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Isocratic ion-pair high-performance liquid chromatographic method for the determination of various iron(III) chelates

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Abstract

The micronutrient iron, an essential element for plant growth, is usually added as fertilizer in chelated form. An isocratic ion-pair chromatographic method was developed to identify and determine the total amount of chelate in fertilizers. Iron(III) chelates containing ethylenediaminetetraacetic acid, diethylenetriaminepentaacetic acid, *trans*-1,2-cyclohexanediaminetetraacetic acid, ethylenediaminedi(*o*-hydroxyphenylacetic) acid (EDDHA), also known as N,N′-ethylenebis-2-(*o*-hydroxyphenyl)glycine, ethylenediaminedi(*o*-hydroxy-*p*-methylphenylacetic) acid, N,N′-bis-(2-hydroxybenzyl)ethylenediamine-N,N′-dipropionic acid and N,N-bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid were well separated by this method. The mobile phase contained 0.03 *M* tetrabutylammonium chloride and 30% acetonitrile at pH 6.0. The stationary phase was a LiChrospher RP-18 column, the injection volume was 20 μl and the flow-rate was 1.5 ml/min. For the iron(III)–EDDHA chelate, linear range studies showed that the method is capable of determining Fe concentrations between 0.5 and 150 μg/ml, which permits the determination of the concentrations found in commercial fertilizers. With this method, separation and identification of the iron(III) complexes were obtained with good resolution and selectivity, including the separation of the geometric isomers of the complexes, in 15 min.

Keywords: Fertilizers; Metal chelates; Iron; Ethylenediaminetetraacetic acid; Diethylenetriaminepentaacetic acid; *trans*-1,2-Cyclohexanediaminetetraacetic acid; Ethylenediaminedi(*o*-hydroxyphenylacetic) acid; Ethylenediaminedi(*o*-hydroxy-p-methylphenylacetic) acid; N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-dipropionic acid; N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid

1. Introduction

For many crops, iron chlorosis is a major obstacle to crop production in calcareous soils. Iron normally exists in nature in either iron(II) or iron(III) form. The solubility of Fe^{3+} changes 1000-fold with each pH unit change [1]. Soil application of soluble inorganic Fe salts (e.g., $FeSO_4$) is therefore only rarely effective since, in the presence of O_2 and $CaCO_3$, the applied Fe^{2+} salts are rapidly oxidized and precipitated as Fe(III) oxides, which are unavailable to plants [2]. Among all methods used to correct iron chlorosis, synthetic iron chelates are currently, but for their cost, the first choice for remediation

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[1] The research described was carried out in this laboratory.

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of iron deficiencies in plants [3]. Metal chelates are used for micronutrient fertilization in foliar, trunk and soil applications and in hydroponic cultures. Iron is by far the most common element used in fertilizers in chelated form [4].

Highly stable chelates of Fe(III), Ga(III) and In(III) are of considerable interest because the complexes formed with the radioactive isotopes of Ga(III) and In(III) may be employed as radiopharmaceuticals for diagnostic imaging applications [5–9], i.e., the corresponding gallium(III) and indium(III) chelates of HBED (see Table 1 for abbreviations) [7,9,10], and the high-spin Fe(III) complexes have possible applications for magnetic resonance imaging (MRI) [11,12]. Also, ligands that form highly stable iron complexes may be of interest as drugs for the removal of iron from the body in cases of iron overload [13]. Owing to the efficient chelation to iron, HBED and its esters have been found to be very effective in removal of iron from test animals [14–17].

This paper addresses the separation of the Fe(III) chelates of EDTA, DTPA, CDTA, EDDHA, EDDHMA, HBED and HBEP. Spatial structures are shown in Fig. 1. As an improvement upon earlier aliphatic aminocarboxylic acids, hard phenolate donors are employed to impart high thermodynamic stability of the metal complexes formed with metal ions [18]. The sexadentate chelating agents EDDHA and HBED have two phenolic groups replacing two of the carboxylates of EDTA and have very high affinities for Fe(III), with stability constants for racemic EDDHA of 10^{35} and HBED 10^{39} [19,20]. HBEP is a variation of the structure of HBED which has a pK_a similar to those of HBED, but has a lower stability constant of the iron(III) chelate (10^{31}) than that of HBED [21]. This indicates that the carboxylate donor groups in HBED are placed in more suitable position for coordination to the metal ion than those in HBEP, which has two β-propionate donor groups. However, the convenient and high-yield preparation of HBEP may make it an attractive chelating agent for iron(III) [21]. The stability constants for the Fe(III) chelates are given in Table 2.

In Europe, the Directive 76/116/EC allows chelates of the elements Fe, Mn, Cu, Zn, and Co to be used as such or incorporated in mixed fertilizers. Six chelating agents are allowed to be used for this purpose. They all belong to the group of aminocarboxylic acids, and are commonly abbreviated as EDTA, DTPA, HEEDTA (hydroxy-2-ethylenediaminetriacetic acid), EDDHA, EDDHMA and EDDCHA [ethylenediaminedi(5-carboxy-2-hydroxyphenyl)-acetic acid].

While developing an HPLC technique for determining FeEDDHA in solutions containing dissolved soil organic matter, it was found that the two stereoisomers of FeEDDHA were separated by anion chromatography [22]. Separation of the isomers by paper chromatography [23] and crystallization of Fe(*rac*-EDDHA) [24] permitted the identification by HPLC of the violet band found in paper chromatography as the *meso* complex and the red band as the racemic complex. Given the great similarity in size and structure of the two isomers, differences in elution time found by anion chromatography sug-

Table 1
Chelating agents chromatographically separated as their Fe(III) chelates in this study

Chelating agent	Abbreviation
Ethylenediaminetetraacetic acid	EDTA
Diethylenetriaminepentaacetic acid	DTPA
trans-1,2-Cyclohexanediaminetetraacetic acid	CDTA
Ethylenediaminedi(*o*-hydroxyphenylacetic) acid	EDDHA
Ethylenediaminedi(*o*-hydroxy-*p*-methylphenylacetic) acid	EDDHMA
N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-dipropionic acid	HBEP
N,N′-Bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid	HBED

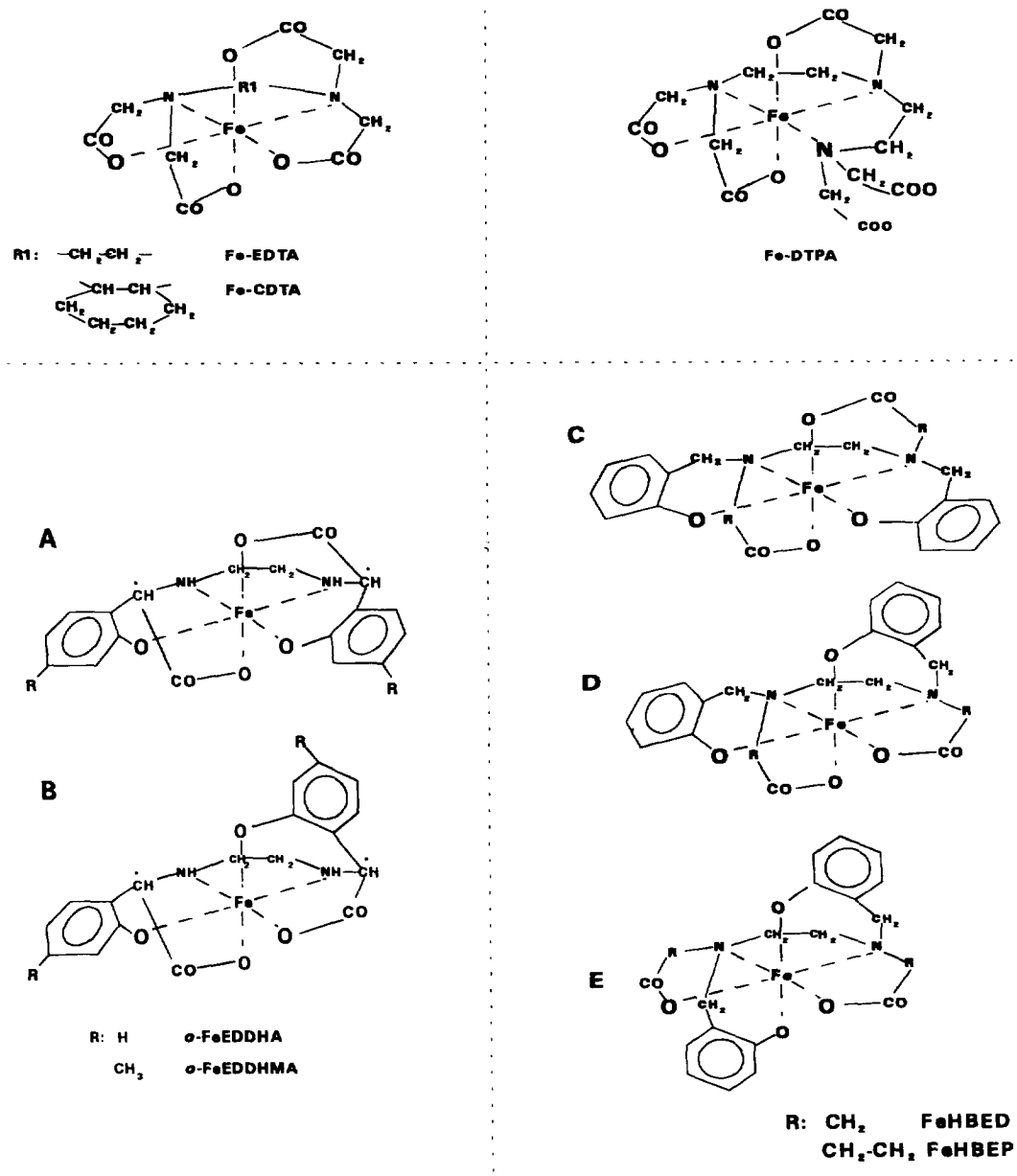

Fig. 1. Spatial structure of Fe(III) chelates of EDTA, CDTA, DTPA, *o*-EDDHA (A = racemic isomer, B = *meso* isomer), *o*-EDDHMA (A = racemic isomer, B = *meso* isomer), HBED (C, D, E = isomers) and HBEP (C, D, E = isomers).

gested that the racemic isomer may chelate Fe more strongly than the *meso* complex, thereby imparting a stronger anionic nature to the racemic complex [22]. Then it was found that the stability constant for FeEDDHA was 2.26 log units greater for the racemic complex than the *meso* complex, indicating a 500-fold difference in iron chelating ability [20].

Several HPLC methods are available to separate Fe(III) chelates of EDTA [25–30], DTPA [29–31], EDDHA [22,30] and EDDHMA [30] using ion-pair chromatography. Owing to the

Table 2
Stability constants of the aromatic Fe(III) chelates ($\mu = 0.100$ *M* at 25°C)

	Log *K*					
	HBED[a]	EDDHA[b] racemic	EDDHA[b] *meso*	EDDHMA[c] racemic	EDDHMA[c] *meso*	HBEP[d]
ML/M·L	39.01	35.54	33.28	37.90	39.00	31.16

[a] Ref. [18].
[b] Ref. [20].
[c] Ref. [38].
[d] Ref. [21].

very nature of the metal chelate equilibrium and the pH, buffer and solvent conditions imposed on this metal chelate equilibrium during the analysis by a chromatographic method, chromatographic methods can only be used for the determination of the amount of chelated metal in a sample containing a single metal in combination with a single chelating agent [32].

In studies of the determination of the chelates, TBA^+ has been the most common ion-pairing reagent used. The method proposed in this paper uses tetrabutylammonium chloride to achieve the separation of the Fe(III) chelates of EDTA, DTPA, CDTA, EDDHA, EDDHMA, HBED and HBEP.

2. Experimental

2.1. Reagents

Analytical-reagent grade $Fe(NO_3)_3 \cdot 9H_2O$ and NaOH were obtained from Merck, hydrochloric acid and acetonitrile of HPLC grade from Fisher Chemical, tetrabutylammonium hydroxide (40% solution in water, 1.5 *M*) from Sigma, HBED and CDTA from Strem Chemicals, EDTA from Pfaltz and Bauer, DTPA from Aldrich and EDDHA from Sigma. HBEP was kindly provided by Dr. P.A. Ganeshpure in its methyl ester form and was subsequentially hydrolysed [21]. As the chelating agent EDDHMA could not be found commercially but is manufactured industrially as the Fe(III) form, several commercial products were chosen. Based on the quality of the chromatograms obtained and on the previous results concerning the purity of various commercial iron chelates [33], the chromatogram shown in this paper corresponds to the purest of the formulations tested. A commercial fertilizer, Sequestrene 138 Fe G-100 (nominal 6% Fe as Fe–EDDHA, Lot 718592) was obtained from Ciba-Geigy. All solutions were prepared with deionized water obtained by passing distilled water through a Waters Milli-Q water-purification system.

2.2. Preparation of standard and sample solutions

For preparing the standard solutions, ligands were dissolved in sufficient NaOH (normally a 1:3 molar ratio). Then an amount of $Fe(NO_3)_3$ that was calculated to be 5% in excess of a molar amount of ligand was added, the pH was adjusted to 7 and the solutions were left to stand overnight to allow excess Fe to precipitate as oxides. The final solutions, with an Fe concentration of 100 mg/l, was filtered through Whatman No. 2 filters and made up to volume with water.

Solutions of the commercial products containing ca. 100 mg/l of Fe based on the Fe content indicated by the manufacturer were prepared by dissolving the formulations in deionized water. The solutions were left to stand overnight, filtered through Whatman No. 2 filters and made up to volume. For both standard and sample solutions, exposure to light was avoided during

their preparation process because of the potential photodecomposition of chelates [25].

Standards and samples for analysis were filtered through Millipore 0.22-μm filters, type GV, prior to injection. The injection volume was 20 μl.

2.3. Preparation of mobile phase for ion-pair chromatography

A 20-ml volume of 1.5 *M* tetrabutylammonium hydroxide solution was added to 650 ml of water, the pH was adjusted to 6.0 with HCl (1:1) and 300 ml of acetonitrile were added and made up to 1 l in a volumetric flask. Finally the solution was filtered through 0.22-μm Millipore filters and degassed during the entire process with helium. The flow-rate of the mobile phase was 1.5 ml/min.

The pH of the mobile phase was adjusted based on the stability constants of the iron(III) complexes. All the chelates considered are mainly in the ferrated ligand form (FeL) at pH 6. Higher pH could lead to decomplexing of some iron chelate (Eq. 1) or hydroxylation of the chelate (Eq. 2):

$$3\,OH^- + FeL^{3-n} \rightleftharpoons Fe(OH)_3 + L^{n-} \quad (1)$$

$$FeL^{3-n} + yOH^- \rightleftharpoons FeL(OH)_y^{3-n-y} \quad (2)$$

Lower pH could lead to the protonation and decomplexing of some chelate (Eq. 3) or the presence of the protonated iron(III) form (Eq. 4):

$$nH^+ + FeL^{3-n} \rightleftharpoons Fe^{3+} + LH_n \quad (3)$$

$$FeL^{3-n} + xH^+ \rightleftharpoons FeH_xL^{3+x-n} \quad (4)$$

At pH 6, the iron chelate is stable under the experimental conditions.

2.4. Ion-pair chromatographic system

A Waters Model 600E multisolvent delivery system, a Waters Model 700 Satellite WISP autosampler and a Waters Model 486 tunable absorbance detector were used. A LiChrospher RP-18 column (150 × 4.6 mm I.D., $d_p = 5$ μm) was used. Data were processed using a Baseline 810 chromatography data system.

3. Results and discussion

3.1. Influence of TBACl concentration

The influence of the TBACl concentration in the mobile phase was investigated (Fig. 2). TBACl concentrations in the mobile phase of 0.01, 0.02 and 0.03 *M* were tested for the iron(III) complexes of EDTA, DTPA, EDDHA and EDDHMA. Fig. 2 shows the retention times with respect to the TBACl concentrations added to the mobile phase. As the TBACl concentration in the mobile phase decreased, the retention time of these solutes increased. These data support previous findings for this Fe(III) chelate with the same ion-pair reagent [30]. The

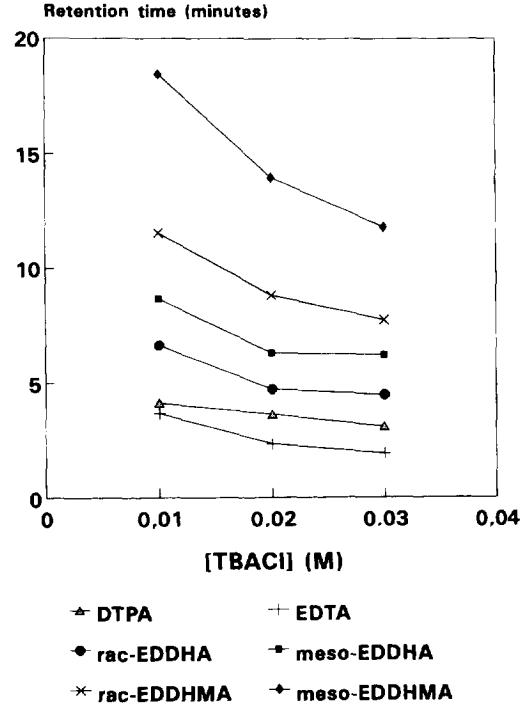

Fig. 2. Influence of TBACl concentration on the retention time of the iron(III) chelates studied. Column, LiChrospher RP-18; eluent, 0.03 *M* TBACl–30% acetonitrile (pH 6.0); flow-rate, 1.5 ml/min; injection volume, 20 μl; detection wavelength, 280 nm [225 nm for Fe(III)–CDTA].

behavior of the Fe–DTPA chelate showed a slightly different tendency that we presume is related to the double negative charge of the complex. It has been shown that the mechanism occurring was a combination of ion exchange and desolvation [34]. As the concentration of ion-pairing reagent increases in the mobile phase, either the saturation of the C_{18} surface area available for desolvation by the ion-pairing reagent, or the counter ion (in this case chloride) behavior as an anion-exchange competing ion which is more effective in this role at higher concentrations, will cause a decrease in the retention time of the solutes. In this study, the separation was achieved with 0.03 *M* TBACl and using acetonitrile to reduce the retention time of the aromatic complexes. The TBACl concentration was chosen owing to the low retention time and the good resolution obtained for the separation.

3.2. Influence of percentage of acetonitrile

The percentage of acetonitrile used (30%) was chosen for similar reasons, i.e., lower retention time for the aromatic compounds, good resolution between isomer peaks and short analysis time. Gradient separations were tested with increasing acetonitrile concentration from 0 to up to 30%, but the results were not satisfactory because a TBACl peak appeared during the chromatographic run, even when only eluent was injected. The addition of an organic modifier to bring the retention time values to reasonable magnitudes will alter the adsorption isotherm shape of the ion pairing in the stationary phase [35], which would explain the presence of the TBACl peak when the percentage of acetonitrile was increased in a gradient sequence, even though its absorption at 280 nm is relatively small (0.1281 A.U. for 0.03 *M* TBACl solution). Other conditions for the separation were tested [30], even though the column was not exactly the same, with a flow-rate of 1.5 ml/min. The TBACl peak appeared under such conditions, and also when the concentration used was 0.03 *M*, which was lower than that used by Deacon et al. [30]. Subsequently, an isocratic separation was tested with good results. Since under isocratic conditions there is a unique mobile phase, it is unnecessary to re-equilibrate the system after each sample because the initial and final eluents have the same composition. This makes the method shorter than other proposed.

3.3. Separation of Fe(III) chelates

For all the iron(III) chelates, a spectrophotometric scan between 200 and 600 nm was performed in order to locate absorbance maxima and minima. UV detection at 280 nm was found adequate for the determination of all the iron(III) complexes except Fe–CDTA, which was better detected at 225 nm.

The chromatograms obtained with the method proposed in this paper show that the Fe(III) complexes of the aliphatic EDTA, CDTA and DTPA eluted within 3 min, with all three peaks well separated, and only 13 min were required to elute Fe(III)–HBED, which has the longest retention time of the aromatic chelates tested (Table 3). Table 3 also gives some characteristics of the method proposed to determine the iron(III) complexes studied.

To the best of our knowledge, formulations intentionally containing more than a single Fe(III) chelate are unavailable, with the exception of a mixture of Fe(III)–EDTA and Fe(III)–EDDHA, so it is not really useful to resolve all the peaks of the chelates in one isocratic run, even though, in one isocratic run, the method described in this paper resolved the peaks of the Fe(III) chelates of EDTA, DTPA, EDDHA and EDDHMA, which are the most commonly present in fertilizers. For the Fe(III)–HBED and Fe(III)–HBEP chelates another isocratic run was necessary, owing to the overlaping of the peaks. The detection of Fe(III)–CDTA was performed at 225 nm, so if a diode-array spectrophotometer is not available, it will be necessary to determine it in another run.

The chromatograms presented good resolution and high selectivity for the determination of Fe(III) chelates of EDTA–DPTA ($R_s = 2.11$, selectivity = 1.97), *rac*-EDDHA–*meso*-EDDHA ($R_s = 3.08$, selectivity = 1.69), *meso*-EDDHMA–

Table 3
Retention times (t_R, min), number of theoretical plates (N), capacity factor (k') and sensitivity (S) based on the peak area (absorbance/mM) obtained for the Fe(III) chelates with the method described

No.	Parameter	EDTA	CDTA	DTPA	EDDHA	EDDHMA	HBED	HBEP
1	t_R	1.92 (±0.02)	2.00 (± 0.04)	3.10 (±0.15)	4.93 (±0.06)	7.75 (±0.07)	8.28 (±0.18)	5.47 (±0.09)
	N	218	554	1330	1438	3554	2445	3908
	k'	1.74	0.29	3.43	0.90	3.51	3.25	1.8
	S	41.83	30.00	41.83	25.54	41.27	107.33	48.21
2	t_R				6.53 (±0.11)	11.78 (±0.16)	10.83 (±0.37)	12.26 (±0.20)
	N				2523	3002	2860	5692
	k'				1.52	5.85	4.55	5.29
	S				25.98	45.15	12.00	79.12

Mobile phase, 0.03 M TBACl–30% acetonitrile (pH 6); flow-rate, 1.5 ml/min; injection volume, 20 μl. Numbers 1 and 2 correspond with the number of isomers of the chelates. Each retention time value represents the t_R(±S.D.) ($n = 8$).

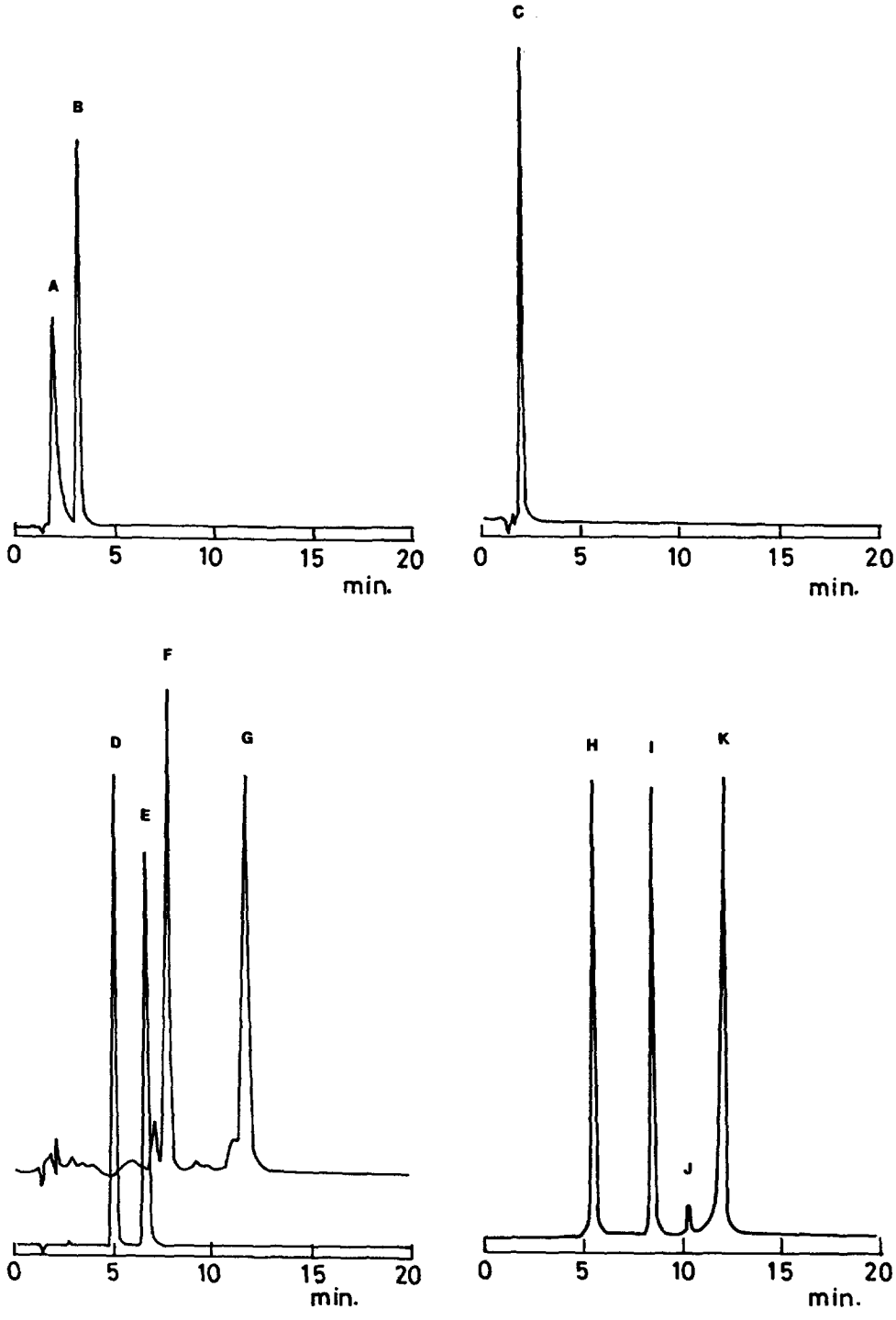

Fig. 3. Chromatograms of (A) Fe–EDTA, (B) Fe–DTPA, (C) Fe–CDTA, (D) Fe–*rac*-EDDHA, (E) Fe–*meso*-EDDHA, (F) Fe–*meso*-EDDHMA, (G) Fe–*rac*-EDDHMA, (H) Fe–HBEP(1), (I) Fe–HBED(1), (J) Fe–HBED(2) and (K) Fe–HBEP(2). Conditions as in Fig. 2.

rac-EDDHMA ($R_s = 5.84$, selectivity = 1.67), HBEP–HBED (Fig. 3) (peaks H–I, $R_s = 5.51$, selectivity = 1.80; peaks I–J, $R_s = 3.45$, selectivity = 1.40; peaks J–K, $R_s = 1.96$, selectivity = 1.16). This method shows two major advantages. First, only 13 min are necessary to elute the Fe(III)–HBEP chelate, which has the longest retention time of the complexes tested. Moreover, because the method is isocratic, a re-equilibration time between one injection and the next is not necessary.

3.4. Comparison among aromatic Fe chelates

The iron(III) chelates of HBED and HBEP were included in this study because of the structural similarity of these compounds with EDDHA and EDHMA. All are sexadentate and have two phenolate donors, two nitrogen donors and two carboxylate donors. The higher metal ion affinity of HBED relative to that of EDDHA (Table 2) is due to the more favorable steric orientation of donor groups and the higher stability constants of *rac*-EDDHA compared with that of *meso*-EDDHA is due to a geometric effect [20]. The number of atoms in the rings is an important factor for the stability of the chelates, mostly when we compare the three equatorial rings that are in the plane formed by the Fe and the two nitrogens. Increasing the chelate ring size from five members (HBED) to six members (HBEP) causes the stability constants of the Fe(III) chelates to decrease from ca. 10^{39} to 10^{31} (Table 2). Steric factors strongly affect stability constants [18].

The aromatic ligands EDDHA and EDDHMA contain two chiral carbons. Hence four possible isomers are (*R*,*R*), (*R*,*S*), (*S*,*R*) and (*S*,*S*), but owing to the internal symmetry of the molecules, the (*R*,*S*) and (*S*,*R*) enantiomers are the same molecule denoted the *meso* isomer (Fig. 1B). The (*R*,*R*) (Fig. 1A) and (*S*,*S*) (not shown) enantiomers are mirror images, so they differ only in the direction of polarized light deviation. Since the affinity of the (*R*,*R*) and (*S*,*S*) isomers for H^+ or metals is the same, they cannot be separated by chemical processes and generally they are called a racemic mixture.

When EDDHA or EDDHMA complexes a metal, e.g., iron, different spatial arrangements can be formed. For the *meso* diasteroisomer (Fig. 1B), only one arrangement is possible that has one phenolic group in an equatorial position with respect to the plane formed among the Fe and the two nitrogens, and the other phenolic group in an axial position [arrangement (6,5,5), where the numbers indicate the atoms that form the three equatorial rings]. This arrangement must be 50% of the total Fe–EDDHA or Fe–EDDHMA that is present. However, for each of the enantiomers of the racemic mixture, two spatial arrangements are possible, one with both phenolic groups in the equatorial position [arrangement (6,5,6), Fig. 1A], and the other with the two phenolic groups in the axial position [arrangement (5,5,5), figure not shown]. Both arrangements are in equilibrium, but for Fe–EDDHA the most significant is the first one with the phenolic groups in the equatorial positions because the presence of both six-membered rings in the equatorial positions seems to be more stable than a constrained arrangement of three five-membered rings in the equatorial plane [36]. It was calculated that for Fe–EDDHA, the arrangement with the two phenolic groups in polar positions (5,5,5) contributes less than 0.5% to the total amount of the racemic mixture (50% of the total amount of Fe–EDDHA). Crystallographic evidence for the dominant presence of the arrangement (6,5,6) (Fig. 1A) for *rac*-$Mg(Fe\text{–}EDDHA)_2$ confirms this hypothesis [24]. It must be remembered that, as shown in Table 2, the *meso* isomer of Fe–EDDHA [(6,5,5), one phenolic group in a polar position] is less stable than the racemic isomer [mostly (6,5,6)].

The chelate Fe–EDDHMA shows an opposite behavior. The *meso* isomer, a combination between a phenolic six-membered ring and two five-membered rings in the equatorial position (6,5,5), is more stable than the racemic isomer, also with the two possible forms, (6,5,6)- or (5,5,5)-membered rings in the equatorial position. Since only two principal peaks are obtained in the chromatographic separation of Fe–EDDHMA, we presume that only one racemic arrangement exists or that the contribution of the

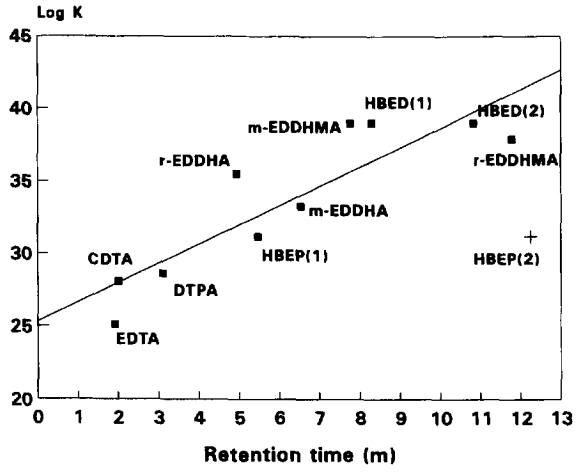

Fig. 4. Relationship between retention times and the stability constants of the iron chelates.

other possible one is not particularly significant. Owing to the similar behavior of the racemic isomer of the Fe–EDDHMA and the racemic isomer of the Fe–EDDHA in the precipitation of the Mg salts (data not published), we presume that the (6,5,6) combination (both phenolic groups in equatorial positions) is prefered to the (5,5,5) combination for the racemic Fe–EDDHMA.

HBED and HBEP chelating agents are good tools for a better understanding of the behavior of EDDHA and EDDHMA isomers. The stabilities and the spatial conformations of the Fe chelates of HBED and HBEP, in contrast with the EDDHA and HPED (N,N′-bis(2-hydroxyphenyl)-N,N′-ethylenediaminediacetic acid) were compared [18], but some errors in the considerations of the size of the rings make that discussion confusing.

HBED and HBEP as ligands do not have optical isomers, since no chiral carbons occur. However three spatial arrangements appear when they complex Fe. In these cases the nitrogens act as asymmetric centers. Previously reported stability constants (Table 2) for these chelates give only one value per chelating agent, which means that no separatable isomers of the molecules have yet been found. Since the probable isomers are in equilibrium, it is not possible to isolate them and study their stability constants separately. Despite the thermodynamics of this equilibrium, it is most likely very slow since it implies the liberation of the Fe (slow) and the formation of the new chelate. Consequently, it seems probable that the different geometrical isomers can be separated during the chromatographic process. For both Fe–HBED and Fe–HBEP, two different peaks are obtained, so it seems that two isomers can be present in the equilibrium mixture.

The retention of an ionized solute in the presence of pairing ion on a C_{18} reversed-phase system may be considered as occurring by two separate mechanisms. One is the desolvation of the solute on the C_{18} surface. This may be minimal in the case of a highly ionized solute, resulting in short retention times, but may be appreciable if the solute is hydrophobic even when ionized. It is suggested that an ion-exchange reaction occurs between the ionized solute and the adsorbed pairing ion followed by desolvation of the neutralized solute on to the C_{18} surface. This desolvation will be proportional

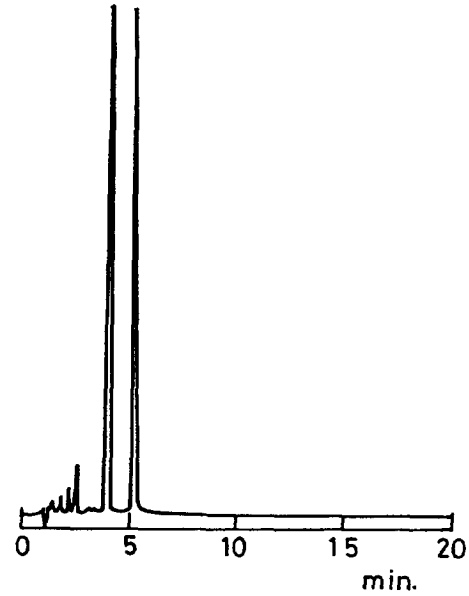

Fig. 5. Chromatogram of an Fe(III)–EDDHA fertilizer sample (Sequestrene 138 Fe G-100, 6% Fe as Fe–EDDHA; Ciba-Geigy) containing 100 mg/l of Fe based on the percentage of iron chelate indicated by the manufacturer. Conditions as in Fig. 2.

to the hydrophobicity of the solute, which will act to increase the electrostatic effect of ion exchange. The retention of the bound species in ion exchange will also be proportional to the area of the stationary phase available for desolvation [34]. The stability constants of the Fe complexes play an important role in the chromatographic separation of such chelates. As the stability constant of the Fe complex increases, the binding between the Fe chelate and the ion-pairing reagent becomes greater, and when the equilibrium constant of the formation of the ion pair increases, the retention time increases [37]. Fig. 4 shows this relationship between the stability constants of the chelate and the retention time. In general, a longer retention time implies a large log K, although this is not true when we compare the racemic and *meso* isomers for Fe–EDDHA or Fe–EDDHMA, perhaps owing to the hydrophobic effect. The geometric isomer of Fe–HBEP with the longer retention time (Fig. 3, peak k) also does not follow the same behavior. It may be noted that the reported log K value for Fe–HBEP is an average for a mixture of isomers and it will be necessary to establish the intrinsic stability constants for these geometric isomers in order to perform a correct evaluation.

3.5. Linear range of the method for Fe(III)–EDDHA

The ion-pair chromatographic method described gave rise to a linear calibration for the determination of the iron(III) chelates in the range 0.5–150 μg/ml of Fe in the form of the EDDHA chelate. The calibration graphs for the racemic and *meso* isomers of Fe–EDDHA were studied. The concentrations used were 0.5, 1.0, 5.0, 10, 25, 50, 100 and 150 μg/ml of Fe chelated by EDDHA and peak area were measured. The correlation coefficients obtained were $r = 0.9998$ for the racemic isomer and $r = 0.9995$ for the *meso* isomer.

3.6. Commercial Fe–EDDHA fertilizer

No interferences were encountered in the analysis of real samples and the sample retention times agreed with those of standards. Fig. 5 shows a chromatogram obtained for a 100 mg/l of Fe solution of commercial Fe(III)–EDDHA fertilizer injected (Sequestrene 138 Fe G-100, 6% Fe as Fe–EDDHA). The two isomers peaks, racemic and *meso* Fe(III)–EDDHA, were detected in addition to impurity peaks.

The percentages of active ingredients (racemic and *meso* isomers) were calculated with respect to the peak areas of the Fe(III)–EDDHA standard. The values obtained were 1.227% for the racemic isomer and 1.307% for the *meso* isomer, which means 2.534% of Fe chelated as Fe–EDDHA.

Comparing the areas of the two isomer peaks, for Fe–EDDHA standard the *meso*:racemic ratio is 1.01, but for the commercial product Sequestrene it is higher (1.10), perhaps owing to the impurities found for the commercial product. Commercial Fe(III) chelates contain not only the isomers of the nominal chelate, but also unreacted reagents and unintended byproducts as impurities, which may complex Fe but not sufficiently strongly to have elution times as long as the isomers of the nominal chelates.

4. Conclusions

An ion-pair chromatographic method was developed to allow the separation of the chelates FeEDTA, FeDTPA, FeCDTA, FeEDDHA, FeEDDHMA, FeHBED and FeHBEP and their diastereoisomers. Two possible geometric isomers of the FeHBEP and FeHBED chelates not previously described in the literature have been found.

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