method previously published by us (*16*) has recently been considered as a European standard for the determination of *o,o*-EDDHA/Fe^{3+} and *o,o*-EDDHMA/Fe^{3+} in commercial fertilizers. Also we have reported a gradient ion pair HPLC method for the quantification of Fe chelated by an EDDHSA chelating agent (*18*). However, when we applied the ion pair chromatographic method described by Lucena et al. (*16*), to confirm the presence of *o,p*-EDDHA/Fe^{3+} (*8*), an optimum chromatographic elution was not achieved. *o,p*-EDDHA/Fe^{3+} presents only five bonds between the Fe and the chelating agent, which represents a structural difference with respect to the aforementioned chelates, which have six bonds. This makes the research for an adequate method for its determination a new challenge for our research group.

As a part of our ongoing work focused on determining the chelated Fe content in synthetic Fe^{3+} fertilizers (*6*, *7*, *16*−*19*), here we report an ion pair HPLC method that allows the determination of *o,p*-EDDHA/Fe^{3+} in EDDHA/Fe^{3+} commercial fertilizers. To evaluate the applicability of the proposed method, in addition to the chromatographic behavior, the detection limits, linear dynamic detection range, and repeatability were studied through the separation of several commercial EDDHA/Fe^{3+} fertilizers.

MATERIALS AND METHODS

Reagents and Standards. Analytical reagent grade NaOH, $Fe(NO_3)_3 \cdot 9H_2O$ (Merck, Mollet del Vallés, Spain), HCl (Panreac, Castellar del Vallés, Spain), acetonitrile, HPLC grade purity (Riedel-de Haën, Sigma-Aldrich, Madrid, Spain), and tetrabutylammonium hydroxide, 40% solution in water (Sigma-Aldrich, Madrid, Spain), were used. All aqueous solutions were prepared with water, type I grade (*20*), using a Milli-Q element water purification system (Millipore, Madrid, Spain).

The *o,o*-EDDHA standard (acid form, 98% purity) was obtained from LGC Promochem (Wesel, Germany). Two different *o,p*-EDDHA standards (acid form) were kindly provided by Syngenta Crop Protection (Basel, Swistzerland) and used in this work. The study of the chromatographic behavior of *o,p*-EDDHA/Fe^{3+} was performed using the *o,p*-EDDHA standard (*o,p*-EDDHA 1) recently described and characterized in ref *9*. Quantification of *o,p*-EDDHA/Fe^{3+} in EDDHA/Fe^{3+} commercial samples was done using a different *o,p*-EDDHA standard (*o,p*-EDDHA 2) characterized by spectroscopic techniques. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 200-AC (200.13 MHz for ^{1}H and 50.03 MHz for ^{13}C) spectrometer. **Table 1** shows chemical shifts (δ) given in parts per million relative to the peak for the corresponding deuterated solvent (D_2O/Na_2CO_3, pH > 10). NMR spectra showed that two stereoisomers could be distinguished in the *o,p*-EDDHA 2 standard compared to one in the *o,p*-EDDHA 1 standard (see Figures 1S−6S, Supporting Information). MS spectra were obtaining using an Esquire-LC system with electrospray as the ionization source operated in both positive and negative modes. Anal. Calcd for $C_{18}H_{20}N_2O_6$: C, 59.94; H, 5.55; N, 7.77. Found: C, 51.54; H, 5.38; N, 6.94. Mass spectrum (ESI, −75.8 eV, negative polarity, *m/z*): $[M - H]^-$ 359.0 (100). Mass spectrum (ESI, 75.8 eV, positive polarity, *m/z*): $[M + H]^+$ 361.0 (38), $[M + Na]^+$ 383.0 (100). All spectra were kindly provided by Dr. Sierra's group.

The complexing capacity of the *o,p*-EDDHA 2 standard was determined following both the photometric methodology described in ref *4* and an HPLC titration using the ion pair chromatographic method described in this paper. In brief, in the photometric method, chelating agent solutions ($\sim 1 \times 10^{-4}$ M) buffered at pH 6 were titrated with an Fe^{3+} standard solution. The formation of the complex was followed measuring the increase of absorbance at 480 nm with a white light spectrode connected to a Metrohm 721 potentiometer. A Metrohm 719 potentiometer, fitted with a combined pH glass electrode, was used to maintain pH 6 during the titration with 0.200 M NaOH. The end point of the titrations was calculated by the linear segments' intersection (*21*). In the HPLC titration, solutions at pH 7, with a concentration of around 4.5 mM for the *o,p*-EDDHA 2 standard and different Fe(III) concentrations (0.00, 0.07, 0.11, 0.14, 0.18, 0.21, 0.25, 0.29, 0.36, 0.43, 0.47, 0.50, 0.54, 0.57, 0.61 0.64, 0.72, 0.82, 0.90, 0.97, 1.04, and 1.11 mM), were prepared. Soluble Fe was assessed by atomic absorption spectrophotometry, and Fe chelated by *o,p*-EDDHA was measured by HPLC. The titration end point was calculated by the linear segments' intersection as in photometric titration.

To prepare the *o,o*-EDDHA/Fe^{3+} and *o,p*-EDDHA/Fe^{3+} standard solutions, ligands were dissolved in NaOH (ligand:NaOH = 1:3 molar ratio). After complete dissolution, a volume of $Fe(NO_3)_3 \cdot 9H_2O$, whose concentration was calculated to be 5% in excess of the molar amount of the ligand and previously assessed by atomic absorption spectrophotometry, was added while the solution pH was kept in the range 6−8 with NaOH or HCl. The solution pH was adjusted to 7.0 at the end of Fe addition. The solution was left to stand overnight to allow excess Fe to precipitate as oxides. The final solutions, with Fe concentrations of 1.79 and 1.54 $mmol \cdot L^{-1}$, respectively, were filtered through a 0.45 μm cellulose membrane and made up to volume with water. These solutions were diluted with water as required. Light exposure of all chelate solutions was avoided during their preparation and storage because of the potential photodecomposition of the chelates (*22*).

Equipment. HPLC analyses were run at room temperature under isocratic elution conditions using a Waters 2695 series Alliance quaternary pump (Waters, Madrid, Spain) equipped with a photodiode array (PDA) detector, model 996. The LC column was a 150 × 3.9 mm i.d. Symmetry C-18 packed with 5 μm particles (Waters, Madrid, Spain). The chromatograms and spectra were collected and processed using the Empower chromatography data system.

Chromatographic Behavior of *o, p*-EDDHA/Fe^{3+}. To establish optimum chromatographic conditions and, on the basis of the parameters

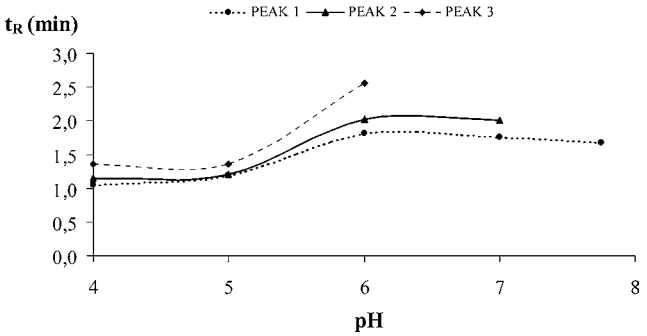

Figure 2. Influence of the pH on the retention time of *o,p*-EDDHA/Fe^{3+} (4×10^{-4} M): column, Symmetry Waters C-18; eluent, 0.03 M TBA^+–30% acetonitrile (variable pH); flow rate, 1.5 mL/min; injection volume, 20 μL; detection wavelength, 480 nm.

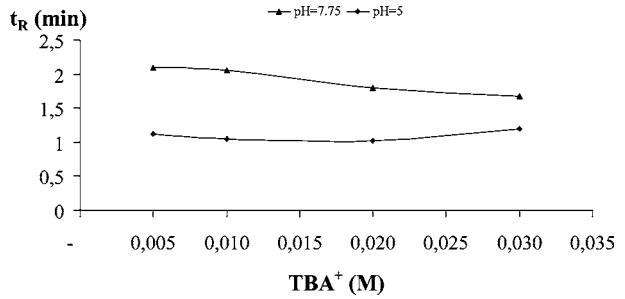

Figure 3. Influence of the TBA^+ concentration on the retention time of *o,p*-EDDHA/Fe^{3+} (4×10^{-4} M) at two different pH values, 5 and 7.75, where FeHL° and FeL^- are, respectively, the predominant species: column, Symmetry Waters C-18; eluent, TBA^+ (variable concentrations)–30% acetonitrile (pH 5.0 or 7.75); flow rate, 1.5 mL/min; injection volume, 20 μL; detection wavelength, 480 nm.

of the method described by Lucena et al. (*16*) (30% acetonitrile, 0.03 M TBA^+, and pH 6.0), factors that affect the retention behavior, such as the pH and concentration of TBA^+ (ion pair reagent) and acetonitrile (organic modifier) of the eluent, were studied.

Fe chelate fertilizers, with ligands having a structure analogous to that of *o,o*-EDDHA, present maximum absorbance (λ_{max}) at approximately 280 and 480 nm, due to the presence of the phenolate group or the Fe^{3+}−phenolate bond, respectively (*14*, *19*). To obtain the highest detectability and the least interference, the wavelength was best set at 480 nm in our studies.

Effect of the pH. The effect of the eluent pH was studied, maintaining the percentage of acetonitrile and the concentration of TBA^+ constant in the isocratic method elaborated by Lucena and co-workers (*16*). pH values of 4.0, 5.0, 6.0, 7.0, and 7.75 were tested for the *o,p*-EDDHA 1 standard and commercial samples.

Effect of the Ion Pair Reagent (TBA^+). The effect of the TBA^+ concentration in the mobile phase was investigated, maintaining the percentage of acetonitrile (30%) and pH (both 5 and 7.75) constant. TBA^+ concentrations of 0.005, 0.01, 0.02, and 0.03 M were tested.

Effect of the Organic Modifier (Acetonitrile). Acetonitrile was selected as the organic modifier throughout all the studies. Different percentages of acetonitrile in the eluent (20%, 25%, 28%, 30%, and 35%) were studied to modify the retention time of *o,p*-EDDHA/Fe^{3+} and separate it from impurities present in commercial samples. The pH and TBA^+ concentration (5 mM) were maintained constant in the eluent.

Analytical Conditions and Analysis of Commercial *o,p*-EDDHA/Fe^{3+} Fertilizers. An isocratic method was used. The mobile phase consisted of 35% acetonitrile in a 0.005 M TBA^+ aqueous solution at pH 6.0 at a flow rate of 1.5 mL·min^{-1}. The injection volume was 20 μL. Spectra were recorded every second between 200 and 600 nm, and chromatograms were acquired at 480 nm. Quantitative analysis of both *o,p*-EDDHA/Fe^{3+} and *o,o*-EDDHA/Fe^{3+} present in EDDHA/Fe^{3+} commercial samples was carried out by performing calibration in the 0.015−1.54 or 0.090−1.79 mM range, respectively.

Four solid commercial EDDHA/Fe^{3+} fertilizers were analyzed. A solution, of each commercial product, containing approximately 1.25 g·L^{-1} product was prepared by dissolving the formulation in water and filtering it through a 0.45 μm cellulose membrane prior to HPLC analysis. These commercial samples were prepared and analyzed in duplicate.

RESULTS AND DISCUSSION

Chromatographic Behavior of *o,p*-EDDHA/Fe^{3+}. *Effect of the pH.* First, the effect of the eluent pH was studied, maintaining all other parameters constant. The retention behavior of the Fe^{3+} complex was influenced by the pH, as shown in **Figure 2** for a commercial sample. One, two, or three chromatographic peaks could be distinguished at the pH tested. The general trend is in good agreement with the behavior of weak acids in ion pair chromatography as described in ref *23*.

In ion pair chromatography, with the sorption of the ion pair reagent on the stationary phase, dynamic ion exchange sites are formed and the higher the charge of the analyte ion, the greater the interaction between the analyte and these ion exchange sites (*24*). At low pH the formation of the FeLH° species corresponds to a low retention by the ion pair reagent. As the pH is increased, a greater fraction of the chelate exists in the negatively charged FeL^- species, which strongly interacts with the positively charged ion pair reagent. This results in a net increase in retention time as the pH is increased. The pK_a of FeLH°, determined spectrophotometrically after base titration, reported in ref *9* is 6.30, but it was calculated for a standard containing only one isomer. It is possible that different isomers present slightly different pK_a values, so the inflection point of the curve can be different for each one of the isomers. This will explain the multiple peaks obtained for different isomers at the pH near the pK_a. Since the maximum retention time is near pH 6, this pH has been considered the most appropriate for the elution in the conditions considered.

Effect of the Ion Pair Reagent (TBA^+). **Figure 3** shows the retention behavior of *o,p*-EDDHA/Fe^{3+} regarding the concentration of TBA^+ added to the eluent at both pH 5 (FeLH°) and pH 7.75 (FeL^-).

At pH 7.75 (over the pK_a of the FeLH°), t_R slightly decreased with increasing amounts of the ion pair reagent. This is in good agreement with previous findings for the EDTA/Fe^{3+}, *o,o*-EDDHA/Fe^{3+}, *o,o*-EDDHMA/Fe^{3+}, and EDDHSA/Fe^{3+} chelates with the same ion pair reagent (*15*, *16*, *18*). In ion pair chromatography, according to the extended thermodynamic model recently described by Cecchi and co-workers (*25*), the retention mechanism of an analyte depends not exclusively on electrostatic interactions, but also on ion pair formation in both the stationary and mobile phases and on adsorption competitions. Adsorption of TBA^+ on the stationary phase offers dynamic ion exchange sites. Hence, retention of oppositely charged complexes is directly related to the surface charge arising from the adsorption of the ion pair reagent, and increases with increasing amounts of ion pair reagent adsorbed until the surface is saturated. At higher concentrations of TBA^+, interaction between the analyte and TBA^+ in the eluent is stronger than that between the analyte and TBA^+ adsorbed in the stationary phase and the retention time decreases (*25*). According to this model, the retention plot of an analyte with a charge opposite that of the ion pair reagent should show a foldover; the analyte retention should first increase with increasing TBA^+ concentration, but TBA^+ concentrations lower than those reported in **Figure 3** have not been tested.

As the TBA^+ concentration increased in the eluent, the chromatographic peak corresponding to the *o,p*-EDDHA/Fe^{3+} chelate became broader and a tail appeared. This fact made difficult its determination, and for this reason, a relatively low concentration of TBA^+ was preferred.

At pH 5, FeLH° is the predominant species ($pK_a = 6.30$ according to ref *9*), and results show that its retention behavior did not apparently change with increasing amounts of TBA^+ in the eluent. The retention time (t_R) remained low at ~1.1 min.

According to the extended thermodynamic approach (*25, 26*) for neutral analytes, it is expected that adsorption competitions between the analyte and ion pair reagent are more important than ion pair formation, because strong electrostatic interactions are not operating. The course of the dependence of neutral molecule retention on the ion pair reagent concentration in the eluent can be very complex and variable (increases or decreases of retention are possible) since it depends on several factors. Their balance may also result in an apparent lack of retention dependence on the ion pair reagent concentration in the eluent (*26*).

If commercial samples were analyzed using these eluents, several chromatographic peaks appeared close to that of *o,p*-EDDHA/Fe^{3+} that may interfere with its determination.

Effect of the Organic Modifier (Acetonitrile). An organic modifier is needed to change the polarity in the eluent and compete with the TBA^+ in the adsorption equilibria to control the elution rate. The retention time of *o,p*-EDDHA/Fe^{3+} decreased as the acetonitrile content increased.

In commercial samples both Fe^{3+} chelates *o,p*-EDDHA/Fe^{3+} and *o,o*-EDDHA/Fe^{3+} are present. Low percentages of acetonitrile increased, until 30 min, the retention time of the chromatographic peaks corresponding to *o,o*-EDDHA/Fe^{3+} (racemic and meso isomers); therefore, the use of 20% and 25% acetonitrile should be avoided in an isocratic separation. Gradient separations with increasing acetonitrile percentage (from 15% to 40%) were also tested, but the results were not satisfactory owing to the large tail of the *o,p*-EDDHA/Fe^{3+} chromatographic peak. Subsequently, an isocratic separation with a high concentration of acetonitrile (35%) was chosen.

Chromatographic Method for the Determination of *o,p*-EDDHA/Fe^{3+} in Commercial EDDHA/Fe^{3+} Fertilizers: Description and Quality Parameters. According to the studied effects of the pH, TBA^+, and acetonitrile, an isocratic method with a mobile phase made up of 35% acetonitrile in a 0.005 M TBA^+ aqueous solution at pH 6.0 should be adequate for the determination of *o,p*-EDDHA/Fe^{3+}. This method simplifies routine analysis of commercial Fe^{3+} chelates, because it is quite similar to the chromatographic method recently described for EDDHSA/Fe^{3+} determination (*18*). This pH was adequate for the chromatographic determination of *o,p*-EDDHA/Fe^{3+} and preferred over pH 7.75 because of the possible silica hydroxylation in the column.

The synthetic *o,p*-EDDHA 2 standard was characterized prior to its use. The ^1H NMR spectrum shows two couples of lines at 3.9 (3.91 and 3.94) and 4.3 (4.33 and 4.38) ppm that can be assigned to the benzylic protons of the *ortho* and *para* systems, respectively. The splitting of these signals suggests the presence of two *o,p*-EDDHA diastereoisomers. The titrimetic purity of this standard, obtained by photometric titration with Fe^{3+} solution, was 75.4 ± 1.4% determined using the method (*21*) described in the Materials and Methods. This purity was compared with that obtained by titration using the HPLC method described above (73.0%).

Once this compound was characterized, we used it as a standard in the analysis of commercial EDDHA/Fe^{3+} fertilizers. With these chromatographic conditions, the *o,p*-EDDHA/Fe^{3+} possible stereoisomers coelute in one peak.

This HPLC method gave rise to a linear calibration for *o,p*-EDDHA/Fe^{3+} in the range of 0.015−1.54 mM Fe with a correlation coefficient r^2 of 0.9997. The concentrations used were 0.015, 0.077, 0.154, 0.384, 0.769, and 1.54 mM *o,p*-EDDHA/Fe^{3+}, and the calibration line obtained was area = 1025094(concentration) − 12169, where the concentrations are presented in millimolar units and the area is presented in area units. For the slope, the standard deviation was $S_b = 8703$ area units·mM^{-1}, and for the intercept, $S_a = 6290$ area units. The confidence interval at 95% for the slope was 1025094 ± 24195 area units·mM^{-1}, and that for the intercept was −12168 ± 17486 area units. The detection limit ($3S_B/b$, where S_B is the standard deviation of the blank and *b* the slope) was 0.033 mM. This implies that the determination could be successfully done in commercial samples containing as low as 0.15% Fe as *o,p*-EDDHA/Fe^{3+}, when they are prepared as indicated in the Materials and Methods. The repeatability in the quantification of *o,p*-EDDHA/Fe^{3+} was also determined by analyzing, under the same chromatographic conditions and in a short period of time, the same *o,p*-EDDHA/Fe^{3+} standard injected eight times, and a relative standard deviation of the repeatability (RSD_r) of 1.1% was obtained.

The separation achieved with this isocratic method is shown in **Figure 4**, where chromatograms of an EDDHA/Fe^{3+} commercial sample (**A**) and *o,o*-EDDHA/Fe^{3+} (**B**) and *o,p*-EDDHA/Fe^{3+} (**C**) standards at 480 nm are presented. The chromatogram of the commercial sample shows three chromatographic peaks at 1.15, 2.11, and 2.60 min (t_R). On comparison of this one with the chromatograms of the standards, the first one can be assigned to *o,p*-EDDHA/Fe^{3+} and the next two peaks to *o,o*-EDDHA/Fe^{3+} diastereoisomers (racemic and meso, repectively). The *o,p*-EDDHA/Fe^{3+} compound has an absorbance spectrum in the UV−vis range (**Figure 4**) similar to that of *o,o*-EDDHA/Fe^{3+}. It exhibits peaks at around 204, 274, and 492 nm. The broad band of absorption found in the visible range is typical of Fe^{3+}−phenol binding, and in this case, its relative height is almost half that of the bands for the *o,o*-EDDHA/Fe^{3+} diastereoisomers, consistent with a structure in which only one Fe^{3+}−phenolate is present. This result agrees with the molar absorptivity (ϵ) of *o,p*-EDDHA/Fe^{3+} at 480 nm obtained in previous works by Gómez-Gallego et al. (*8*) and Yunta et al. (*9*); this value (2044 ± 34) was almost half of the ϵ observed for *o,o*-EDDHA/Fe^{3+}.

For the analysis of commercial chelates the quantification of total Fe chelated by *o,p*-EDDHA is required, but for research and development purposes it is preferred to distinguish the diastereoisomers of the chelate. Since using ion pair chromatography the peaks are separated by the relative stability of the molecules (*16*), in the case of *o,p*-EDDHA/Fe^{3+} two chromatogram peaks should be found, one corresponding to the more favored forms of the *RR* and *SS* mixture and the other corresponding to those of the *RS* and *SR* mixture. In the method presented in this paper only one peak is obtained, so other methods should be prepared to distinguish the diastereoisomers.

Analysis of Commercial *o,p*-EDDHA/Fe^{3+} Fertilizers. Four EDDHA/Fe^{3+} commercial fertilizers were analyzed, and the percentage of Fe chelated by *o,p*-EDDHA and *o,o*-EDDHA was calculated by estimation of the peak areas at 480 nm wavelength in comparison with those of the standard solutions (**Table 2**).

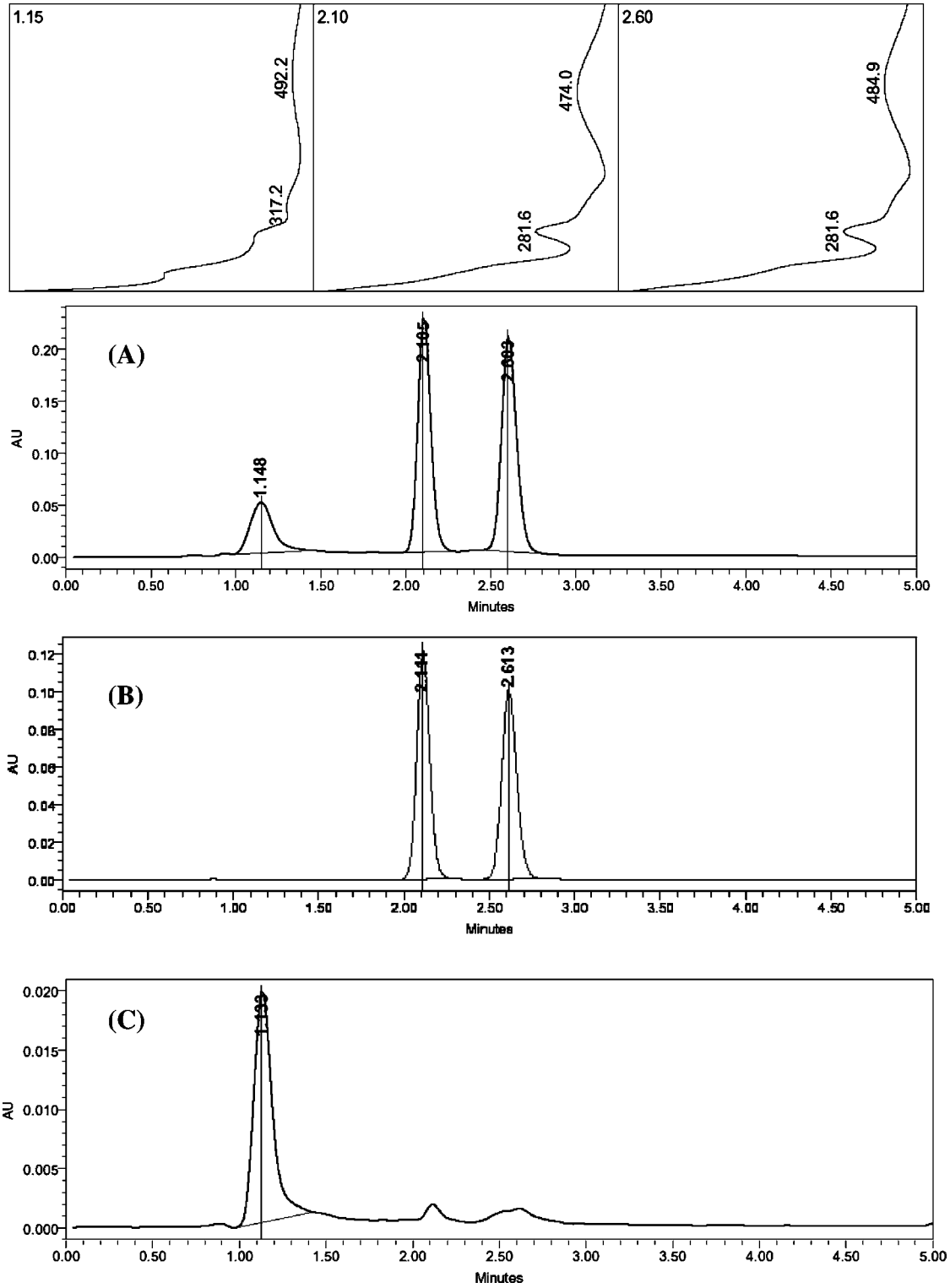

Figure 4. Chromatograms and UV–vis spectra at 480 nm for (**A**) a commercial EDDHA/Fe^{3+} fertilizer, (**B**) an *o,o*-EDDHA/Fe^{3+} standard, and (**C**) an *o,p*-EDDHA/Fe^{3+} standard: column, Symmetry Waters C-18; eluent, 0.005 M TBA^+–35% acetonitrile (pH 6.0); flow rate, 1.5 mL/min; injection volume, 20 μL; detection wavelength, 480 nm.

The chelated Fe:water-soluble Fe ratio in the commercial samples is 76%, 85%, 98%, and 82%. For the third commercial sample, most of the Fe can be explained as Fe chelated by *o,p*-EDDHA and *o,o*-EDDHA chelating agents, while for the other three samples between 15% and 24% of the soluble Fe cannot be explained by these chelating agents. Cremonini et al. (*7*) detected the presence of a condensation product of the EDDHA ligand (**Figure 1**, structure **4**) that could form a 2:1 (Fe^{3+}:ligand) complex. Molecules of this type could chelate the rest of the Fe as occurs in EDDHSA/Fe^{3+} commercial samples with the presence of condensation products (*18*). In fact, if the gradient chromatographic method described for the determination of EDDHSA/Fe^{3+} (*18*) is used for EDDHA/Fe^{3+} samples, a fourth peak appears in addition to those of *o,p*-EDDHA and the two diastereoisomers of *o,o*-EDDHA that could correspond to molecules of this type. Their structure, stability, potential use

Table 2. Percentage of Chelated Fe by *o,p*-EDDHA and *o,o*-EDDHA (Racemic and Meso Isomers) and Percentage of Water-Soluble Fe [g of Fe·(100 g of product)$^{-1}$] (±SD, $N = 2$)

		o,o-EDDHA/Fe^{3+}			
sample	*o,p*-EDDHA/Fe^{3+}	racemic	meso	EDDHA/Fe^{3+}	water-soluble Fe
1	2.07 ± 0.02	1.53 ± 0.00	1.67 ± 0.00	5.26 ± 0.02	6.90 ± 0.13
2	1.37 ± 0.02	2.09 ± 0.01	2.25 ± 0.01	5.71 ± 0.04	6.68 ± 0.12
3	0.84 ± 0.00	2.48 ± 0.00	2.77 ± 0.00	6.09 ± 0.01	6.23 ± 0.03
4		2.36 ± 0.01	2.36 ± 0.01	4.72 ± 0.02	5.77 ± 0.01

as a source of Fe, and chromatographic determination are currently under investigation in our laboratories.

In conclusion, an isocratic ion pair HPLC method has been developed to determine the total Fe chelated by the *o,p*-EDDHA ligand in commercial EDDHA/Fe^{3+} samples. This method also permits the analysis of *o,o*-EDDHA/Fe^{3+}. All the soluble Fe in some of the commercial samples still cannot be explained by the two chelates. As much as 24% of the soluble Fe could be chelated by other molecules as dimers or polymers of these ligands.

ABBREVIATIONS USED

EDDHSA, ethylenediamine-*N*,*N*′-bis(2-hydroxy-5-sulfophenylacetic) acid; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; *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; TBA^+, tetrabutylammonium; t_R, retention time.

ACKNOWLEDGMENT

We thank Dr. M. Gómez-Gallego for NMR and MS analysis.

Supporting Information Available: Spectra of *o,p*-EDDHA 2 standard. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Mengel, K.; Kirby, E. A.; Kosegarten, H.; Appel, T. Iron. *Principles of Plant Nutrition*, 5th ed.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2001; pp 849.

(2) Chen, Y.; Barak, P. Iron nutrition of plants in calcareous soils. *Adv. Agron.* **1982**, *35*, 217−240.

(3) Lucena, J. Iron fertilizers in correcting iron deficiencias in plants. In *Iron Nutrition in Plants and Rhizospheric Microorganisms: Iron in Plants and Microbes*; Barton, L. L., Abadía, J., Eds.; Kluwer Academic Publishers: Dordrecht, The Netherlands, 2005; pp 103−128.

(4) Yunta, F.; García-Marco, S.; Lucena, J.; Gómez-Gallego, M.; Alcázar, R.; Sierra, M. A. Chelating agents related to ethylenediamine bis(2-hydroxyphenil)acetic acid (EDDHA): síntesis, 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.

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

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

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

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

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

(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*, in press.

(11) Hill-Cottingham, D. G. The paper chromatography of some complexones and their iron chelates. *J. Chromatogr.* **1962**, *8*, 261−264.

(12) Boxema, R. Analysis of iron chelates in commercial iron fertilizers by gel chromatography. *Pflanzenernaehr. Bodenkd.* **1979**, *142*, 824−835.

(13) Patch, M. G.; Simolo, K. S.; Carrano, C. J. The cobalt (III), chromium (III), copper (II) and manganese (III) complexes of ethylenebis(o-hydroxyphenyl) glycine: Models for metallotransferrins. *Inorg. Chem.* **1982**, *21*, 2972−2977.

(14) Barak, P.; Chen, Y. Determination of Fe-EDDHA in Soils and Fertilizers by Anion Exchange Chromatography. *Soil Sci. Soc. Am. J.* **1987**, *51*, 893−896.

(15) Deacon, M.; Smyth, M. R.; Tuinstra, L. Chromatographic separations of metal chelates present in commercial fertilizers. II. Development of an ion-pair chromatographic separation for the simultaneous determination of the Fe(III) chelates of EDTA, DTPA, HEEDTA, EDDHA and EDDHMA and the Cu(II), Zn-(II) and Mn(II) chelates of EDTA. *J. Chromatogr. A* **1994**, *659*, 349−357.

(16) Lucena, J.; Barak, P.; Hernández-Apaolaza, L. Isocratic ion-pair high-performance liquid chromatographic method for the determination of various iron(III) chelates. *J. Chromatogr. A* **1996**, *727*, 253−264.

(17) Hernádez-Apaolaza, L.; Álvarez-Fernández, A.; Lucena J. J. Chromatographic determination of commercial Fe(III)-chelates. *J. Plant Nutr.* **2000**, *23*, 2035−2045.

(18) 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.

(19) Álvarez-Fernández, A.; Cremonini, M. A.; Sierra, M. A.; Placucci, G.; Lucena, 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.

(20) Standard Specification for Reagent Water. In *Annual Book of ASTM Standards*; American Society for Testing Materials: West Conshohocken, PA, 1999; p 11.01.

(21) Willard, H. H.; Merritt, L. L., Jr.; Dean, J. A.; Settle, F. A., Jr. *Instrumental Methods of Analysis*; Wadsworth Publishing Co.: Belmont, CA, 1988; p 884.

(22) Hill-Cottingham, D. G. Photosensitivity of iron chelates. *Nature* **1955**, *175*, 347−348.

(23) Kong, R. C.; Sachok, B.; Deming, S. N. Combined effects of pH an surface-active-ion concentration in reversed-phase liquid chromatography. *J. Chromatogr.* **1980**, *199*, 307−316.

(24) Jen, J.-F.; Chen, C.-S. Determination of metal ions as EDTA complexes by reversed-phase ion-pair liquid chromatography. *Anal. Chim. Acta* **1992**, *270*, 55−61.

(25) Cecchi, T.; Pucciarelli, F.; Passamonti, P. Extended thermodinamic approach to ion interaction chromatography. *Anal. Chem.* **2001**, *73*, 2632−2639.

(26) Cecchi, T.; Pucciarelli, F.; Passamonti, P. Ion interaction chromatography of neutral molecules. Use of a potential approximation to obtain a simplified retention equation. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24* (3), 291−302.

Received for review July 20, 2005. Revised manuscript received November 23, 2005. Accepted December 2, 2005. This work was made possible by the financial support provided by Syngenta Crop Protection and Ministerio de Educación y Ciencia Project AGL 2004/07849/C02-01.

JF051745X